Microscale Canopy Interactions in Aquatic Phototrophs

PhD Thesis
Mads Lichtenberg
Microscale Canopy Interactions in Aquatic Phototrophs

PhD thesis by
Mads Lichtenberg

Supervisor
Professor Michael Kühl

Marine Biological Section
Department of Biology
University of Copenhagen
Cover page

Front cover:

Underwater photo of a massive coral colony in the sheltered lagoon ‘Shark Bay’ on the reef of Heron Island, Australia. Photo by Mads Lichtenberg.

Back cover:

Top: Field microsensor setup for diurnal measurements of O\(_2\), pH, H\(_2\)S, and H\(_2\) dynamics in the famous Aggersund microbial mat during the 2016 Microsensor course in Rønbjerg, Denmark. Photo by Mads Lichtenberg.

Lower left: The apical tip of a serrated wrack (Fucus serratus) with hyaline hairs (white dots) protruding. Photo by Erik Trampe.

Lower right: A coral polyp showing host pigment fluorescence during measurements of a scalar irradiance profile through the polyp tissue on Heron Island Research Station. Photo by Mads Lichtenberg.

Erik Trampe is thanked for help with the design of the cover.
Preface

You often hear how life as a PhD student is filled with disagreements and conflicts with supervisors and enduring stressful working conditions. I can honestly say that I have not experienced any of this. The journey over the last 3 years have been a genuinely good trip filled with the wonders of science, meeting new friends and getting to explore nature in some very exotic places around the world while always being in good company. If I had the chance, I would do it all again!

First and foremost I would like to thank my supervisor Michael Kühl. Michael you have been a solid support throughout my project and your empathetic nature, good humour and excellent guidance has made the last 3 years some of the most fun and rewarding in my life. You have been a great mentor and the best supervisor one could hope for. I sincerely thank you for this experience.

Erik Trampe is thanked for being a good friend, colleague and field-companion. It is always a pleasure to be in your company whether in the lab, snorkelling the outskirts of the blue pools on Heron Island, scaring off Grizzly bears in the Yellowstone wilderness or calibrating sulphide sensors in the snowy Japanese Alps. I can honestly say that we have shared some amazing experiences that I will never forget.

Kasper Elgetti-Brodersen is thanked for being a close friend and colleague. We started the scientific adventure together and it is always a pleasure to engage in scientific (as well as less scientific) discussions with you and I have enjoyed all of our projects together.

I thank Klaus Koren, Maria Moßhammer, and Daniel Wangpraseurt for editorial help with the thesis but also Sofie Jakobsen without whom the lab simply would not work, Johannes Gössling, Lars Rickelt, Verena Schrameyer, Paulo Cartaxana, Sonia Cruz, Lars Behrendt and everyone else from the Microenvironmental Ecology Group and at MBS is thanked for being the best colleagues. With you, it was always a pleasure to come to work and I have enjoyed your company for the last 3 years.

I would also like to express my gratitude to Anthony Larkum and Thomas Vogelmann. Tony you are a true original and it has been a pleasure to work with you. Adventure is always guaranteed in your company, whether on reef-walks at low tide, striding through thick mangrove or snorkelling in murky waters to find interesting algae. Tom I thank you for taking the long trip to Denmark to discuss my project and for the excellent time I had in your lab in Vermont.

Lastly, my family and partner is thanked for their support throughout this journey. Simone, I know that I have pushed the limit at times when adventures abroad presented themselves, but you have been very patient and a true support. Your reluctance to stay behind have given us some unique experiences together.

Mads Lichtenberg, Copenhagen 2017
“Adapt or perish, now as ever, is nature’s inexorable imperative”
- H.G. Wells
Summary

Photosynthetic production and light utilization efficiencies in aquatic organisms and microbial communities is determined by the irradiance incident on the system, which on a macroscale is dependent on factors such as, water depth and turbidity. However, on a microscale the light field inside phototrophic tissues and communities is determined by interactions between the incident light and the optical properties of the system, which is influenced by pigmentation, organization of tissue structural components, and the intracellular organisation of phytoelements. Our current understanding of how photosynthesis is influenced by light interactions is largely based on studies of terrestrial plants where canopy interactions have been described across scales; from landscape-level down to the organization of individual chloroplasts. How light interactions and photosynthetic efficiencies are influenced by microstructural heterogeneities in the organization of aquatic tissues and communities is largely unexplored although a few papers have described the importance of community structure on whole-community production. In this thesis, it was the aim to investigate if fundamental links exists between the microscale organization of aquatic photosynthetic tissues and biofilm communities, their optical properties, and photosynthetic efficiencies and to investigate whether canopy-like effects are relevant for the microscale regulation of aquatic phototrophs similar to what is found in terrestrial plants.

This was investigated in a range of aquatic phototrophs such as macroalgae, reef-building corals, and photosynthetic biofilms. As a first step, we demonstrate that a microscale stratification of the internal light- and chemical environment exists across the investigated systems, with concomitant internal gradients of photosynthesis and respiration. We further investigate this by compiling a closed radiative energy budget of a coral and find that corals are highly efficient light collectors that can display photosynthetic quantum efficiencies close to the theoretical limit. Using a similar approach, we then investigate i) how community composition affects energy budgets in photosynthetic sediments, ii) the role of incident light field angularity (diffuse/collimated) on radiative energy conservation, and iii) how light-induced migration of cyanobacteria change community-structure and photosynthetic efficiencies in a natural biofilm. We develop methods for measuring quantum yields inside tissues while considering the actual light availability. Furthermore, physical structures protruding from the surface of a system can change both the light- and chemical microenvironment and the consequence of such changes on plant fitness was studied. In the final chapter, a comparative analysis between terrestrial and aquatic photosynthetic systems is given and it is discussed if aquatic microscale structure/function relationships can be described conceptually similar to terrestrial canopy interactions.
Resumé

Fotosyntetisk produktion and lysudnyttelses effektiviteter i akvatiske organismer og mikrobielle samfund er afhængigt af det indfaldende lys hvilket, på en makro-skala, bliver bestemt af faktorer såsom vanddybde og sigtbarhed. På en mikro-skala vil lysfeltet inde i fotosyntetiske væv og –samfund dog være bestemt af interaktioner mellem det indfaldende lys og de optiske egenskaber af systemet som bliver påvirket af pigmentindhold, organisering af strukturelle komponenter samt den intracellulære organisering af de fotosyntetiske elementer. For nuværende er vores forståelse af hvordan fotosyntesen bliver påvirket af lys interaktioner primært baseret på studier af terrestriske planter hvor 3-dimensionelle interaktioner er blevet beskrevet på tværs af niveauer; fra landskab ned til organiseringen af enkelte kloroplaster. Hvordan lys interaktioner og fotosyntetiske effektiviteter bliver påvirket af mikrostrukturerne heterogeniteter i organisationen af akvatiske væv og samfund er derimod stort set ikke undersøgt (indtil videre?) selvom nogle få studier har vist at vandplanters 3-dimensionelle struktur har stor indflydelse på den samlede fotosyntese. I denne afhandling var målet at undersøge hvorvidt der findes fundamentale sammenhænge mellem den mikrostrukturerne organisering af fotosyntetiske væv og –samfund, deres optiske egenskaber og fotosyntetiske effektiviteter samt at undersøge hvorvidt nogle af de 3-dimensionelle effekter der er blevet beskrevet i terrestriske planter er relevante i mikroskala i akvatiske fototrofe organismer.

Dette blev undersøgt i en række forskellige systemer såsom makroalger, rev-byggende koraller og fotosyntetiske biofilm. Til at starte med, beskriver vi hvordan der på en mikroskala er en intern lagdeling af både lys og kemiske forhold på tværs af de undersøgte systemer med tilhørende interne gradierter af fotosyntese og respiration. Vi undersøger dette nærmere ved at måle afstemte lys-energibudgetter af koraller og konkluderer at disse er højst effektive til at indfange lys og kan operere med et kvanteudbytte der nærer sig det teoretiske maksimum. Ved at bruge en lignende metode undersøger vi derefter i) hvordan sammensætningen af fotosyntetiske sampioner påvirker energibudgetter, ii) hvilken rolle retningen af det indfaldende lys (diffust/retningsbestemt) har på energi omsætningen og iii) hvordan lysinducerede bevægelser af cyanobakterier ændrer samfund sammensætningen og den fotosyntetiske effektivitet i en naturlig biofilm. Vi udvikler metoder til at måle kvanteudbytter internt i fotosyntetiske væv der tager højde for den faktiske lysstilgængelighed. Derudover kan fysiske strukturer på overfladen af væv ændre både lys og kemiske forhold inde i vævet og konsekvensen af sådanne ændringer bliver beskrevet.

I det sidste kapitel bliver der givet en sammenlignende analyse mellem terrestiske og akvatiske fotosyntetiske systemer og det bliver diskuteret hvorvidt mikroskala forhold mellem struktur og funktion konceptuelt kan beskrives som de tilpasninger der er beskrevet i 3-dimensionelle terrestriske systemer.
Table of Contents

List of Publications 7

Chapter 1: General introduction and thesis outline 10
    Terrestrial photosynthesis 11
    Structural regulations 11
    Photoadaptation 14
    Aquatic photosynthesis 16
    Macro- vs. microscale canopy 17
    Methodology 18
    Microsensors 18
    Fiber-optic microprobes 19
    Pulse-amplitude modulation (PAM) fluorometry 21
    Thesis outline 24

Chapter 2: Radiative energy budget reveals high photosynthetic efficiency in symbiont-bearing corals 31

Chapter 3: Photosynthetic acclimation of *Symbiodinium in hospite* depends on vertical position in the tissue of the scleractinian coral *Montastrea curta* 58

Chapter 4: Radiative energy budgets of phototrophic surface-associated microbial communities and their photosynthetic efficiency under diffuse and collimated light 85

Chapter 5: Pronounced gradients of light, photosynthesis and O$_2$ consumption in the tissue of the brown alga *Fucus serratus* 124

Chapter 6: Vertical migration optimizes photosynthetic efficiency of motile cyanobacteria in a coastal microbial mat 150

Chapter 7: Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O$_2$ loss from the below-ground tissue 173

Chapter 8: Diffusion or advection? Mass transfer and complex boundary layer landscapes of the brown alga *Fucus vesiculosus* 197

Chapter 9: Fiber-optic probes for small-scale measurements of scalar irradiance 221

Chapter 10: Multicolor light sheet microscopy-based imaging of absorption- and photosynthesis distribution in plant tissue 248

Chapter 11: Nanoparticle-based measurements of pH and O$_2$ dynamics in the rhizosphere of *Zostera marina* L.: Effects of temperature elevation and light-dark transitions 280

Chapter 12: Discussion and future directions 314
    Redistribution of light is affected by tissue/community structure 316
    Dynamic modulation of internal light environment 318
    Photoadaptation on a microscale 321
    Technical advances and future directions 322
    Thesis conclusion 325
Publications included in PhD thesis


5. Lichtenberg M, Cartaxana P, Kühl, M. Vertical migration optimizes photosynthetic efficiency of motile cyanobacteria in a coastal microbial mat. (Intended for submission to Environmental Microbiology)


Other publications arising from the PhD candidature:


Conference presentations arising from the PhD candidature


2. Lichtenberg M, Brodersen KE, Carlota-Paz L, Kühl M. Epiphytic microalgae on seagrass leaves impede photosynthesis and radial O₂ loss from the roots. November 2015; Poster presentation at PhD day conference, University of Copenhagen, Denmark. (tinyurl.com/epihyteposter)


Chapter 1

General introduction and thesis outline

by

Mads Lichtenberg
Photosynthesis is, put simply, the process of acquiring biomass by converting inorganic carbon to complex sugars driven by solar energy and electrons from water and can be compressed into the simple reaction:

$$6\text{CO}_2 + 6\text{H}_2\text{O} \xrightarrow{\text{light}} \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2$$

However, the process of photosynthesis is far from simple and involves a breathtaking amount of proteins, enzymes, alternative pathways, regulating factors etc. In this thesis, I have investigated some of the basic environmental factors that influences photosynthesis, such as light availability and gas exchange, in aquatic photosynthetic systems such as tropical corals, temperate macrophytes and photosynthetic biofilms from both temperate and hot-spring ecosystems. The aim of the thesis was to investigate if such diverse systems display similarities in light handling properties in order to optimize, and/or counteract sub-optimal conditions for; photosynthesis.

Terrestrial photosynthesis

By far, the largest body of knowledge on photosynthesis comes from studies of terrestrial systems. The largest difference in terrestrial and aquatic photosynthesis is that in aquatic photosynthesis the organism is submerged whereas terrestrial organisms are in a gaseous environment. This means that terrestrial systems have the opportunity to freely exchange gases, such as O₂ and CO₂, with their environment. However, essential growth-factors such as nutrients and water are not readily available from the atmosphere which creates the need for specialized structures, such as roots and stomata, to maintain water balance and acquisition of nutrients. Because of the high permeability of CO₂ in leaves, due in part to internal gas filled spaces (Evans et al., 2009), internal gradients are usually small (Evans & von Caemmerer, 1996). However, as leaves are opaque, internal gradients of light exists (Vogelmann et al., 1996b) which is often considered to be the main regulating factor of production in photosynthetic systems (Sand-Jensen, 1989; Vogelmann, 1993). Due to this light limitation, plants have evolved complex structures to counteract some of the limitations imposed by uneven illumination, where parts of the plant can be light-saturated, or can be in deep shade while other parts experience a highly changing light environment, e.g. by sun flecks, over short time scales (Chazdon & Pearcy, 1991; Way & Pearcy, 2012).

Structural regulations

The amount of light available at a depth ($x$) in a canopy can be described as (Terashima & Hikosaka, 1995):

$$11$$
\[ I_x = I_0 \exp(-KF_x) \]

where \( I_0 \) is the irradiance over the canopy, \( K \) is the leaf inclination and \( F_x \) is the cumulative leaf area.

Thus, on a macroscale plants can regulate the amount of light they receive by regulating the cumulative surface area of leaves or by changing the leaf inclination. Studies have shown that, depending on light regime, leaves in the upper canopy are often vertically oriented (McMillen & McClendon, 1979), while leaves in the lower canopy are horizontally oriented (Vogelmann & Björn, 1983). This mechanism serves to minimize light capture in the upper leaves that are fully sun-exposed while it transmits light to leaves deeper in the canopy (Raabe et al., 2015). As light travels through a canopy it is scattered and becomes progressively more diffuse (Brodersen & Vogelmann, 2010). The effect of diffuse light has been shown to cause lower leaf level photosynthesis compared to direct light (Brodersen et al., 2008) but an overall increase in canopy photosynthesis, due to the more even illumination of the canopy (Gu et al., 2002).

**Figure 1: Structural regulations on macroscales in terrestrial systems.**

A) In sunlight the upper part of the canopy will experience collimated light whereas leaves in the lower canopy will perceive a more diffuse light field due to scattering and absorption inside the canopy. B) Conceptual drawing of leaf inclination as function of depth in the canopy where leaves high in the canopy are more vertically oriented than leaves deep in the canopy to improve penetration and interception of light on a whole canopy scale.
On a leaf scale, structures have evolved to compensate for this difference in angular light distribution. Sun leaves, which are often located high in the canopy, are usually thicker than their shaded counterparts (Lichtenthaler et al., 1981) which limits equal distribution of light through the leaf layers. As an anatomical adjustment to this, sun leaves have more well-developed palisade layers that are columnar cells between the epidermis and the mesophyll layers. The palisade layer has been shown to be less light scattering than mesophyll layers, and may thus function as a light-funnel in direct light (Vogelmann & Martin, 1993). When illuminated with diffuse light, however, the light gradients were found to be similar to gradients seen in leaves without palisade layers, which underlines its functional significance in leaves exposed to high proportions of direct light (Vogelmann, 1993; Vogelmann & Martin, 1993).

In the lower part of the canopy, leaves will often be light limited with reduced tissue thickness probably to avoid self-shading and minimize respiratory load (Lichtenthaler et al., 1981). In this low light environment, some plants have evolved plano-convex, conical or other convex shaped epidermal cells that can act as a focusing lens (Fig. 2A; Vogelmann et al. (1996a)). It has been shown that these epidermal cells can concentrate light up to 15 – 20 fold in the absence of underlying tissue layers and up to 5-fold when a scattering and absorbing mesophyll layer was present underneath (Vogelmann et al., 1996a).

Figure 2: Structural regulations on microscales in terrestrial systems. A) Structural regulation on a microscale where convex shaped epidermal cells can focus and concentrate light into the mesophyll layers where the chloroplasts are harbored. B) Simplified schematic of chloroplast movement which is the dynamic modulation of absorptive properties for a leaf depending on the light exposure.

The abovementioned factors describe regulations in leaves found in the extreme scenarios of high- and low light. But leaves, e.g. in the central parts of a canopy, will experience a range of different light environments over a diurnal cycle where direct sunlight or shade can occur as a function of the solar angle. In these cases a ‘static’ adaptation like elongated palisade cells will be disadvantageous; or at least
only advantageous during short time-scales. To utilize this variable light environment, plants have evolved mechanisms such as chloroplast movement, where the chloroplast position within the plant cells can be dynamically modulated to alter the amount of light delivered to individual chloroplasts depending on incident light (Fig. 2B). Other terminology has been proposed where the terms ‘avoidance’ and ‘accumulation’ describes the opposing directions of movement away from- and towards light to either avoid high levels of photodamage or maximize photosynthesis (Kasahara et al., 2002). In any case, the advantages of being able to dynamically regulate absorptive properties are clear, and can serve to create a more equal light distribution among chloroplasts, and increase/decrease transmission of light (Wada et al., 2003). As examples, the light utilization was in some cases estimated to increase by 20% due to dynamic chloroplast movement (Brugnoli & Björkman, 1992) and faster induction of photodamage and leaf necrosis was shown in mutant-plants, defective in chloroplast movement (Kasahara et al., 2002).

**Photoadaptation**

In the previous section, only structural changes that can regulate the amount of light delivered to the photosynthetic units were described. However, as light finally intercepts the light absorbing pigments a range of adaptations have evolved to regulate the biochemical pathways ultimately leading to carbon assimilation. The photosynthetic apparatus is composed of a range of different components where the reaction centers, or photosystem I and II (PSI and PSII), constitutes the core of the photosynthetic reaction. Briefly, PSII is excited by absorbing a photon which creates a high-energy electron that is ultimately transferred via a range of electron acceptors to PSI. The now oxidized PSII is neutralized by extracting an electron from the oxygen evolving complex where water is split to $O_2$ and protons. This creates a charge separation across the thylakoid membrane that drives ATP synthesis that together with NADPH, created in PSI, provides the energy for the Calvin cycle where inorganic carbon is fixed and stored as simple sugars (Falkowski & Raven, 2007). The reaction centers are surrounded by light harvesting complexes (LHC) which are membrane bound pigment molecules that can transfer energy of absorbed photons to the reaction center Chl $a$ molecules. LHCs thus increase the cross section for photon absorption making sure that, in subsaturating light fluxes, the reaction center pigments receive as much energy as possible.
The LHCs can contain both pigments, compensatory (Fig. 3A) and dissipatory (Fig 3B), meaning that they can, depending on light history, either supplement the reaction center Chl a with energy or dissipate excess energy by vibrational energy dissipation and thus shading reaction centers from a harmful energy bombardment. Regulating the amount of light that goes to photochemical or non-photochemical reactions can occur via different routes. High light photosystems are generally adapted to having higher photosynthetic rates with lower absorptive properties. This can be achieved by having a lower content of LHCs and chlorophylls per chloroplast and having a higher content of sun-protective xanthophylls and carotenoids and more electron transport chains per total chlorophyll. Low light adapted photosystems, which are adapted to maximize absorption, display the opposite properties (Lichtenthaler & Babani, 2004; Lichtenthaler et al., 2007). In addition, the ultrastructure of chloroplasts change according
to light adaptation where shade adapted chloroplasts have higher numbers of thylakoids than light adapted chloroplasts (Lichtenthaler et al., 1981).

Aquatic photosynthesis

The light reactions, i.e. the absorption of photons, energy transfer and charge separation, is practically similar in all photosynthetic organisms (Falkowski & Raven, 2007). However, a major difference between terrestrial and aquatic photosynthetic organisms is the external environment they are surrounded by. As mentioned earlier, terrestrial systems can freely exchange gases with their environment and, while this also holds true for aquatic systems, diffusion of e.g. O₂ is ~10,000 times slower in water than in air (Cussler, 2009). This presents a major limitation in acquisition and release of essential gases and nutrients in aquatic systems. The term ‘diffusion’ is essential in this context since all submerged surfaces are surrounded by a diffusive boundary layer (DBL).

The diffusive boundary layer is a thin layer surrounding submerged surfaces where diffusion is the dominant transport form of dissolved materials (Jørgensen & Des Marais, 1990). It is created by the friction of moving water, which decreases the water velocity as the surface is approached. This creates a viscous sublayer at the solid-water interface where molecular diffusion becomes the dominant transport form relative to advection and eddy-diffusion (Jørgensen & Revsbech, 1985). This limits the mass transfer of essential gases, such as O₂ and CO₂, and nutrients between the water column and the photosynthesizing organism. The thickness of the DBL is dependent on flow velocity and the surface topography, where higher flow velocity decreases the DBL thickness by imposing a higher shear stress on the viscous water layers, while increasing topography will lead to an overall thicker DBL (Jørgensen & Revsbech, 1985). The rate of mass transfer (flux; J) across the DBL is dependent on the concentration gradient of the diffusing species (\(\partial C\)), the pathlength of diffusion (\(\partial z\)) and from the temperature and salinity dependent diffusion coefficient (\(D_0\)), as seen from Fick’s first law of diffusion:

\[
J = D_0 \frac{\partial C}{\partial z}
\]

In addition to these chemical gradients, aquatic ecosystems are characterized by gradients of light on the macro-meso scale, with exponential attenuation of light as function of water-depth (Kirk, 1994). Also the spectral composition changes as a function of depth, leaving a more blue/green light field in deeper waters due to the molecular absorption of water itself, which absorbs primarily in the red part of the visible spectrum (Stomp et al., 2007). This simple attenuation of light, however, is only characteristic for open-ocean oligotrophic systems where very little biomass is present. As turbidity
increases, either by dissolved matter or suspended particles (biotic and abiotic), dramatic changes in the spectral composition occur. This effect can be both temporally and spatially dynamic (Bricaud et al., 1981; Morel & Bricaud, 1981; Bricaud & Morel, 1986; Bricaud et al., 1998) and will change the depth of the photic zone (Stomp et al., 2007). Thus, the composition and depth of the water column can affect the light field incident on sessile aquatic macrophytes/corals/benthic phototrophic biofilms. However, in order to understand the microscale structure/function relationship between anatomical/physiological regulations and photosynthetic performance it is essential that the relevant parameters, such as light field and chemical environments, are approached on similar spatial scales.

Macro- vs. microscale canopy

Macroscale canopy effects in aquatic communities have previously been studied, e.g. in a range of excellent papers by Sand-Jensen and co-workers (Binzer & Sand-Jensen, 2002; Middelboe & Binzer, 2004; Binzer & Middelboe, 2005; Binzer et al., 2006; Sand-Jensen et al., 2007). The theory that the canopy structure affects community photosynthesis is well accepted and was supported by some of their main findings. As an example, due to the drag- and shearing forces in moving water, sessile aquatic macrophytes cannot achieve the same spatial organisation of photosynthetic elements as terrestrial systems which have been proposed to be a main reason for an observed lower community photosynthesis in aquatic systems (Sand-Jensen & Krause-Jensen, 1997). Binzer and Sand-Jensen (2002) found that community photosynthesis under high light markedly increased when thalli was vertically oriented indicating that the inability to evenly distribute photons limits high community production rates. The photosynthesis-irradiance relationship between individual phytoelements and the community is markedly different since light is diminished through the canopy; an effect that is strongly affected by the angle of individual phytoelements to the sun angle (Binzer et al., 2006).

Community photosynthesis is generally higher than photosynthesis of individual thallus fragments since the 3D structure of macrophyte assemblages display overall higher light use efficiencies, as photons which are not absorbed in the upper canopy will reach phytoelements with unused photosynthetic potential further below (Binzer et al., 2006). Thus, the spatial arrangement of phytoelements and the underlying optical properties, such as absorption and scattering dependent on phytoelement angle, seem tightly linked to the photosynthetic performance on a macroscale.

It was the aim of this thesis to investigate whether some of the same fundamental macroscale effects also apply on a microscale, i.e. to investigate if the microscale structural organization regulates photon absorption and photosynthetic efficiencies across different systems.
Methodology
In this PhD I have used a range of techniques to study the microenvironmental ecology of different aquatic photosynthetic systems. The most frequently used techniques will be briefly introduced below.

Microsensors
Microsensors were introduced in the 1970’s (e.g. Baumgärtl et al. (1974)), and later improved significantly by Revsbech and co-workers at Aarhus University (Revsbech et al., 1980; Revsbech & Jørgensen, 1983; Revsbech, 1989). By a new method, Clark-type O$_2$ microelectrodes were constructed implementing a guard-cathode (Fig. 4A) in addition to the measuring cathode, which removes internal O$_2$ and thus provides very stable signals; far preceding the performance of sensors developed at that time (Revsbech, 1989).

**Figure 4:** A) Schematical drawing of a O$_2$ microelectrode tip and the measuring principle (not drawn to scale; see text body for explanations; partly redrawn from Revsbech (1989)) B) A typical O$_2$ concentration profile from the bulk water towards an O$_2$ producing surface. The predominant modes of mass transport are shown in different color shadings where advection is dominating in the well stirred water, while molecular diffusion dominates inside the diffusive boundary layer. In the transient zone advection and diffusion equals each other. The diffusive flux (outflux = net photosynthesis; influx = dark respiration) can be calculated from Fick’s 1$^{st}$ law of diffusion (see text) from the concentration gradient on the linear slope in the DBL. The strictly linear part is termed the ‘true DBL’ while the zone indicated on the graph, i.e. where the slope intercepts the water O$_2$ concentration, is termed the ‘effective DBL’.
The application of these new microelectrodes made it possible to investigate microscale O₂ dynamics very precisely (and later other metabolic products such as H₂, H₂S, N₂O etc.) in e.g. sediments. Groundbreaking work has been published ever-since improving our knowledge on photosynthetic- and microbial metabolic processes (see e.g. (Gundersen et al., 1992; Kuypers et al., 2003; D’Hondt et al., 2004; Ettwig et al., 2010; Nielsen et al., 2010; Pfeffer et al., 2012).

The measuring principle is as follows (Fig. 4A): O₂ from the external environment diffuses through a silicone membrane and is reduced at the measuring cathode, which is a gold-plated platinum wire polarized to approximately -0.7V. The current generated by the reduction of O₂ flows through a KCl electrolyte to the positive anode, which is a chlorinated silver wire where Ag is oxidized and precipitates as AgCl. The generated current is directly proportional to the influx of O₂ which is dependent on the O₂ concentration of the external medium. A build-in guard cathode removes all residual O₂ from the electrolyte, thus giving better zero current and signal stability (Revsbech, 1989).

By the use of these O₂ microsensors, very precise (temporal and spatial) profiles of O₂ can be constructed to characterize metabolic processes in e.g. photosynthetic systems (Fig. 4B). By considering the flux calculated with Fick’s 1st law of diffusion (see above) it is possible to determine the steady state net photosynthesis rate (NPP) which is proportional to the out-flux of O₂ through the DBL. Similarly, the dark respiration rate can be determined from the in-flux of O₂ from the bulk water to the surface of the system. In addition, the high temporal resolution allowed the development of a method for measuring gross photosynthesis rates (GPP). This method, called the light/dark shift method was invented by Revsbech and Jørgensen (1983) and relies on measurements of the decreasing O₂ concentration after a short intermittent dark period. The assumption here is that during steady state at a given light level, a balance between photosynthesis and respiration is established. By applying a short dark period (usually 2-5 s.), respiration is assumed to be unchanged while photosynthesis is stopped. Thereby the decrease in O₂ concentration must be equal to the GPP just before the darkening.

A range of other numerical procedures to analyze O₂ concentration profiles exists, but will not be further introduced here (see e.g. Kühl et al. (1996); Berg et al. (1998); De Beer and Stoodley (2013)).

**Fiber-optic microprobes**

In studies of photosynthesis it is paramount to know the light field that the photosynthetic units perceive. The invention of fiber-optics, and later implementation of these tools in photobiology, made it possible to investigate the light environment within optically dense systems such as microbial mats (Kühl & Jørgensen, 1994), terrestrial leaves (Vogelmann & Björn, 1984), macroalgae (Lassen et al., 1994), corals
(Wangpraseurt et al., 2012) and it has been an important tool in e.g. biomedical applications such as photodynamic therapy (Star et al., 1988) and studies of embryogenesis (Li et al., 2014).

In principle, the light field can be calculated, but such calculations requires information about cell size distribution, scattering phase function (Privoznik et al., 1978; Berberoglu et al., 2009) and the inherent optical properties, such as scattering and absorption coefficients which are not easily derived, and the calculations are not for the faint-hearted (see e.g. Seyfried and Fukshansky (1983); Richter and Fukshansky (1996b); Richter and Fukshansky (1996a); Richter and Fukshansky (1998)). Alternatively, with the use of small optical fibers, the light-field can be directly measured using only some basic knowledge about light field parameters. Basically, three different geometries of light are relevant in photosynthesis studies (Fig. 5B): i) the field radiance, which is the light collected from a given area,
defined by the distance from the fiber-tip to the surface and from the acceptance angle of the fiber; ii) the downwelling irradiance, which is the light incident on a flat surface from all directions in a hemisphere and iii) the scalar irradiance, which is the light incident to a sphere from all direction.

The optical fiber itself can be made from a range of different materials and with different designs (Chapter 9). The fibers used during this PhD were all multimode, step-index optical fibers which guide light via internal reflection, created by a refractive index mismatch between the core and cladding material of the fiber. A field radiance microprobe can be used to map the light field isotropy (see e.g. Kühl and Jørgensen (1994)), i.e. the relationship between forward- and backscattered light. These probes can also be used to quantify e.g. reflection/backscattered light from photosynthetic surfaces when considering radiative balances (Chapter 2, 4 and 6). The most relevant parameter in photobiological studies is the scalar irradiance since photosynthetic cells perceive light from all directions. The scalar irradiance microprobes (Fig. 5A) used in this PhD were all based on the type invented by Lassen et al. (1992) and later modified by Rickelt et al. (2016) (Chapter 9). They consist of a single optical fiber which is tapered and flat-cut. An integrating sphere is cast onto the tip by first dip-coating a clear layer of polymethyl-methacrylate (PMMA) and subsequently a layer of titanium dioxide (TiO₂) mixed with PMMA (Fig. 5A). These microprobes (down to 30 µm sphere diameter) display good isotropic light collection properties and low attenuation of light in the visible-NIR spectrum and thus are ideal for use in photosynthetic studies.

Pulse-Amplitude-Modulation (PAM) Fluorometry

Chlorophyll a quenching analysis has now been an important measuring technique for decades. It has increased in popularity since its introduction in phytoplankton research (by the pump-and-probe technique (Mauzeral, 1972)) and in terrestrial photosynthesis (by the PAM technique (Schreiber, 1986)) since it is a fast and non-invasive technique that is directly related to the quantum yield of CO₂ assimilation (Genty et al., 1989). The basis for measurements of photosynthesis rates using chlorophyll fluorescence relies on the principle that the processes of PSII photochemistry, fluorescence and heat loss competes for excitation energy in the pigment antennae of PSII (Fig. 6A; Butler (1978)).

The measurements take place as follows (Fig. 6B): in dark-adapted state the electron acceptor plastoquinone (Qₐ) pool is fully oxidized, i.e. fully ready to receive electrons from PSII. This minimal fluorescence level is assessed with weak modulated measuring light. The measuring light is modulated in order to distinguish the fluorescence arising from the measuring light alone and not from other types of light reaching the photodetector. The variable fluorescence (Fᵥ), or the difference between the
fluorescence emitted with all reactions centers being either ‘closed’ or ‘open’, can be assessed with the saturation pulse method (Schreiber, 2004) where a brief (µs) pulse of strong light will fully reduce the Qa pool and yield a maximum fluorescence signal (Fm). The ratio Fv/Fm denotes the maximum quantum yield of PSII photochemistry, i.e. how efficiently absorbed photons are used to reduce Qa.

**Figure 6: PAM measurement principle**

A) The possible fates of energy dissipation from an excited reaction center chlorophyll. An electron can either be used for photochemistry where the electron is transferred to an electron acceptor, plastoquinone (Qa), or the energy can be lost from PSII as fluorescence or heat (redrawn from Baker (2008)). B) Plot showing the basic parameters used in PAM fluorometric calculations of photosynthetic parameters. Weak measuring light (black line) which is low enough to not induce any photochemistry gives rise to a baseline fluorescence (F0), i.e. dark-adapted state. As a saturating pulse is applied (yellow line) the max fluorescence of the system (Fm) is reached. The difference between these two parameters is denoted Fv. The ratio Fv/Fm is termed the max quantum efficiency of PSII. As actinic light is applied (blue line), the fluorescence rises to a higher level (F′) which after a saturating pulse is applied reaches the maximal fluorescence of light adapted state (Fm′). The difference between F′ and Fm′ is termed Fq′ and indicates the photochemical quenching and the ratio Fq′/Fm′ is termed the PSII operating efficiency (ϕPSII) (partly redrawn from Baker et al. (2008)).

If actinic light is applied (light adapted), a new level of fluorescence is reached (F′) denoting the partial closure of PSII reaction centers. When a saturating pulse is applied the fluorescence rises to the maximum fluorescence level (Fm′) in light adapted state. The difference between F′ and Fm′ (Fq′) is an indication of the photochemical quenching of fluorescence by open PSII reaction centers and the ratio Fq′/Fm′ (also denoted ϕPSII) estimates how efficiently absorbed photons are used for Qa reduction, i.e. an estimate of the quantum yield of the linear electron flux through PSII. The electron transport rate through PSII is dependent on the operating efficiency (ϕPSII), the quantum flux and the absorption factor and can be calculated as:

\[ ETR = \phi_{PSII} \times AF \times PAR \]
The absorption factor is determined by the amount of photons absorbed by the photosystems and by the balance between PSI and PSII photochemistry. Thus, practically it describes the amount of absorbed photons by PSII; however it is not a trivial parameter to estimate although it is possible (Szabó et al., 2014). An empirical value of 0.42 (assuming a 1:1 absorption by PSI and PSII) was suggested by Demmig and Björkman (1987) for terrestrial vascular plants but will be very different in many cases; probably depending on species and environmental conditions. Therefore, a more conservative calculation of the relative electron transport rate can be estimated as (Ralph et al., 2002):

\[ rETR = \phi_{PSII} \times PAR \]

Here, only the operating efficiency and the quantum flux are needed for calculations of the rETR.

A range of different PAM instruments are commercially available which, essentially can be categorized into fiber-optic or imaging type systems. The fiber-optic systems basically collects emitted fluorescence via an optical fiber which goes to a photodetector while the imaging systems collects emitted fluorescence on a CCD camera. Other than this, there is a multitude of different system variables including different fields-of-view, excitation sources and casings (underwater, leaf-clips, cuvettes etc.) making it possible to assess photosynthesis (even the relationship between PSI and PSII) for virtually any photosynthetic system. In this thesis I have mainly used a customized Microfiber PAM system which consists of a standard PAM control box, external actinic LED light sources (blue, red and white), and a photomultiplier with built-in measuring light LEDs (blue, green, yellow, red) for application of measuring light through the fiber. The Microfiber PAM system can give measuring light and saturation pulse from either the external light source or through the fiber. This gives the advantage that information on the photosystem performance can be gathered deep inside e.g. tissues. Such high resolution measurements are inherently prone to reflect heterogeneities in the organization of phototrophs, but also enables precise measurements in particular tissue layers. Non-invasive PAM measurements typically monitor a larger area and are limited in signal retrieval to the upper layers due to the steep light gradients often predominant in these systems. In addition, they have the analytical complication that signals detected by the fluorometer will be from an unknown volume with chlorophyll fluorescence from surface layers typically contributing more than layers further away. This relationship depends on the optical properties of the system investigated i.e. the ability of measuring light to penetrate tissue layers, and on the ability of fluorescence to escape the tissue and reach the detector.

In Chapter 3 and 5 a method for combining measurements of local operating efficiencies with local measurements of the scalar irradiance to very precisely estimate the gradients of light utilization in tissues
dominated by steep light gradients is proposed. Further, in Chapter 10 a method for combining profiles of light absorption with local operating efficiencies is developed to reveal gradients of photosynthetic efficiencies in densely pigmented systems.

**Thesis outline**

A central aim in this thesis was to investigate if some of the fundamental macroscale effects mentioned in the Introduction is also present on a microscale. In this pursuit I have explored the structure/function relationship across different phototrophic systems to elucidate regulations of photon absorption and photosynthetic efficiencies on a microscale. The thesis has been divided into three parts:

**Part 1 – Gradients of light utilization**

In this part containing 3 chapters, the central aim was to investigate light utilization and photosynthetic efficiencies in dense phototrophic systems and to explore whether the systems display stratification in their ability to capture and utilize light for photosynthesis.

*Chapter 2 “Radiative energy budget reveals high photosynthetic efficiency in symbiont-bearing corals”* present the first balanced light energy budget for a symbiont-bearing coral based on a fine-scale study of the microenvironmental photobiology of the massive coral *Montastrea curta*. By characterizing the incoming irradiance, light attenuation, heat dissipation and photosynthesis we could calculate local quantum efficiencies as a function of depth in the tissue.

*Chapter 3 “Photosynthetic acclimation of Symbiodinium in hospite depends on vertical position in the tissue of the scleractinian coral Montastrea curta”* explores the photophysiology of the oral and aboral symbiont band of scleractinian coral *Montastrea curta* to investigate if different acclimations to light exist in hospite on a polyp scale. Based on our findings we present a conceptual model on the photophysiology of *Symbiodinium* residing inside living coral tissue under natural gradients of light and chemical parameters.

*Chapter 4 “Radiative energy budgets of phototrophic surface-associated microbial communities and their photosynthetic efficiency under diffuse and collimated light”* investigates how the directionality of light influences photosynthetic efficiencies in a compact uniform cyanobacterial biofilm and a heterogeneous coral reef sediment by compiling closed radiative energy budgets accounting for the distribution of incident irradiance into photochemical conservation or heat dissipation.
Part 2 – Adaptations to life in gradients and physiological consequences of microscale canopies

In this part containing 4 chapters, adaptations to life in gradients are explored including structural- and physiological regulations to optimize photon capture and how this affects the chemical microenvironment. In addition to the changes in the microenvironment imposed by the organisms themselves, physical structures protruding from the surface of a system can change both the light- and chemical microenvironment. Such effects were additionally explored in this part.

Chapter 5: “Pronounced gradients of light, photosynthesis and $O_2$ consumption in the tissue of the brown alga *Fucus serratus*” presents a detailed study of the internal microenvironment of *Fucus serratus* and show that the thallus exhibits a highly stratified balance of production and consumption of $O_2$. High incident irradiance levels on the upper cortex did not saturate photosynthesis in the lower thallus and we discuss possible photoadaptive responses and consequences for optimizing photosynthetic activity.

Chapter 6: “Vertical migration optimizes photosynthetic efficiency of motile cyanobacteria in a coastal microbial mat” explores how radiative energy budgets change in a migrating cyanobacterial biofilm depending on light- or shade adaptation state. We find that by migrating, the light levels perceived by individual cells can change more than 25-fold. Thus, by movement the cyanobacteria can change their photophysiological status and optimize their photosynthetic efficiencies.

Chapter 7: “Epiphyte-cover on seagrass (Zostera marina L.) leaves impedes plant performance and radial $O_2$ loss from the below-ground tissue” investigates how the plant performance of the seagrass *Zostera marina* is affected by a leaf cover of epiphytes. We show how this affects both the light-quality and quantity and ultimately leads to a reduced plant fitness due to lower internal $O_2$ concentrations and thus a reduced ability to avoid sulphide intrusion through the roots.

Chapter 8: “Diffusion or advection? Mass transfer and complex boundary layer landscapes of the brown alga *Fucus vesiculosus*” gives a very detailed description of the diffusive boundary landscapes and $O_2$ fluxes around hyaline hairs on the thallus of the macroalgae *Fucus vesiculosus*. We find indications that diffusional $O_2$ uptake can be supplemented by advection and discuss the biophysical basis for this phenomenon. Further, we speculate that our findings could play a role in epibiotic niche differentiation and discuss how this would affect algal physiology.
Part 3 – Methodological developments

This part evolves around technological- and methodological advances to study microenvironmental gradients around a suite of different phototrophic systems, which I have been involved in. The most frustrating aspect of science, in my opinion, is the restriction in our ability to view and comprehend the microbial life and the processes associated with them. Therefore, methodological developments which improves our ability of “seeing” life is crucial and should follow naturally to all the unanswered questions arising during experiments.

Chapter 9: “Fiber-optic probes for small-scale measurements of scalar irradiance” develops improved scalar irradiance microprobes that are unprecedented small (down to <30 µm in sphere diameter) while still displaying good optical characteristics. Simultaneously, similar existing sensors are reviewed and applications of the new microprobes are demonstrated.

Chapter 10: “Multicolor light sheet microscopy-based imaging of absorption- and photosynthesis distribution in plant tissue” demonstrates a new method for quantifying how quantum yields of PSII photochemistry change under natural tissue light gradients compared to conventionally measured quantum yields. In addition, we present a method for using chlorophyll fluorescence profiles in combination with integrating sphere measurements of reflection and transmission to calculate depth-resolved photon absorption profiles, which can be used to calculate apparent PSII electron transport rates corrected for photons absorbed by PSII. This was achieved by using a combination of integrating spheres, fluorescence microscopy and an advanced super-continuum laser light source, and combining them with microscope variable chlorophyll fluorescence imaging under tissue light gradients.

Chapter 11: “Nanoparticle-based measurements of pH and O2 dynamics in the rhizosphere of Zostera marina L.: Effects of temperature elevation and light-dark transitions” investigates the pH and O2 heterogeneity around the roots of the seagrass Zostera marina using novel nanoparticle based optical O2 and pH sensors. The study showed that the seagrass can actively alter its rhizosphere pH microenvironment alleviating the local H2S toxicity and enhancing nutrient availability in the adjacent sediment via geochemical speciation shift.
References


Chazdon RL, Pearcy RW. 1991. The importance of sunflecks for forest understory plants - photosynthetic machinery appears adapted to brief, unpredictable periods of radiation. Bioscience 41: 760-766


Chapter 2

Radiative energy budget reveals high photosynthetic efficiency in symbiont-bearing corals

Published in *Journal of the Royal Society Interface*

by

Kasper Elgetti Brodersen, Mads Lichtenberg, Peter J. Ralph, Michael Kühl and Daniel Wangpraseurt
Radiative energy budget reveals high photosynthetic efficiency in symbiont-bearing corals

Kasper Elgetti Brodersen1,a, Mads Lichtenberg1,a, Peter J. Ralph2, Michael Kühl1,2 and Daniel Wangpraseurt2

1Marine Biological Section, University of Copenhagen, Denmark
2Plant Functional Biology and Climate Change Cluster, University of Technology Sydney, Australia

aThese authors contributed equally to this work.

Abstract

The light field on coral reefs varies in intensity and spectral composition, and is the key regulating factor for phototrophic reef organisms such as scleractinian corals harboring microalgal symbionts. However, the actual efficiency of light utilization in corals and the mechanisms affecting the radiative energy budget of corals are underexplored. We present the first balanced light energy budget for a symbiont-bearing coral based on a fine-scale study of the microenvironmental photobiology of the massive coral Montastrea curta. The majority (>96%) of the absorbed light energy was dissipated as heat, whereas the proportion of the absorbed light energy used in photosynthesis was ~4.0% under an irradiance of 640 µmol photons m⁻² s⁻¹. With increasing irradiance, the proportion of heat dissipation increased at the expense of photosynthesis. Despite such low energy efficiency, we found a high photosynthetic efficiency of the microalgal symbionts showing high gross photosynthesis rates and quantum efficiencies of ~0.1 O₂ photon⁻¹ approaching theoretical limits under moderate irradiance levels. Corals thus appear as highly efficient light collectors with optical properties enabling light distribution over the corallite/tissue microstructural canopy that enables a high photosynthetic quantum efficiency of their photosynthetic microalgae in hospite.
Introduction

Coral reefs are among the most productive and diverse ecosystems on Earth despite situated mainly in oligotrophic tropical waters. The evolutionary success of this important ecosystem is largely attributed to the successful symbiosis between the coral host (a cnidarian) and their photosynthetic microalgal endosymbionts (dinoflagellates in the genus *Symbiodinium*). These so-called zooxanthellae excrete photosynthates, which can provide up to 95% of the energy demand of their cnidarian hosts (Muscatine *et al.*, 1981; Edmunds & Davies, 1986). Photosynthesis is the process where solar energy is converted into chemical energy and stored as biomass in phototrophic organisms. It is driven by photons absorbed by pigment-protein complexes resulting in a charge separation at the two reaction centers of the photosystems (Kirk, 1994; Falkowski & Raven, 2007). At low irradiance, the photosynthesis is limited by the rate of energy supply to the photosystems, whereas at higher irradiances enzymatic reactions limit the rate of energy transformation and thus lead to increasing saturation of photosynthesis with irradiance (Falkowski & Raven, 2007). Excess absorbed light energy that is not used for photosynthesis, especially at high photon fluxes, is dissipated as heat and fluorescence. Dissipation of excess light energy as heat is in part due to various photo-regulatory mechanisms termed non-photochemical quenching (NPQ). NPQ is used by photosynthetic cells under high light to avoid photodamage such as degradation of pigments and enzymes by reactive oxygen species produced in de-excitation of the triplet state of Chl (*3Chl*) (Müller *et al.*, 2001; Falkowski & Raven, 2007).

Photosynthetic organisms employ various mechanisms to avoid photodamage, where heat dissipation through NPQ is just one effective short-term way to get rid of excess energy. Long-term regulation, and thereby protection, can be achieved by regulating the amount of light harvesting and carotenoid pigments (Bartley & Scolnik, 1995; Nymark *et al.*, 2009). Corals acclimatized to high irradiance often appear more transparent than those acclimatized to low-light conditions due to lower pigment concentrations per cell or spatial organization of chloroplasts ensuring a lower absorption cross section (Falkowski & Dubinsky, 1981). Another long-term regulatory mechanism in corals is to up-regulate the expression of protective coral host pigments, which absorb light in the blue to orange region of the PAR spectrum without inducing oxygenic photosynthesis (Salih *et al.*, 2000; D’Angelo *et al.*, 2008). Host pigment absorption and subsequent energy dissipation via reflection or fluorescence of photons may thus result in lower photosynthetic quantum efficiencies but host pigments may also ensure photoprotection in high irradiance environments (Dove *et al.*, 2008; Smith *et al.*, 2013) and might give rise to scattering phenomena and wavelength transformations that could enhance photosynthesis (Schlichter & Fricke, 1990; Dove *et al.*, 2008). However, some other members of the large family of
GFP-like host pigments fulfill presumably different as yet unresolved functions in reef corals (Alieva et al., 2008).

Absorbed solar radiation can also drive an increase in the surface temperature of corals relative to the ambient seawater (Jimenez et al., 2008; Jimenez et al., 2012b; Jimenez et al., 2012a) and such warming correlates linearly with incident irradiance (Jimenez et al., 2008). The increase in surface temperature is counter-balanced by convective heat transfer to the surrounding water, leading to the establishment of a thermal boundary layer (TBL; (Jimenez et al., 2008)). The presence of a TBL limits the rate of convective heat dissipation from the coral surface, as the TBL acts as an insulating barrier, the thickness of which is decreasing with the inverse power of the flow velocity (Jimenez et al., 2011). The TBL behaves analogous to the diffusive boundary layer (DBL), which at low flow velocity impedes mass transfer and thereby affects coral gas exchange and nutrient uptake (Atkinson & Bilger, 1992; Kühl et al., 1995; Wangpraseurt et al., 2012b) as well as the rate of photosynthesis and respiration (Edmunds, 2005; Finelli et al., 2006; Jimenez et al., 2008).

Corals in shallow reef habitats are exposed to high downwelling irradiance of >2000 µmol photons m\(^{-2}\) s\(^{-1}\), especially during mid-day low tide periods (Jimenez et al., 2012b). The variability of light in the reef environment is not only underlying diurnal dynamics modulated by tides but corals also experience pulses of high intensity light exposure due to wave focusing producing short duration flashes of >9000 µmol photons m\(^{-2}\) s\(^{-1}\), with up to >350 light flashes min\(^{-1}\) in shallow waters (Veal et al., 2010). Excess absorbed light energy can lead to photoinhibition and damage the photosystems as well as coral light absorption can increase the temperature in the coral microenvironment, potentially aggravating negative responses to elevated seawater temperatures (Jimenez et al., 2008; Jimenez et al., 2011). High irradiance in combination with elevated water and coral tissue temperatures can induce a cascade of stress responses in corals ultimately leading to the breakdown of the algal-cnidarian symbiosis (due to excretion of symbionts and/or pigment degradation of the algal symbiont), which is termed coral bleaching (Lesser, 1996; Jones et al., 1998; Warner et al., 1999; Weis, 2008). Moreover, nutrient starvation reduces the photosynthetic efficiency of zooxanthellae and renders corals more susceptible to bleaching (Wiedenmann et al., 2013). This demonstrates the importance of understanding the mechanisms regulating radiative energy dissipation and the fate of absorbed light energy within corals.

It has been shown that corals are highly efficient at collecting (Enriquez et al., 2005; Stambler & Dubinsky, 2005) and using solar radiation (Rodriguez-Roman et al., 2006; Pinchasov-Grinblat et al., 2013). It is known that >90% of incident photosynthetic active radiation (PAR, 400-700 nm) can be absorbed by corals and calculations have suggested that their quantum efficiencies are close to theoretical limits (i.e.,
0.125 O$_2$ photon$^{-1}$ as 8 photons are needed to separate the electrons required to produce one O$_2$ molecule (Rodriguez-Roman et al., 2006; Falkowski & Raven, 2007; Hochberg & Atkinson, 2008). There is also mounting evidence that corals have unique optical properties that could relate such high efficiency to efficient light capture (Enriquez et al., 2005; Wangpraseurt et al., 2012a; Wangpraseurt et al., 2012b; Wangpraseurt et al., 2014). However, a closed radiative energy budget for corals and direct microscale measurements of the photosynthetic quantum efficiency of zooxanthellae in hospite are lacking.

In this study, we apply an experimental approach developed for photosynthetic biofilms (Al-Najjar et al., 2010) to determine the first balanced light energy budget of a coral as a function of incident irradiance and flow velocity. This was obtained by combining fibre-optic and electrochemical microsensor measurements of light reflectance and absorption, rates of gross photosynthesis (GPP), and coral tissue surface warming. Such detailed measurements of the main energy dissipating mechanisms in coral tissue allowed us to estimate the proportion of the absorbed light energy used by photosynthesis and dissipated as heat, respectively. Furthermore such measurements provided the first measurements of the local quantum efficiency of zooxanthellae photosynthesis at depth within coral tissue.

Materials and Methods

Coral samples

Coral specimens were collected from shallow waters (<3 m depths) on the reef flat of the Heron Island lagoon, Great Barrier Reef, Australia (152°06′E, 20°29′S). The coral Montastrea curta was chosen as highly suitable for intra-tissue microsensor measurements due to its thick tissue and minimal mucus secretion. Specimen were transported to the coral holding facility at the University of Technology, Sydney, where corals were acclimated and maintained under continuous flow at 25°C, salinity of 33 and moderate levels of downwelling irradiance (150-200 µmol photons m$^{-2}$ s$^{-1}$; 400-700 nm; 12/12h light-dark cycle).

Experimental setup

Small fragments of M. curta were placed in a custom-made black acrylic flow-through chamber for at least 45 min prior to the microsensor measurements to ensure steady state O$_2$ and temperature conditions (as confirmed from repeated microprofile measurements). Corals were illuminated with a defined irradiance regime and were continuously flushed with aerated seawater (25°C and a salinity of
at an average flow velocity of either ~0.4 or ~0.8 cm s\(^{-1}\) as maintained by a submerged water pump in a 20L thermostated aquarium reservoir. Illumination was provided by a fibre-optic tungsten halogen lamp (KL-2500, Schott GmbH, Germany) equipped with an internal heat filter and a collimating lens positioned vertically above the flow-through chamber (Fig. 1). The light intensity of the lamp could be regulated without spectral distortion by a built-in filter wheel with pinholes of various sizes. The downwelling quantum irradiance in the PAR range (400-700 nm) \((E_d\text{ in } \mu\text{mol photons m}^{-2}\text{ s}^{-1})\) was measured with a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a planar cosine collector (LI-192S, LiCor, USA).

Figure 1. Experimental setup. (a) Schematic drawing of the experimental setup visualizing the relative position of light source, microsensors and coral fragment. (b) A scalar irradiance microsensor inserted into the coenosarc tissue of a *Montastrea curta* coral. (c) Conceptual diagram showing the fate of light energy (abbreviations explained in text).
The experimental irradiances (160, 320, 640, 1280 and 2400 µmol photons m\(^{-2}\) s\(^{-1}\)) were achieved by adjusting the aperture on the fibre-optic halogen lamp without any spectral distortion. The downwelling spectral irradiance at the above-mentioned quantum irradiance levels was also measured in radiometric energy units (in W m\(^{-2}\) nm\(^{-1}\)) with a calibrated spectroradiometer (Jaz A0523, Ocean Optics, Dunedin, Florida, USA). The coral fragment was positioned in the center of the light beam. The complete set-up was covered with black cloth to avoid stray light.

**Microsensor measurements**

Spectral scalar irradiance, \(E_0(\lambda)\), was measured with a fibre-optic scalar irradiance microprobe (integrating sphere diameter ~100 µm; (Lassen et al., 1992b; Lassen et al., 1992a) connected to a fibre-optic spectrometer (USB 2000+, Ocean Optics, USA). We used a black non-reflective light well to measure the incident downwelling spectral irradiance, \(E_d(\lambda)\) at the same distance from the light source as done with the measurements on the coral surface; in a collimated light beam the downwelling scalar irradiance and the downwelling irradiance are identical (Kühl & Jørgensen, 1994). The spectral reflectance was measured with a fibre-optic field radiance microprobe (Jørgensen & Des Marais, 1988; Kühl, 2005).

Oxygen concentrations were measured with Clark-type microelectrodes (tip diameter ~25 µm, OX-25, Unisense AS, Aarhus, Denmark) with a fast response time (<0.5 s) and a low stirring sensitivity (<1-2%) (Revsbech, 1989a; Revsbech, 1989b). The microsensor was connected to a pA-meter (Unisense A/S, Aarhus, Denmark) and was linearly calibrated, at experimental temperature and salinity, from measurements in aerated (free-flowing part of the flow chamber) and anoxic seawater (flushed with N\(_2\)).

Temperature measurements were performed with a thermocouple microsensor (tip diameter ~50 µm; T50, Unisense A/S, Aarhus, Denmark) connected to a thermocouple meter (Unisense A/S, Aarhus, Denmark). The temperature microsensors were linearly calibrated against a high precision thermometer (Testo 110, Testo AG, Germany; accuracy ±0.2°C) in seawater at different temperatures. Both temperature and O\(_2\) microsensors were connected to an A/D converter (DCR-16, Pyroscience GmbH, Germany) interfaced with a PC running data acquisition software (ProFix, Pyroscience GmbH, Germany).

Microsensor measurements of spectral scalar irradiance, spectral radiance, O\(_2\) and temperature were done with the sensors approaching the coral surface at a 45° angle relative to the vertically incident light.
beam to avoid self-shading. The microsensors were mounted on a PC-interfaced motorized micromanipulator (MU-1, PyroScience, GmbH, Germany) controlled by dedicated data acquisition and positioning software (ProFix, PyroScience GmbH, Germany); the software automatically corrected for the sensor inclination and all depths are given in vertical distances.

The microsensor was positioned at the coral tissue surface (defined as 0 µm depth) by means of the micromanipulator and observed using a stereo-microscope (7x – 90x, AmScope, Irvine, CA, USA). Microsensor measurements of temperature, O2, and scalar irradiance on and within coral coenosarc tissue were performed as described previously (Jimenez et al., 2008; Wangpraseurt et al., 2012a) (see Fig. 1). All profiles were measured in vertical steps of 100 µm. Within the tissue, microprofiles were performed from the tissue surface until the skeleton was reached, which could be observed by a slight bending of the microsensors and enhanced noise in the O2 signal.

### Table 1. Terms, definitions and units. PAR denotes photosynthetically active radiation (400-700 nm).

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_d(\lambda)</td>
<td>Downwelling spectral scalar irradiance</td>
<td>(µmol photons m(^{-2}) s(^{-1}) nm(^{-1}))</td>
</tr>
<tr>
<td>E_0(\lambda)</td>
<td>Spectral scalar irradiance at depth, z</td>
<td>(µmol photons m(^{-2}) s(^{-1}) nm(^{-1}))</td>
</tr>
<tr>
<td>E_E(\lambda)</td>
<td>Absolute downwelling irradiance</td>
<td>(W m(^{-2}) nm(^{-1}))</td>
</tr>
<tr>
<td>E_0(PAR)</td>
<td>Photon scalar irradiance (PAR)</td>
<td>(µmol photons m(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>J_ABS</td>
<td>Absorbed light energy, vector irradiance (PAR)</td>
<td>(J m(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>E_ABS(z)</td>
<td>Local density of absorbed light</td>
<td>(µmol photons m(^{-3}) s(^{-1}))</td>
</tr>
<tr>
<td>R(PAR)</td>
<td>PAR irradiance reflectance</td>
<td>(W m(^{-2}) nm(^{-1}))</td>
</tr>
<tr>
<td>K_0(PAR)</td>
<td>Diffuse attenuation coefficient (PAR)</td>
<td>(mm(^{-1}))</td>
</tr>
<tr>
<td>A(PAR)</td>
<td>Absorption coefficient (PAR)</td>
<td>(mm(^{-1}))</td>
</tr>
<tr>
<td>K_ABS(\lambda)</td>
<td>Spectral attenuation coefficient</td>
<td>(mm(^{-1}))</td>
</tr>
<tr>
<td>GPP_A</td>
<td>Areal rates of GPP</td>
<td>(nmol O(_2) cm(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>PS_A</td>
<td>Areal rates of GPP, in energy units</td>
<td>(J m(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>P(_{max})</td>
<td>Photochemical energy conservation</td>
<td>(J m(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>PS(z)</td>
<td>Volumetric rate of GPP at depth z</td>
<td>(nmol O(_2) cm(^{-3}) s(^{-1}))</td>
</tr>
<tr>
<td>PS(_{max})</td>
<td>Local photosynthesis maximum</td>
<td>(nmol O(_2) cm(^{-3}) s(^{-1}))</td>
</tr>
<tr>
<td>J_H</td>
<td>Energy dissipation as heat</td>
<td>(J m(^{-2}) s(^{-1}))</td>
</tr>
<tr>
<td>η(z)</td>
<td>Local photosynthetic quantum efficiency</td>
<td>(O(_2) photon(^{-1}))</td>
</tr>
</tbody>
</table>

### Irradiance calculations

The spectral scalar irradiance, E\_0(\lambda), was measured in vertical depth steps throughout the coral tissue and calculated as the fraction of the incident downwelling irradiance, E\_d(\lambda)/E\_d(\lambda). By multiplying the normalized scalar irradiance spectra with the measured spectra of absolute downwelling irradiance at
the coral tissue surface (in W m\(^{-2}\) nm\(^{-1}\)) as measured by a calibrated spectrometer (Jaz, Ocean optics, USA), we obtained the absolute energy levels of scalar irradiance at the different measuring depths. We converted the absolute scalar irradiance spectra to photon scalar irradiance spectra (in µmol photons m\(^{-2}\) s\(^{-1}\) nm\(^{-1}\)) by using Planck’s equation:

\[ E_\lambda = h \cdot \frac{c}{\lambda} \]

where \( E_\lambda \) is the energy of a photon, \( \lambda \) is the wavelength, \( h \) is Planck’s constant (6.626 \times 10^{-34} \text{ W s}^2) and \( c \) is the speed of light in vacuum (in m s\(^{-1}\)). The light attenuation in the tissue was calculated by integrating the spectral photon irradiance over PAR (420-700nm) yielding a measure of PAR photon scalar irradiance (in µmol photons m\(^{-2}\) s\(^{-1}\)), i.e., light energy available for oxygenic photosynthesis at a given tissue depth. Light <420 nm was strongly absorbed in the upper tissue and light measurements in this wavelength range exhibited increasing amounts of straylight from within the spectrometer and were therefore not included.

The PAR irradiance reflectance of the coral tissue surface was calculated as

\[ R(PAR) = \frac{\int_{420}^{700} E_u(\lambda) \lambda d\lambda}{\int_{420}^{700} E_d(\lambda) \lambda d\lambda} \]

where \( E_u(\lambda) \) is the upwelling irradiance at the coral tissue surface, here estimated as the backscattered spectral radiance measured at the coral tissue surface (Kühl, 2005) and \( E_d(\lambda) \) is the downwelling irradiance estimated as the backscattered spectral radiance measured over a white reflectance standard (Spectralon; Labsphere, North Sutton, NH, USA). R(PAR) measurements rely on the assumption that the backscattered light from the tissue surface was entirely diffused (Kühl & Jørgensen, 1994; Enriquez et al., 2005; Wangpraseurt et al., 2012a).

The absorbed light energy (\( J_{ABS} \); in J m\(^{-2}\) s\(^{-1}\)) within the coral tissue and thus available for photosynthesis, was calculated by subtracting the downwelling and upwelling irradiance at the tissue surface, as calculated by:

\[ J_{ABS}(PAR) = \int_{420}^{700} E_d(\lambda)(1 - R(\lambda)) \lambda d\lambda \]

where \( E_d(\lambda) \) and \( R(\lambda) \) are the downwelling spectral irradiance and irradiance reflectance, respectively.

The parameter \( J_{ABS} \) is equivalent to the so-called vector irradiance, which is a measure of the net downwelling energy flux (Kühl & Jørgensen, 1994).

39
Temperature and O$_2$ calculations

Gross photosynthesis (GPP) was measured with O$_2$ microsensors using the light-dark shift method, which allows photosynthesis estimates independent of light respiration (Revsbech & Jørgensen, 1983). Areal rates of GPP ($J_{GPP}$ in nmol O$_2$ cm$^{-2}$ s$^{-1}$) were calculated by depth integration of the volumetric rates (in nmol O$_2$ cm$^{-3}$ s$^{-1}$) measured in different depths over the euphotic zone, i.e., throughout the photosynthetic coral tissue.

The total amount of energy used by photosynthesis in the coral tissue ($J_{PS}$ in J m$^{-2}$ s$^{-1}$) was calculated by multiplying the areal gross photosynthesis rate with the Gibbs free energy (482.9 kJ (mol O$_2$)$^{-1}$), i.e., the energy released through O$_2$ and ATP formation (Thauer et al., 1977; Al-Najjar et al., 2010):

\[ J_{PS} = J_{GPP} E_G \]

Light energy that was not used in photosynthesis, resulted in a local increase of the coral tissue surface temperature leading to the establishment of a TBL (Jimenez et al., 2008). The heat dissipation ($J_{H,up}$ in J m$^{-2}$ s$^{-1}$), i.e., the heat flux from the coral tissue into the water column, was estimated from the temperature flux across the TBL and was calculated by Fourier’s law of conduction:

\[ J_{H,up} = k \frac{\partial T}{\partial z} \]

where $k$ is the thermal conductivity in seawater (0.6 W m$^{-1}$ K$^{-1}$; (Young et al., 1996)) and $\frac{\partial T}{\partial z}$ is the measured linear temperature gradient in the TBL.

As microsensor measurements of heat conduction into coral skeleton are very challenging due to high risk of sensor breakage, the downward flux of heat into the coral skeleton was calculated as $J_{H,down} = J_{ABS} - (J_{H,up} + J_{PS})$. The total amount of energy dissipated as heat in the coral tissue ($J_H$ in J m$^{-2}$ s$^{-1}$) was then calculated as $J_H = J_{H,up} + J_{H,down}$.

Light Energy budget and photosynthetic efficiency calculations.

To estimate the overall radiative energy utilization efficiency of the system, the balanced light energy budget was determined by the following equations:

\[ J_{IN} = J_H + J_{PS} + R \text{ and } J_{IN} - R = J_{ABS} = J_{PS} + J_H \]

where $J_{IN}$ is the total incoming light energy flux; $J_H$ is the amount of the incoming light energy dissipated as heat; $J_{PS}$ is the amount of the incoming light energy used by photosynthesis; $R$ is the amount of incident light energy backscattered from the coral tissue surface and thus lost from the system; and $J_{ABS}$
is the amount of the incoming light energy absorbed by the system. The final balanced light energy budget characterized the tissue as a homogenous layer, ignoring any vertical microheterogeneity in symbiont distribution and was defined as

$$J_{ABS} = J_{PS} + J_H$$

assuming a 1:1 stoichiometry of CO₂ fixation and O₂ production, i.e., the energy stored in the light-dependent reaction is completely used for CO₂ fixation in the dark reaction, and that autofluorescence from the tissue is negligible (Al-Najjar et al., 2010).

$\varepsilon_{PS}$ and $\varepsilon_H$ represent the efficiencies of photosynthetic energy conservation and heat dissipation at a given absorbed light energy ($J_{ABS}$), respectively, and were calculated as (Al-Najjar et al., 2010):

$$\varepsilon_{PS} = \frac{J_{PS}(J_{abs})}{J_{abs}} \quad \text{and} \quad \varepsilon_H = \frac{J_H(J_{abs})}{J_{abs}}$$

The photon scalar irradiance of PAR within the coral tissue $E_0(PAR)$, was fitted to an exponential decay function to estimate the diffuse attenuation coefficient of $E_0(PAR)$, $K_0(PAR)$ (in units of mm⁻¹):

$$E_0(PAR, z) = E_0(PAR, z_0)e^{(-K_0(PAR)z)}$$

The absorption coefficient of PAR within the coral tissue was estimated from the coral tissue irradiance reflectance ($R$) and the scalar irradiance attenuation coefficient, $K_0(PAR)$ as (Al-Najjar et al., 2010):

$$A(PAR) = K_0 \frac{1 - R}{1 + R}$$

assuming that photons at each depth have equal probability to propagate in all directions, i.e., a totally diffuse light field within the tissue.

The spectral attenuation coefficient ($K_0(\lambda)$) of scalar irradiance with depth ($z$), was calculated as:

$$K_0(\lambda) = -\frac{\ln(E_0(\lambda)_1)}{z_2 - z_1}$$

where $E_0(\lambda)_1$ and $E_0(\lambda)_2$ are the spectral scalar irradiance measured at depths $z_1$ and $z_2$, respectively (Kühl & Jørgensen, 1994). We used these data to identify depths within the coral tissue with the strongest light attenuation and spectral signatures of photopigments.

The local density of absorbed light $E_{ABS}(z)$ (in µmol photons m⁻³ s⁻¹) at particular depths in the corals tissue was calculated as:
assuming a totally diffuse light field inside the tissue, i.e., photons at the given depths had equal probabilities of propagation in all directions (Al-Najjar et al., 2010).

Finally, the local photosynthetic quantum efficiency, $\eta(z)$ (O\textsubscript{2} photon\textsuperscript{-1}) was calculated by dividing the locally measured volumetric gross photosynthesis rates with $E_{ABS}(z)$:

$$\eta(z) = \frac{PS(z)}{E_{ABS}(z)}$$
Results

Spectral light microenvironment

In the uppermost coral tissue layers (~0-0.3 mm) there was a local enhancement in scalar irradiance relative to the incident irradiance with maximum values at the coral surface reaching 135% and 191% of the incident downwelling irradiance at 640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\), respectively (Fig. 2a,b).

![Figure 2. Photon scalar irradiance and spectral attenuation in coral tissue. (a, b) photon scalar irradiance spectra measured at 2 different downwelling photon irradiances (640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\)). The dashed line is the incident downwelling spectral irradiance. (c, d) Spectral attenuation coefficient of scalar irradiance \(K_0(\lambda)\) (mm\(^{-1}\)) measured at 2 different downwelling photon irradiances (640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\)). Legends show the measurement depth below the tissue surface (0 mm = coral tissue surface). (Spectra represent mean values; \(n=3\)).

Scalar irradiance attenuation spectra showed that the highest light attenuation occurred at the tissue-skeleton interface, especially around the absorption maxima of the predominant photopigments Chl \(a\) (440 & 675 nm), Chl \(c\) (635 nm) and the dinoflagellate carotenoid peridinin (490 nm) (Fig. 2c,d).
Below 0.1 mm depth, $E_0$(PAR) was attenuated exponentially with depth in the coral tissue (Fig. 3) with attenuation coefficients of 0.79 mm$^{-1}$ and 1.18 mm$^{-1}$ at an incident irradiance of 640 and 1280 µmol photons m$^{-2}$ s$^{-1}$, respectively. This corresponds to a decrease from 135% to 90% and 191% to 91% of the incident irradiance over a tissue thickness of 0.4 mm and 0.5 mm, respectively. The absorption coefficient, $A$(PAR) in the coral tissue was calculated to be 0.628 mm$^{-1}$ and 0.949 mm$^{-1}$ under a downwelling irradiance of 640 and 1280 µmol photons m$^{-2}$ s$^{-1}$, respectively.

The coral tissue irradiance reflectance, $R$(PAR), was ~12% and 11% at 640 and 1280 µmol photons m$^{-2}$ s$^{-1}$, respectively.

Reflectance levels were constant over an irradiance range of 160-2400 µmol photons m$^{-2}$ s$^{-1}$ and no significant correlation between reflection and incident irradiance was found ($p > 0.05$, Fig. S1).

**Temperature microenvironment:**

A slight surface heating of the coral tissue relative to the ambient seawater was observed, which increased with irradiance. A thermal boundary layer (TBL) could only be identified at irradiance levels >320 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 4a), reaching a thickness of ~3 mm at a flow velocity of 0.4 cm s$^{-1}$.
There was a positive linear correlation between the coral surface warming, $\Delta T$, and the incident irradiance with a heating slope of 0.0023 °C (J m$^{-2}$ s$^{-1}$)$^{-1}$, and increasing temperature gradients of 0.24-0.98°C between coral tissue and the ambient water under increasing irradiance ($R^2=0.98$; Fig. 4b).

**O$_2$ microenvironment and photosynthesis:**

Local volumetric rates of gross photosynthesis ranged between 7-25 nmol O$_2$ cm$^{-3}$ s$^{-1}$ and O$_2$ production was detected at all vertical measurement positions within the 0.5-0.7 mm thick coenosarc tissue (Fig. 5).
Generally, gross photosynthesis peaked ~0.1–0.3 mm below the tissue surface, however the vertical distribution of photosynthesis differed under the different experimental irradiance regimes. The O$_2$ microenvironment within the tissue ranged between 500 and 900 µM (240–430% air saturation) and showed in most cases an increasing trend towards the tissue-skeleton interface (Fig. 5).

Energy budget:

Based on detailed measurements of light, photosynthesis and temperature, we calculated a balanced light energy budget in % of the incident light energy for incident downwelling photon irradiances of 640 and 1280 µmol photons m$^{-2}$ s$^{-1}$ (equivalent to a vector irradiance of 116 and 234 J m$^{-2}$ s$^{-1}$, respectively) (Fig. 6).
About 3.5% and 2.2% of the incident irradiance was conserved by photosynthesis, while 84.9% and 86.9% was dissipated as heat under an incident photon irradiance of 640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\), respectively (flow velocity of ~0.4 cm s\(^{-1}\)). The remaining 11.6% and 10.9% of the incident light energy was backscattered by the tissue surface and thus not absorbed.

At increased flow velocity (~0.8 cm s\(^{-1}\)), the proportion of the incident light energy that was photochemically conserved decreased to 2.6% and 1.0% under an incident photon irradiance of 640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\), respectively. Generally, the energy budget was dominated by heat dissipation and the proportion of energy conserved by photosynthesis decreased with increasing incident irradiance favoring dissipation of heat (Fig. 6, Table 2). The maximum efficiency of photochemical energy conservation, \(\varepsilon_{PS,max}\), and the minimum efficiency of heat dissipation, \(\varepsilon_{H,min}\), were 0.04 and 0.96, respectively (Table 2).
Based on detailed light and photosynthesis measurements, we estimated the quantum efficiency of photosynthesis in particular depths in the coral tissue (Fig. 7). The local quantum efficiencies (QE) varied over depth, with an increasing trend towards the tissue-skeleton interface and showing decreasing QE values at increasing incident irradiances. The maximum photosynthetic QE was 0.102 O₂ photon⁻¹ (320 µmol photons m⁻² s⁻¹), approaching the theoretical maximum of 0.125 O₂ photon⁻¹.

Discussion

This fine-scale study of the energy budget and photosynthetic efficiency of the symbiont-bearing scleractinian coral Montastrea curta presents the first detailed account of the fate of incident and absorbed light within coral tissues. Despite the finding that a relative high proportion of the incident irradiance (~11%) was backscattered at the tissue surface and thus not absorbed (Fig. 6, S1) we found that corals are highly efficient at using solar radiation (Fig. 7).

Within the coral tissue, the absorbed light differed from the incident irradiance with respect to both spectral composition and intensity, where scalar irradiance was enhanced (135% and 191% for incident irradiances of 640 µmol photons m⁻² s⁻¹ and 1280 µmol photons m⁻² s⁻¹, respectively) at and just below the coral tissue surface (Fig. 2a, b). Such enhancement suggests intense scattering and redistribution of photons in the upper layers of the tissue (Wangpraseurt et al., 2012a). Tissue scattering increases the local density and residence time of photons due to increased photon pathlength per vertical distance traversed (Kühl & Jørgensen, 1994). These findings are similar to recent studies by Wangpraseurt et al. (Wangpraseurt et al., 2012a; Wangpraseurt et al., 2014) observing scalar irradiance levels reaching up to 200% of the incident downwelling irradiance in the upper coral tissue layers (0-100 µm).
We observed the highest attenuation of scalar irradiance in the lowest tissue depth interval, i.e., at the tissue-skeleton interface (Fig. 2c, d), where strong backscatter would further enhance light absorption efficiency by symbionts in the coral tissue (Enriquez et al., 2005). We found a pronounced positive correlation between increasing incident irradiance and dissipation of heat, leading to the establishment of a ~3 mm thick thermal boundary layer (TBL) at incident irradiances >320 µmol photons m⁻² s⁻¹ (Fig. 4a). We also measured a linear increase in tissue surface temperature, resulting in convective heat dissipation over a TBL, with an average slope of 2.3·10⁻³ °C (J m⁻² s⁻¹)⁻¹ and a maximum temperature difference of +0.98°C (Fig. 4b). Hemispherical corals studied by Jimenez et al (Jimenez et al., 2008; Jimenez et al., 2011) showed a similar maximum surface warming, ∆T, between the surface tissue and the ambient water of +0.9°C, with a TBL thickness of ~3 mm and an average slope of ~1.7·10⁻³ °C (J m⁻² s⁻¹)⁻¹ at equivalent flow-velocities and absorbed irradiances.

The rate of photosynthesis within the illuminated coenosarc coral tissue showed a relatively uniform distribution of photosynthesis throughout the tissue and O₂ concentrations ranged from 500 µM - 900 µM (240-430% air saturation; Fig. 5). This correlated with the attenuation of PAR observed within the coenosarc tissue (Fig. 2 & 3), where ~90% of the incident irradiance remained at the tissue-skeleton interface.

The coral skeleton acts as a diffusion barrier for chemical species, leading to a relative build-up of solutes (such as O₂) in the lower tissue layers (Fig. 5). This has recently been shown to increase the O₂ concentration up to ~400% of air saturation at the tissue-skeleton interface (Wangpraseurt et al., 2012a). Saturation of photosynthetic activity at high irradiance led to decreased photosynthetic efficiencies, i.e., a decreased proportion of the absorbed light energy was conserved by photosynthesis with increasing incident irradiances (Fig. 6, Table 2). At increased flow velocity (~0.8 cm s⁻¹), the

<table>
<thead>
<tr>
<th>J₂abs</th>
<th>J₂ps</th>
<th>J₂h</th>
<th>ε₂ps</th>
<th>ε₂h</th>
<th>ε₂ps, ε₂h</th>
</tr>
</thead>
<tbody>
<tr>
<td>(J m⁻² s⁻¹)</td>
<td>(J m⁻² s⁻¹)</td>
<td>(J m⁻² s⁻¹)</td>
<td>0.4 cm s⁻¹</td>
<td>0.8 cm s⁻¹</td>
<td>0.4 cm s⁻¹</td>
</tr>
<tr>
<td>116</td>
<td>4.55</td>
<td>3.39</td>
<td>111.0</td>
<td>112.2</td>
<td>0.04</td>
</tr>
<tr>
<td>(3.9%)</td>
<td>(2.9%)</td>
<td>(96.1%)</td>
<td>(97.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>234</td>
<td>5.77</td>
<td>2.71</td>
<td>228.1</td>
<td>231.2</td>
<td>0.02</td>
</tr>
<tr>
<td>(2.5%)</td>
<td>(1.2%)</td>
<td>(97.5%)</td>
<td>(98.8%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Balanced light energy budget (in energy units), overall photosynthetic energy use and heat dissipation (in % of absorbed light energy), and efficiencies of photochemical energy conservation and heat dissipation (ε₂ps and ε₂h) at two different incident irradiances and flow velocities.
proportion of the absorbed light energy conserved by photosynthesis decreased as compared to the lower flow velocity (~0.4 cm s⁻¹; Fig. 6). This was unexpected as a decrease in boundary layers thickness due to increased flow rates, normally leads to increased rates of photosynthesis and respiration as a response to alleviation of mass transfer resistance for O₂ and DIC exchange (Kühl et al., 1996; Larkum et al., 2003). However, we also saw a decreased euphotic zone (i.e., the photosynthetic tissue layer) from ~0.6 mm to 0.4 mm, possibly due to coenosarc tissue contraction at higher flow. Recently, tissue contraction has been found to reduce the amount of lateral light transfer through coral tissue and could thus explain the observed reduction in local gross photosynthesis rates (Wangpraseurt et al., 2014).

The volumetric photosynthesis rates at higher flow were in the same order as at the lower flow velocity, i.e., $P_{\text{max}}$ of 24.2 nmol O₂ cm⁻³ s⁻¹ (data not shown). At a flow velocity of ~0.8 cm s⁻¹, the proportion of the absorbed light energy that was used in photosynthesis still represents a high energy efficiency (Fig. 6, Table 2) as compared to other photosynthetic systems such as biofilms and microbial mats, where <1.8 % of the absorbed light energy was conserved by photosynthesis at equivalent vector irradiances (Al-Najjar et al., 2010).

In comparison with previous studies of the radiative energy budget of benthic photosynthetic systems, such as biofilms and microbial mats (Al-Najjar et al., 2010; Al-Najjar et al., 2012), we found much higher photosynthetic efficiencies in coral tissue. Locally measured volumetric rates of photosynthesis in the coral tissue were very high (up to 25 nmol O₂ cm⁻³ s⁻¹; Fig. 5) as compared to what has been reported in microbial mats and biofilms (Australian mat 6-13 nmol O₂ cm⁻³ s⁻¹, Abu Dhabi mat 2-6 nmol O₂ cm⁻³ s⁻¹; Al-Najjar et al., 2010; Al-Najjar et al., 2012)) at equivalent absorbed irradiances. Even when considering the relatively restricted spatial extension of photosynthesis in corals (Montastrea curta ~0.5 mm tissue thickness) as compared to a photic zone of ~1-2 mm in microbial mats and biofilms, the depth integrated areal rates of photosynthesis, i.e., the photosynthetic energy conservation of the coral system ($P_{\text{max}} = 5.8 \text{ J m}^{-2} \text{s}^{-1}$; Table 2) was about 3 times higher than what has been reported in biofilms and microbial mats at equivalent absorbed irradiances.

A high light utilization efficiency in corals was also supported by our measurements of the local quantum efficiency of photosynthesis (QE) showing much higher values than in biofilms or other compact photosynthetic systems of similar thickness and degree of compaction (Al-Najjar et al., 2012). The local QE generally decreased with increasing incident downwelling irradiances but the maximum QE of 0.102 O₂ per photon under a photon irradiance of 320 µmol photons m² s⁻¹ (Fig. 7) approached the theoretical maximum of 0.125. Such high efficiency values are about a magnitude higher than previous estimates that were based on tissue extracts and thus ignored the role of coral tissue and skeleton
optics in light absorption efficiency (Dubinsky et al., 1984; Wyman et al., 1987; Dubinsky et al., 1990; Lesser et al., 2000). More similar but still lower quantum efficiencies were reported when coral absorptance was based on skeleton reflectivity (≈0.07 mol O₂ per absorbed mol photons; (Rodriguez-Roman et al., 2006)). Likewise, a study of intact colonies of the coral Montipora monasteriata in shaded environments also showed high QE values of 0.071-0.096 O₂ per incident photon (Anthony & Hoegh-Guldberg, 2003). Such high efficiencies are comparable to macroalgal stands and terrestrial communities such as forests, which exhibit a canopy distribution of the photosynthetic active components facilitating a more uniform availability and use of light throughout the photic zone (Sand-Jensen & Krause-Jensen, 1997; Krause-Jensen & Sand-Jensen, 1998). It is thus intriguing to speculate whether microscale canopy effects are at play in corals that facilitate the observed high photosynthetic efficiency.

Light regulation on a micro-scale occurs through several mechanisms in corals. Photons incident on the coral surface will be strongly scattered in the coral tissue. This enhances photon pathlength per vertical distance traversed and thus the average residence time of photons at a given point within the tissue increasing the probability of absorption for wavelengths overlapping with absorption maxima of symbiont photopigments or coral host pigments (Wangpraseurt et al., 2012a). In combination with refractive index mismatches between coral tissue and water such photon trapping leads to near-surface maxima in scalar irradiance and enhanced spectral filtering (Kühl & Jørgensen, 1994). Light can also be transferred laterally through coral tissue thereby leading to a more homogeneous distribution of incident irradiance over the coral (Wangpraseurt et al., 2014). Additionally, photons that have passed through tissue undergo multiple scattering in the coral skeleton, which acts as a Lambertian-like diffuser and thus facilitates further enhancement of light capture by zooxanthellae (Enriquez et al., 2005; Marcelino et al., 2013). Corals thus have several distinct micro-scale mechanisms that can optimize light capture and utilization.

Light regulating mechanisms also operate on larger scales. It is known that individual colonies show a plastic response to the ambient light climate, where for instance coral orientation, branch spacing and corallite architecture are regulated by the ambient light climate (Helmuth et al., 1997; Anthony et al., 2005; Hoogenboom et al., 2008; Ow & Todd, 2010; Kaniewska et al., 2014). The concerted action of the mentioned micro- and macro-scale light regulating mechanisms indeed indicates that symbiont-bearing corals have canopy-like optical properties not only at larger colony- and reef-scales but also at the scale of single polyps, where optical properties of tissue and skeleton act in with behavioral modulation of tissue contraction/expansion (Levy et al., 2003; Wangpraseurt et al., 2014) to maintain high quantum efficiencies and metabolic rates.
In conclusion, we present the first balanced radiative energy budget of a symbiont-bearing coral. The majority (>96%) of absorbed light energy was dissipated as heat and the proportion of the absorbed light energy that was photochemically conserved decreased with increasing incident irradiance favoring heat dissipation. Yet, coral symbionts are able to retain a very high photosynthetic activity and efficiency in hospite, and we propose that canopy-like effects involving the interplay between tissue and skeleton optical properties of the coral holobiont are important but largely unexplored factors affecting the successful algal-cnidarian symbiosis and its ability to adapt to different light regimes.

Acknowledgements

We thank the staff at Heron Island Research Station for excellent assistance during our field work and Dr. V. Schrameyer and Dr. D. Nielsen for thoughtful discussions. We acknowledge the help and assistance from the Aquatic Processes Group at the University of Technology, Sydney (UTS). The research was conducted under research permits for field work on the Great Barrier Reef, Australia (G11/34670.1 and G09/31733.1) and was funded by grants from Knud Høigaards Fond, Oticon Fonden, Thorsons Rejselegat, Københavns Universitets Fælleslegat (Pastor Peter Albert Raashous legat) (KEB & ML), the Danish Council for Independent Research | Natural Sciences (MK), the Australian Research Council (PJR, MK) and the University of Technology, Sydney (KEB & DW).
References


Supplementary information

The coral tissue irradiance reflectance, R(PAR), was ~12% and 11% at 640 and 1280 µmol photons m\(^{-2}\) s\(^{-1}\), respectively. Reflectance levels were quasi-constant over an irradiance range of 160-2400 µmol photons m\(^{-2}\) s\(^{-1}\) with no significant correlation between reflection and incident irradiance (p>0.05, Fig. S1).

The linear relationship between the vector irradiance and the measured downwelling photon irradiance (slope of 𝛼 = 0.19; R\(^2\) = 0.99; Fig. S2) justifies our light energy calculations.

**Fig. S1.** Reflection measurements of PAR over a white reflectance standard (black circles in A) and immediately above the coral surface (red circles in A) measured under 5 different downwelling photon irradiance levels (160, 320, 640, 1280 and 2400 µmol photons m\(^{-2}\) s\(^{-1}\)). (B) The corresponding irradiance reflectance, R (PAR) (in %). R\(^2\) of slope in panel A = 0.98. p>0.05 panel B. n=3.

The linear relationship between the vector irradiance and the measured downwelling photon irradiance (slope of 𝛼 = 0.19; R\(^2\) = 0.99; Fig. S2) justifies our light energy calculations.

**Fig. S2.** Vector irradiance as a function of the downwelling photon irradiance at 5 different light intensities (160, 320, 640, 1280 and 2400 µmol photons m\(^{-2}\) s\(^{-1}\)). n=3, R\(^2\)=0.99.
Chapter 3

Photosynthetic acclimation of *Symbiodinium in hospite* depends on vertical position in the tissue of the scleractinian coral *Montastrea curta*

Published in *Frontiers in Microbiology*

by

Mads Lichtenberg, Anthony W. Larkum and Michael Kühl
Photosynthetic acclimation of *Symbiodinium in hospite* depends on vertical position in the tissue of the scleractinian coral *Montastrea curta*

Mads Lichtenberg¹, Anthony W. Larkum² and Michael Kühl¹²

¹Marine Biological Section, University of Copenhagen, Denmark
²Plant Functional Biology and Climate Change Cluster, University of Technology Sydney, Australia

Abstract

Coral photophysiology has been studied intensively from the colony scale down to the scale of single fluorescent pigment granules as light is one of the key determinants for coral health. We studied the photophysiology of the oral and aboral symbiont band of scleractinian coral *Montastrea curta* to investigate if different acclimation to light exist *in hospite* on a polyp scale. By combined use of electrochemical and fiber-optic microsensors for O₂, scalar irradiance and variable chlorophyll fluorescence, we could characterize the physical and chemical microenvironment experienced by the symbionts and, for the first time, estimate effective quantum yields of PSII photochemistry and rates of electron transport at the position of the zooxanthellae corrected for the in-tissue gradient of scalar irradiance. The oral- and aboral *Symbiodinium* layers received ~71% and ~33% of surface scalar irradiance, respectively, and the two symbiont layers experience considerable differences in light exposure. Rates of gross photosynthesis did not differ markedly between the oral- and aboral layer and curves of PSII electron transport rates corrected for scalar irradiance *in hospite* showed that the light use efficiency under sub-saturating light conditions were similar between the two layers. However, the aboral *Symbiodinium* band did not experience photosynthetic saturation, even at the highest investigated irradiance where the oral layer was clearly saturated. We thus found a different light acclimation response for the oral and aboral symbiont bands *in hospite*, and discuss whether such response could be shaped by spectral shifts caused by tissue gradients of scalar irradiance. Based on our experimental finding, combined with previous knowledge, we present a conceptual model on the photophysiology of *Symbiodinium* residing inside living coral tissue under natural gradients of light and chemical parameters.
Introduction

Coral reefs form one of the most diverse and productive ecosystems on Earth. The high productivity relies on the relationship between the endosymbiotic zooxanthellae (dinoflagellates in the genus *Symbiodinium*) and the coral host (Muscatine & Porter, 1977). The endosymbiont gains protection and nutrients, while the coral host relies on the energy supplied as carbohydrates by its phototrophic partner (Muscatine & Porter, 1977; Edmunds & Davies, 1986; Edmunds & Davies, 1989). Symbiont-bearing corals are limited to habitats with appropriate light conditions, but inhabit a wide span of light-exposed habitats ranging from shallow reef flats where mid-day solar irradiance reaches >2000 µmol photons m\(^{-2}\) s\(^{-1}\) to shaded caves (Anthony & Hoegh-Guldberg, 2003) and >150 m deep waters (Bridge et al., 2013) in virtual darkness. Colonization of such a wide range of habitats is facilitated by the ability of corals to modulate and optimize their tissue light environment and thereby the light exposure of the zooxanthellae. The regulation of internal light field serves to either filter out excess light that can be harmful to the algae, or to increase the photon flux reaching the algae in sun-exposed or shaded environments, respectively. There are several mechanisms by which the coral host optimizes the light environment for its endosymbionts, e.g. by i) screening out harmful UV-radiation by chromoproteins (Smith et al., 2013) or other fluorescent host pigments (Salih et al., 2000), ii) host pigment conversion of short-wave radiation to longer wavelengths, which are more efficient for photosynthetic conversion (Schlichter & Fricke, 1990; Gilmore et al., 2003), or iii) increasing the internal photon flux density in the tissue by scattering and skeleton reflection (Enriquez et al., 2005; Wangpraseurt et al., 2014a). On a larger scale, Anthony et al. (2005) investigated irradiance levels inside foliaceous (leaf-like) corals and found that structural elements (colony plates) can regulate the light regime towards the maximum sub-saturation irradiance (*E*\(_{k,\text{max}}\)). The light field in contrasting colony growth forms (branching vs. massive) has been shown to be the same at the level of the endosymbionts, despite different surface light environments (Kaniewska et al., 2011). In addition, corals with different tissue configuration (relaxed vs. contracted) show different light microclimates (Wangpraseurt et al., 2014a), and tissue plasticity may thus be very important for regulating the internal light regime towards the optimal conditions for coral endosymbionts.

Besides host-induced regulation of the light microclimate, the zooxanthellae can employ different strategies to regulate photon absorption, e.g. by regulating the concentration of light harvesting pigments and photoprotective pigments, and it has been shown that photoprotection can be achieved by varying
the PSII antennae size, i.e., the functional absorption cross-section of PSII (Hill & Ralph, 2006; Hill et al., 2012).

Normally, shade adapted corals display high light use efficiencies but low maximal photosynthesis rates. This strategy involves employing a higher amount of light harvesting pigments and thus a greater absorption cross-section (Dubinsky et al., 1984), while the opposite is the case for corals adapted to high irradiance that often appear more transparent due to downregulation of light harvesting pigments and organization of chloroplasts to minimize light capture (Dubinsky et al., 1984). In shade, the photochemical conversion is limited by the supply of photons to the photosystems, whereas in full sunlight the enzymatic processes limit the energy transformation and as a result the coral is left with a surplus of photons. This surplus energy can be dissipated via non-photochemical quenching processes in order to avoid photoinhibition (Brown et al., 1999; Gorbunov et al., 2001; Cooper et al., 2011).

Reef-building corals have developed a number of strategies to succeed in highly variable light environments. Clearly one passive option is to adapt the light harvesting pigments to the average incident radiation (Ramus et al., 1977). At the same time, the zooxanthellae have the capacity to entrain non-photochemical quenching mechanisms to avoid high levels of photoinhibition when light levels become high (Brown et al., 1999) and to entrain the water-water cycle (Mehler ascorbate peroxidase pathway (Roberty et al., 2014). Furthermore, the coral host can modulate the light scattering in the coral tissue by i) modifying the calcium carbonate skeleton (Marcelino et al., 2013) enhancing light absorption (Enriquez et al., 2005), and ii) by modulating scattering in the tissue due to contraction and expansion strongly affecting intra tissue light levels (Wangpraseurt et al., 2014a). The coral host can also regulate the number of zooxanthellae engulfed in the endodermal cells as a response to irradiance (Stimson, 1997). Finally, corals produce a range of fluorescent protein-like pigments (FP), which can change the optical properties of the coral tissue in a number of ways. For instance, by i) changing the scattering properties (Salih et al., 2000) (Lyndby et al. submitted), ii) by changing light quality within the host tissue (Salih et al., 2000), and iii) by converting short wave radiation to longer wave radiation (Schlichter et al., 1986; Gilmore et al., 2003). While there is some evidence for all mentioned mechanisms for modifying light-harvesting in corals there is much work left to be done to fully understand the subtle processes involved and how they regulate Symbiodinium photosynthesis.

In this study we explored the photophysiology of Symbiodinium in hospite in the tissue of the massive scleractinian coral Montastrea curta by the combined use of electrochemical and fiber optic microsensors. Electrochemical microsensors for O2 are important tools to unravel biogeochemical
processes in e.g. marine sediments (Revsbech & Jørgensen, 1983), microbial mats from extreme environments (Revsbech & Ward, 1984), aquatic macrophytes (Spilling et al., 2010; Brodersen et al., 2015; Lichtenberg & Kühl, 2015) and corals (Kühl et al., 1995). Combined with fiber-optic probes for either field radiance (directional photon flux) or scalar irradiance (integrated total photon flux) photosynthetic performance can be investigated in high spatial resolution inside phototrophic tissues or communities (Kühl et al., 1996; Brodersen et al., 2014). Measurements of variable chlorophyll fluorescence, using the saturation pulse method with pulse-amplitude-modulated (PAM) fluorometers (Schreiber, 2004), have become increasingly popular to assess photosynthetic performance in aquatic systems. However, only the micro fiber-based PAM system (Schreiber et al., 1996) allows high spatial resolution, intra-tissue measurements of photosynthetic parameters, by either applying actinic light through the fiber to assess potential quantum yields or by applying actinic light externally to measure effective quantum yields as a function of the gradient of light seen by the photosynthetic unit.

The symbiotic algae reside in the gastrodermal tissue and are thus spatially separated by the gastrovascular cavity. This separation means that oral and aboral symbiont layers can experience differences in light quantity and spectral composition due to absorption and scattering of light in the tissue (Wangpraseurt et al., 2012). Most studies of coral photosynthesis have ignored such symbiont stratification, but there is increasing evidence that such stratification can enable differential photoacclimation in the coral tissue (Wangpraseurt et al., 2015). Microniches enabling differential acclimation in different parts of coral tissue may be an important yet overlooked component governing efficient coral photosynthesis over a wide range of irradiance.

In this study, we investigated whether *Symbiodinium* cells located in oral and aboral tissue layers display similar light acclimation properties by characterizing intra-tissue light gradients, the oxic microenvironment and the depth distribution of photosynthetic rates and rates of PSII electron transport within the coral tissue. Such detailed information on photosynthetic performance of *Symbiodinium in hospite* could have important implications for the understanding of symbiont resilience against high-light stress.

**Materials and methods**

**Coral samples**

Coral samples were collected from shallow waters in Shark Bay, Heron Island (Capricornia Cays, Great Barrier Reef, Australia; 23°26'31"S 151°55'30"E). We selected the favid coral *Montastrea curta* due to
its suitability for microsensor studies owing to thick tissue and low mucus production (Wangpraseurt et al., 2012; Brodersen et al., 2014). After collection, coral fragments were transferred to a 50 L aquarium where they were maintained under a continuous flow of filtered seawater from the lagoon (temperature: ~26°C; salinity: ~36). The coral tank was located outdoors under a natural diurnal light cycle, but was shaded such that maximum midday photon irradiance (400-700 nm) was ~500 µmol photons m⁻² s⁻¹. Three coral fragments were chosen for experiments. From these, polyps were randomly chosen across all coral fragments for individual measurements. However, at least one replicate was done on a polyp from each fragment; when n>3, more than one polyp were measured on one of the fragments. Replicates across measurements were thus done on the polyp scale. The measurement points on individual polyps were chosen randomly on the oral disc tissue surrounding the polyp mouth.

**Experimental Setup**

All measurements were conducted with a coral fragment placed in a custom-made black acrylic flow chamber (25x8x8 cm) supplied with aerated seawater (26°C; S=36) at a flow velocity of ~2 cm s⁻¹ as provided by a water pump (Fluval U1, Rolf C. Hagen Ltd., England) in a 25 L aquarium with seawater that was continuously flushed with atmospheric air by an air pump (Sera Air 110 plus, Sera GmbH, Germany). We used fiber-optic and electrochemical microsensors to measure photon scalar irradiance, gross photosynthesis, O₂ concentrations and variable chlorophyll fluorescence in vertical steps through the coral tissue (see details below). Positioning of the microsensors on the coral surface was done visually through a PC-interfaced USB-microscope (AM7013MZT Dino-Lite, AnMo Electronics Corporation, Taiwan). For measurements, the microsensors were mounted on a motorized micromanipulator (MU-1, PyroScience GmbH, Germany) controlled by a PC running dedicated software (ProFix, PyroScience GmbH, Germany).

**Measurements and calculations**

**Light.** Depth profiles of photon scalar irradiance in coral tissue were measured with fiber-optic scalar irradiance microprobes with a sphere diameter of ~45 µm and an isotropic angular response (Rickelt et al., 2016). The scalar irradiance microprobe was connected to a fiber-optic spectrometer (USB2000+, Ocean Optics, USA) interfaced to a PC running spectral acquisition software (Spectra Suite, Ocean Optics, USA). Light was provided at a slight angle by a fiber-optic tungsten halogen lamp (KL2500-LCD, Schott GmbH, Germany) equipped with a collimating lens. All measurements were performed in a dark
room to avoid stray light. Profiles of photon scalar irradiance were measured in vertical steps of 0.1 mm from the surface of the coral tissue towards the skeleton, which was determined as the depth where the fiber of the microprobe bended slightly or retracted into the needle. To penetrate the coral tissue, a small incision in the tissue of the oral disc was carefully made with the tip of a hypodermic needle. During this procedure, the coral tissue contracted and corals were allowed 1-3 minutes to allow tissue relaxation before measurements of light microprofiles.

Incident light was quantified as the downwelling photon scalar irradiance from the fiber optic tungsten halogen lamp with the fiber optic microprobe positioned over a black, non-reflective light-well at a distance and position in the light field similar to the position of the coral surface; in a collimated light field, the downwelling irradiance and the downwelling scalar irradiance is identical (Kühl & Jørgensen, 1994). Absolute incident photon irradiance (PAR, 400-700 nm; in μmol photons m⁻² s⁻¹) was measured with a calibrated photon irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a spherical sensor (US-SQS/L, Walz GmbH, Germany) positioned in the light-well at a distance similar to the position of the coral surface.

The acquired spectra were integrated over the spectral regions of interest, i.e., PAR (400-700 nm), and the integral was related to the absolute incident photon irradiance to obtain the amount of photosynthetic active radiation at each measuring depth expressed as fractions of incident photon scalar irradiance. The photon scalar irradiance attenuation coefficient, K₀ (mm⁻¹), was calculated as the slope of the natural logarithm transformed photon scalar irradiance plotted as a function of depth (Kühl, 2005). The spectral attenuation coefficient, K₀(λ) (mm⁻¹) was calculated as, $K_0(\lambda) = -\ln\left[\frac{E_0(\lambda)_1}{E_0(\lambda)_2}\right]/(z_2 - z_1)$, where $E_0(\lambda)_1$ and $E_0(\lambda)_2$ are the spectral scalar irradiances measured at depth $z_1$ and $z_2$, respectively (Kühl & Jørgensen, 1994; Kühl, 2005).

**Variable Chlorophyll Fluorescence.** Microscale measurements of variable chlorophyll fluorescence using the saturation pulse method (Schreiber, 2004) were done with a sensitive fiber-optic fluorometer (Microfiber PAM, Waltz GmbH, Germany) (Schreiber et al., 1996; Ulstrup et al., 2006). The fiber-optic microprobe consisted of a single strand graded index multimode fiber cable (Radiall Inc., France) mounted in a syringe and needle with the measuring tip tapered and rounded to ~30 μm at the light collecting end (Kühl, 2005) and connected to a sensitive pulse-amplitude modulated detector system at the other end via a fiber-optic beam splitter/coupler (see details in Schreiber et al. 1996). On the system end, one fiber branch of the splitter/coupler was connected to a LED light source providing measuring light and saturating pulses, while the other branch was connected to a sensitive photomultiplier-detector.
equipped with a long-pass filter to screen out the LED excitation light and only detect chlorophyll fluorescence. On the measuring side of the splitter/coupler, one branch was connected to the fluorescence microprobe, while the other branch was not used. To ensure good optical throughput, a small droplet of microscope immersion oil was added in the fiber-fiber connections. Fiber connections used the ST-connector standard.

The PAM control unit was connected to a LED ring (Ulstrup et al., 2006) providing known photon irradiance levels of red light (63, 93, 142, 213, 303, 422, 695 and 1018 µmol photons m\(^{-2}\) s\(^{-1}\); peak emission: 666 nm; Fig. S1). The incident photon irradiance from the red LED ring at different settings was measured with a calibrated irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a spherical sensor (US-SQS/L, Walz GmbH, Germany) positioned over a black light-well at a distance similar to the position of the coral surface. Data were collected using PC controlled data acquisition software (Win Control v. 2.08, Walz GmbH) that controlled the Microfiber PAM system.

We measured rapid light curves (RLC) and steady state light curves (LC) in the two spatially separated endosymbiont layers. Due to inter-polyp differences in the location of these endosymbiotic layers (Fig. 3), we located the layers by monitoring the fluorescence yield signal. By slowly moving the optical fiber vertically through the tissue, the center of the layers was determined as the position showing the largest fluorescence yield. RLC’s (Ralph & Gademann, 2005) were measured with 10 s acclimation to increasing irradiance, while steady state light curves (LC) were measured with 5 min acclimation to each of the increasing irradiance levels. Local rates of relative photosystem II (PSII) related electron transport, \(rETR\), were calculated from the effective quantum yield of PSII ignoring the absorption factor and the factor describing absorption by both photosystems; (Ralph et al., 2002) and by using the actual scalar irradiance measured locally instead of the incident irradiance (Lichtenberg & Kühl, 2015). Fitting of experimental \(rETR\) vs. scalar irradiance curves was done using an exponential function (Webb et al., 1974) yielding the maximum rate of electron transport through PSII (\(rETR_{\text{max}}\)) and the light use efficiency (\(\alpha\); the initial slope of the \(rETR\) vs. scalar irradiance curve). Where data did not reach saturation, the initial slope (\(\alpha\)) was linearly fitted below 150 µmol photons m\(^{-2}\) s\(^{-1}\) of scalar irradiance. The scalar irradiance at the onset of photosynthesis saturation, the so called \(E_k\) parameter, was calculated as \(E_k = \frac{rETR_{\text{max}}}{\alpha}\). Curve fitting was done with the non-linear curve fitting functions of Origin 9.2 (OriginLab Corporation, MA, USA).
O2 concentration and gross photosynthesis. Profiles of O2 concentration and gross photosynthesis were measured in 0.1 mm vertical steps through the coral tissue using a Clark type O2 electrochemical microsensor (OX-25, Unisense, Denmark) (Revsbech, 1989) with a tip diameter of <25 µm, low stirring sensitivity (<1-2 %) and a fast response time (t90<0.5 s). The microsensor was connected to a pA-meter (pA-2000, Unisense, Denmark) and signals were recorded on a strip-chart recorder (BD 12E, Kipp & Zonen B.V., Netherlands). The O2 microsensor was linearly calibrated from signal readings in air saturated seawater and in anoxic seawater (produced by addition of sodium sulphite to seawater at experimental temperature and salinity). Measurements were done at increasing photon irradiance (18, 63, 93, 213, 303, 698 and 1018 µmol photons m-2 s-1) of red light as provided by the red LED ring described above. Volumetric rates of gross photosynthesis (in nmol O2 cm-3 s-1) were calculated from the initial O2 depletion rate after a brief darkening following the light-dark shift method (Revsbech & Jørgensen, 1983). Depth integration of the volumetric rates measured throughout the tissue at each irradiance, yielded areal gross photosynthesis (in nmol O2 cm-2 s-1) vs. photon irradiance curves. Vertical profiles of O2 concentration were obtained during the gross photosynthesis measurements in each depth from the steady-state O2 concentration obtained in each measurement depth just before the brief darkening.

Results

Spectral light regime.

Both PAR (400-700 nm) and red light (630-700 nm) was attenuated exponentially from the coral tissue surface towards the skeleton (Fig. 1A), with photon scalar irradiance attenuation coefficients (K0) of 1.8 mm-1 (R2=0.98) and 1.7 mm-1 (R2=0.99) for PAR and red light, respectively. Over the coral tissue layer, PAR varied from 107% of incident photon irradiance at the tissue surface to 24% 0.7 mm below the tissue surface, while red light was reduced from 124% to 33% of incident photon irradiance over the same tissue thickness. Scalar irradiance transmission spectra measured in the coral tissue showed characteristic minima and shoulders corresponding to absorption peaks of major zooxanthellate pigments such as Chl a (430-440 nm; 675 nm) and Chl c (460 nm; 590 nm; 635 nm) (Halldal, 1968; Shibata & Haxo, 1969; Kühl et al., 1995) (Fig. 1B). In addition, we found indications of host fluorescent pigments that emitted light at longer wavelengths (450-575 nm) when excited with low wavelength blue light (390-410 nm) (Fig. S2). The host fluorescence partly concealed the spectral signature of the dinoflagellate carotenoid peridinin (490 nm). Scalar irradiance attenuation was higher near the tissue
surface (0.1-0.3 mm) and near the skeleton/tissue interface (0.5-0.7 mm) as compared to the middle part of the tissue (0.3-0.5 mm) (Fig. 1C).

Photosynthesis and O$_2$ conditions.

Gross photosynthesis rates generally increased with irradiance and O$_2$ production was measured at all tissue depths. The highest gross photosynthesis rate (>17 nmol O$_2$ cm$^{-3}$ s$^{-1}$) was measured ~0.3 mm inside the tissue in the highest light treatment (Fig. 2). The vertical distribution of production was rather uniform and did not follow the tissue light gradient as would be expected, except for the highest light treatment (1018 µmol photons m$^{-2}$ s$^{-1}$) where production decreased with depth towards the skeleton. Tissue O$_2$ concentration ranged between 59 µM in the lowest light treatment (18 µmol photons m$^{-2}$ s$^{-1}$).
and 755 µM in the highest light treatment (1018 µmol photons m⁻² s⁻¹) and was uniformly distributed with depth.

**Chlorophyll fluorescence.**

Vertical microprofiles of Chl a fluorescence revealed two spatially separated fluorescence bands (Fig. 3). The zones of enhanced fluorescence were of varying intensity, and the vertical position of both bands differed. An upper peak was detected at 0.23 mm ± 0.10 and a lower peak was detected at 0.75 mm ± 0.27 (means ± S.D. n=4). However, depending on the contraction status of the polyp tissue, the bands could be located closer to each other or farther apart. From these measurements, the *Symbiodinium* bands were defined as the zones that exhibited the highest fluorescence (Fig. 3) and in combination had

---

**Figure 2: Photosynthesis in coral tissue.** Depth profiles of gross photosynthesis (nmol O₂ cm⁻³ s⁻¹; blue bars) and O₂ concentration (µmol l⁻¹; red lines; note difference in O₂ concentration scale between panels) in the polyp tissue of *M. curta* at increasing incident photon irradiance (18, 63, 93, 213, 303, 695, and 1018 µmol photons m⁻² s⁻¹). Data represent means ± 1 SD (n = 3). Note that for clarity, only minus SD is shown for gross photosynthesis, and plus SD for O₂ concentration.
the highest spectral attenuation coefficient ($K_0(\lambda)$; Fig. 1C), and thus we defined the oral band to be located around 0.2 mm and the aboral band around 0.7 mm below the coral tissue surface.

Photosynthetic electron transport of zooxanthellae in hospite. 

The measured photon scalar irradiance profiles allowed us to estimate the amount of red actinic light available for photosynthesis in different tissue depths, and thereby to relate these values to the variable chlorophyll fluorescence-derived measurements of relative photosynthetic electron transport rates and effective quantum yields of PSII related photochemistry (Fig. 4). The light available for photosynthesis was 71% and 33% of incident photon irradiance in the oral (0.2 mm) and aboral (0.7 mm) Symbiodinium band, respectively (Fig. 1A). Effective PSII quantum yields ($\phi_{PSII}$) and relative rates of electron transport through PSII (rETR) were calculated for the two Symbiodinium layers, and were related to the actual photon scalar irradiance of red actinic light (630-700nm) in each zone (Fig. 4). $\phi_{PSII}$ and rETR rates were obtained both from rapid light curves (RLC) and steady state light curves (LC). Both types of photosynthesis vs. irradiance curves showed that the oral Symbiodinium layer reached saturation and approached an asymptotic rETR$_{max}$ value of 53 and 83, for RLC and LC respectively. $\phi_{PSII}$ decreased with increasing irradiance in the oral layer and reached a value <0.2 at the highest light treatment. The aboral Symbiodinium layer, however, did not experience sufficient irradiance levels to become saturated, and both RLC and LC measurements showed that rETR continued to increase with irradiance without reaching saturation, with $\phi_{PSII}$ values in the high light treatment, similar to values in low light (Table S1). At the highest irradiance, rETR rates in the aboral layer reached 83 and 106, for the RLC and LC respectively.

Figure 3: Chlorophyll fluorescence in coral tissue. Vertical profiles of Chl a fluorescence in the polyp tissue of M. curta measured from the surface toward the skeleton. Panels show four replicates demonstrating the heterogeneity of the position of the pigmented Symbiodinium layers, in part affected by tissue contraction/relaxation. Solid black lines is the average position of the center of the fluorescent peaks and the gray area depicts ± 1 SD ($n = 4$).
Figure 4: PSII activity in coral tissue. Relative electron transport rates through PSII (rETR) (top), and effective quantum yields of PSII photochemistry ($\phi_{\text{PSII}}$) (bottom). Red and blue triangles represent measurements in the oral and aboral Symbiodinium bands, respectively. Measurements from left panels were acquired with 10 s acclimation to increasing irradiances (RLC), while the right panels show steady state light curves (LC) using a 5 min acclimation period to each increasing irradiance steps. Data were corrected for the actual scalar irradiance measured at the depth horizons of the defined Symbiodinium bands. Curve fits of rETR vs. scalar irradiance were done using either an exponential function (oral tissue layer) (Webb et al., 1974) or a linear curve fit (aboral tissue layer). Data points represent means ± 1 SD (n = 4 (RLC); n = 3 (LC)).
The efficiency of light utilization, i.e., the initial slope ($\alpha$) of the rETR vs. scalar irradiance curves were similar in all treatments, but was generally higher in the oral layer as compared to the aboral layer (Table S1). In addition, steady state light curves exhibited higher $\alpha$ values than RLC measurements.

The scalar irradiance at the onset of light saturation ($E_k$) for the oral layer was calculated as $E_k = \frac{rETR_{max}}{\alpha}$ reaching 156 $\mu$mol photons m$^{-2}$ s$^{-1}$ and 239 $\mu$mol photons m$^{-2}$ s$^{-1}$ for the RLC and LC measurements, respectively. Because rETR in the aboral Symbiodinium layer did not saturate over the investigated irradiance range, it was not possible to calculate the $E_k$ parameter for this layer.

**Discussion**

To the best of our knowledge, we report the first measurements of PSII quantum efficiency and relative electron transport rates (rETR) measured internally in coral tissues, i.e., at the position of the zooxanthellae in hospite. Numerous studies have been published on *Symbiodinium* photophysiology in culture, often kept in exponential growth (Iglesias-Prieto & Trench, 1994; Reynolds *et al*., 2008; Szabó *et al*., 2014). However, the microenvironmental conditions of *Symbiodinium in hospite* within the coral host tissue differ significantly from conditions in the surrounding water or at the coral-water tissue interface (Kühl *et al*., 1995; Wangpraseurt *et al*., 2012; Barott *et al*., 2015) and the proliferation of symbionts is controlled to some extent by the host (Davy *et al*., 2012; Cunning & Baker, 2014; Cunning *et al*., 2015). This study thus provides novel information on the photophysiology of *Symbiodinium in hospite* within coral tissue under natural gradients of chemical parameters and light. Based on our findings combined with existing knowledge, we present a conceptual model on the photophysiology of *Symbiodinium in hospite* (Fig. 5).

**Scalar irradiance.**

Light levels in the coral tissue were similar to values previously found in the same species (Wangpraseurt *et al*., 2012; Brodersen *et al*., 2014), and the small differences in attenuation as compared to these studies can probably be ascribed to either intercolonial differences or specific differences in light adaptation as natural light exposure (i.e. sun towards shade colonies) have been shown to be a determinant of tissue light penetration (Ulstrup *et al*., 2006). The penetration of light is affected by the concentration of light absorbing pigments (Dubinsky *et al*., 1984), the tissue type (coenosarc or polyp (Wangpraseurt *et al*., 2012)) and thickness, which can vary substantially with colony size (Anthony *et al*., 2002) and over time (Wangpraseurt *et al*., 2014a). The coral tissue itself may act as an important
determinant of the *Symbiodinium* light microclimate *in hospite* (Wangpraseurt *et al.*, 2012; Wangpraseurt *et al.*, 2014a; Wangpraseurt *et al.*, 2014b) in addition to the contribution of diffuse backscattered light of the skeleton to the internal tissue light field (Enriquez *et al.*, 2005; Marcelino *et al.*, 2013). The spectral composition of scalar irradiance changed progressively from the tissue surface towards the skeleton, and our spectral data showed distinct absorption signatures of coral photopigments (Chl a, Chl c and the carotenoid, peridinin), thus altering the intra tissue spectral light composition with depth. Consequently, e.g. blue light (400-500 nm) was effectively reduced to <10% in the lower tissue layers, while light outside the spectral region of the major photopigments (550-650nm) only decreased to 32% of the incident irradiance (Fig. 1B). In addition, we showed a stratification of absorption properties, where the spectral attenuation coefficients ($K_0(\lambda)$) were higher in the oral and aboral layers as compared to the central tissue layer (Fig. 1C). This observation correlates with the vertical distribution of the symbiont biomass as approximated by the vertical Chl a fluorescence profiles.

**Photosynthesis and oxic environment.**

The vertical distribution of gross photosynthetic production is typically correlated with the attenuation of scalar irradiance and the distribution of photosynthetic elements (light harvesting biomass) e.g. in biofilms and microbial mats (Kühl *et al.*, 1996; Kühl & Fenchel, 2000). In the investigated coral tissue, production exhibited a relatively uniform vertical distribution and with no apparent correlation with the distribution of scalar irradiance or Chl a fluorescence. This apparent mismatch between biomass and production can be a result of e.g. adaptation of the photosynthetic elements to local quality and quantity of light (Falkowski & Owens, 1980). In higher plants, it has e.g. been shown that the production along a vertical transect through the leaf did not follow the internal light gradient (Nishio *et al.*, 1993; Sun *et al.*, 1998). Recently gradients of carbon fixation were measured within coral tissues suggesting a different light use efficiency of oral and aboral tissue layers (Wangpraseurt *et al.*, 2015), and microgradients of photosynthetic quantum efficiencies (i.e. mol O$_2$ produced per mol photons absorbed) measured in *M. curta* (Brodersen *et al.*, 2014) showed a progressive increase in quantum efficiency with depth. Effectively, this means that aboral tissues, with higher quantum efficiencies, have the ability to produce more O$_2$ per mol quanta absorbed than the oral tissue, and thus contribute equally to O$_2$ production despite the lower light availability. Photosynthetic O$_2$ production increased with increasing photon irradiance and the highest photosynthetic rates were found in the high light treatment. Similarly, the O$_2$ concentration was almost constant at all tissue depths, but increased with increasing incident irradiance.
**Figure 5:** (A) Image of a *M. curta* polyp during measurement of scalar irradiance under red light illumination. Scale bar = 2 mm. (B) Image of host pigment fluorescence centralized around the mouth in a *M. curta* polyp when illuminated with blue light (390–410 nm). Scale bar = 2 mm. (C) Schematic drawing of a tissue cross-section showing anatomical features in coral polyp tissue. From top toward bottom: the mucus layer covering the coral epidermis underneath. Between the two epithelial layers (epiderm, and gastroderm) is the mesoglea. The gastrodermis containing the zooxanthellae surrounds the gastrovascular cavity and underneath the second mesoglea layer is the...
calicoblastic layer which, excretes the CaCO₃ and the organic matrix forming the skeleton. (D) Conceptual model of the photosynthetic parameters in hospite in coral tissue. Light, which is attenuated with depth (blue gradient), drives photosynthesis and O₂ production in the Symbiodinium layers as measured by variable Chl a fluorescence (red line). The O₂ concentration (green line) is a product of photosynthetic production, consumption via respiration of host and symbiont cells and transport by diffusion across the diffusive boundary layer (DBL). The downward flux of O₂ is spatially restricted by the skeleton, which presents a diffusion barrier enhancing the increase in tissue O₂ concentration. The skeleton also acts as a barrier for photon flux and light will partly be diffusely backscattered to the tissue. The quantum yield of PSII photochemistry (ϕPSII; dark blue bars) was higher in the aboral compared to the oral Symbiodinium layer, which gives basis for an equal O₂ production despite lower light availability.

Unlike many other photosynthetic systems (e.g. photosynthetic biofilms, macroalgae and other aquatic macrophytes, etc.) coral tissues are spatially constricted by the skeleton which, can act as a diffusion barrier. This has been shown to create a build-up of O₂ towards the tissue-skeleton interface (Kühl et al., 1995; Wangpraseurt et al., 2012; Brodersen et al., 2014). The rather uniform O₂ concentration across the coral tissue can be explained in terms of i) the higher efficiency of photosynthesis in the aboral band of zooxanthellae, ii) the enhanced scalar irradiance in these lower regions due to back-scattering from the skeleton, and iii) the fact that the skeleton impedes O₂ diffusion into the skeleton matrix.

Quantum yield and photosynthesis of zooxanthellae in hospite.

The external light field is a poor proxy for the internal light microenvironment experienced by the coral symbionts (Kaniewska et al., 2011). Furthermore, the photosynthetic elements, i.e., Symbiodinium cells, in coral polyp tissues are vertically structured as they reside in the gastrodermal tissue layers surrounding the gastrovascular cavity (Fitt & Trench, 1983; Barott et al., 2015) (Fig. 5C). Thus, to characterize photosynthesis under the conditions experienced by the symbionts inside the tissue of living coral, we measured effective quantum yields of PSII (ϕPSII) and derived relative electron transport rates of PSII photochemistry (rETR), and related them to the actual scalar irradiance at the position of the symbionts. The symbionts reside in the gastrodermal tissue near the tissue surface and the tissue-skeleton interface (Fig. 5C). At the coral tissue surface, the photon scalar irradiance is highest and then attenuates exponentially with depth in the tissue. However, the photons reaching the skeleton can also be partly backscattered leading to a photon flux from multiple directions leading to an unexpected level of scalar irradiance in the lower zones (Enriquez et al., 2005; Wangpraseurt et al., 2014a). The layering of photosynthetic elements in combination with strong gradients of irradiance and spectral composition can cause substantial light-driven stratification of light use efficiency and photosynthesis, even over very small distances (Lichtenberg & Kühl, 2015). The vertical attenuation of light in coral tissue is strongest in
the blue region leaving a larger fraction of red light in the lower tissues (Wangpraseurt et al., 2012). In our microscale variable chlorophyll fluorescence measurements, we used red light (630-700 nm; peak wavelength 666 nm) for measuring light, saturating pulses and actinic light driving photosynthesis. Effectively, this could mean that the aboral Symbiodinium band were provided with light that they are naturally adapted to, while the oral layer were given a disproportionately large fraction of red light relative to natural spectral distribution in the upper tissue layers. Recently, PSII absorption cross-sections of Symbiodinium (\(\sigma_{\text{PSII}}\)) were measured, both in culture and in hospite, (Szabó et al., 2014) showing a progressive decrease in \(\sigma_{\text{PSII}}\) from blue towards red light. However, this was done either by surface measurements of intact corals or in culture with symbionts adapted to culture conditions. We propose that Symbiodinium in hospite might display different adaptation to spectral quality depending on their vertical position in the coral tissue. This could explain the observed difference in effective quantum yield and concomitant photosynthetic electron transport in the oral- and aboral symbiont layers (Fig. 4), where the aboral layer did not saturate, even at the highest experimental irradiance. Similar phenomena have been observed in leaves of terrestrial plants, where a clear difference in absorption of monochromatic blue, green and red light was observed in the palisade and spongy mesophyll layers (Vogelmann & Han, 2000), and where others have shown that, deep within leaf tissues, green light drives photosynthesis more effectively than red and blue light (Terashima et al., 2009). In corals, maximum photochemical efficiencies (\(F_v/F_m\)) in the outer- and inner Symbiodinium bands have been estimated by variable chlorophyll fluorescence, albeit by a much more invasive method, i.e., by fracturing the skeleton, and measuring the inner Symbiodinium band perpendicular to the surface, demonstrating higher maximum photochemical efficiencies in the inner- relative to the outer Symbiodinium layer and with similar initial slopes on the subsaturated part of the rETR versus irradiance curve (Edmunds et al., 2012). The findings were supported by Brodersen et al. (2014) who showed that the local quantum efficiency (i.e. mol O\(_2\) produced per mol photons absorbed) increased in deeper lying tissue regions. Corals are able to optimize light conditions for their symbiotic algae leading to high quantum efficiencies (Dubinsky et al., 1984; Brodersen et al., 2014) and the question of how corals optimize their internal light environment for their photosymbionts has been studied intensively in terms of e.g. i) regulation of zooxanthellae pigment density (Falkowski & Dubinsky, 1981), ii) increased internal light absorption due to backscattering from the skeleton (Enriquez et al., 2005), tissue scattering and light guiding phenomena (Wangpraseurt et al. 2012, 2014a), as well as iii) wavelength transformation by fluorescent host pigment complexes (Schlichter et al., 1986; Schlichter & Fricke, 1990; Salih et al., 2000; Gilmore et al., 2003). In high-light exposed areas, coral host pigments can absorb radiation in the harmful UV-A and blue range
and re-emit photons outside the main peaks in the photosynthetic action spectra (Salih et al., 2000), while in shaded and light limited areas it has been speculated that host pigments can transform radiation outside the photosynthetic action spectra into wavelengths that overlap with the main absorption peaks of Chl a, c and accessory pigments (Schlichter et al., 1986). In this study we did not quantify host pigment but we found clear evidence for fluorescent host pigments transforming blue light (390-410nm) into longer wavelengths (460-560nm) (Fig. S2).

We performed the first intra-tissue measurements of variable chlorophyll fluorescence in intact corals using a tapered optical fiber with a rounded tip of ~30 µm; this brings the sampling volume (i.e. spatial resolution) down to approximately the same size around the fiber-tip (Kühl, 2005). Such high resolution measurements are inherently prone to reflect heterogeneities in the organization of phototrophs, i.e., the position of zooxanthellae in the coral tissue, but also enables precise measurements in particular tissue layers. This is in contrast to other fiber- or imaging-based variable chlorophyll fluorescence measurements (see e.g. Schreiber (2004); Szabó et al. (2014)), where a larger surface area is monitored without precise knowledge of the excitation light penetration depth and the relative contributions of different layers to the measured signal; albeit chlorophyll fluorescence from surface layers typically contribute more than layers further away. While correction procedures for variable chlorophyll fluorescence measurements in dense algal cultures have been proposed (Klughammer & Schreiber, 2015), similar corrections in complex stratified tissues such as corals or leaves of higher plants are contrived by their intricate optical properties including the close coupling of scattering and absorption processes affecting light attenuation. In contrast, microfiber-based variable chlorophyll fluorescence analysis can obtain detailed local information on the photosynthetic activity in particular tissue layers under natural light gradients (see e.g. Lichtenberg and Kühl (2015)). Optimally, it requires a sample that does not change anatomical organization as such change might change the relative position of the fiber tip and photosynthetic cells inside the tissue. This was indeed a challenge in the current study in living coral tissue as seen by the large standard deviations in the steady state light curves, where the longer acclimation time to increasing irradiance allowed the coral tissue to relax or expand; such tissue change is probably an important regulatory mechanism of the internal light climate (Wangpraseurt et al., 2014a). The position of the symbiont, may thus have changed during measurements of steady state conditions. Our study of Symbiodinium photosynthesis in hospite under real tissue light gradients was done under red illumination. The role of spectral composition is undeniably important as it affects processes such as light harvesting (Vogelmann & Han, 2000; Szabó et al., 2014), photoinhibition (Oguchi et al., 2011), respiration (Wangpraseurt et al., 2014c), CO2 fixation (Sun et al., 1998), and O2 production (Kühl et
al., 1995), and we note that the results may differ under white light illumination, although the internal light field in coral tissues is red shifted (Wangpraseurt et al., 2014a). There is now a need to further investigate the photophysiology of the separate *Symbiodinium* bands at different tissue contraction states (e.g. using a suitable tissue relaxant) to investigate how tissue distribution may be involved in optimizing light utilization of the coral photobionts. In addition, differential adaptations to not only light quantity but spectral composition in the two endosymbiont layers should be studied.

**Acknowledgements**

This study was supported by a Sapere Aude Advanced grant from the Danish Council for Independent Research ǀ Natural Sciences (MK). The research was conducted under a permit for field work on the Great Barrier Reef (G12/35118.1). We thank Erik Trampe and the staff at Heron Island Research Station (HIRS) for excellent assistance during field work, and Lars F. Rickelt for manufacturing scalar irradiance microsensors.
References


Bridge TCL, Hughes TP, Guinotte JM, Bongaerts P. 2013. Commentary: Call to protect all coral reefs. Nature Climate Change 3: 528-530


Fitt WK, Trench RK. 1983. Endocytosis of the symbiotic dinoflagellate Symbiodinium microadriaticum (Freudenthal) by endodermal cells of the scyphistomae of Cassiopeia xamachana and resistance of the algae to host digestion. Journal of Cell Science 64: 195-212


Supplementary information

Fig. S1 shows the spectral composition of the light sources used in this study as measured with a fiber optic scalar irradiance microprobe (Rickelt et al., 2016) connected to a fiber optic spectrometer (USB2000+, Ocean Optics, FL, USA). Light from the fiber optic tungsten halogen lamp (KL2500-LCD, Schott GmbH, Germany) and the red LED ring (Walz GmbH, Germany; Ulstrup et al. (2006) was measured as the downwelling scalar irradiance with the fiber optic microprobe positioned over a black, non-reflective light-well. The fiber optic halogen lamp was used for measurements of vertical microprofiles of scalar irradiance in the tissue of Montastrea curta and the red LED ring was used for measurements of gross photosynthesis and measurements of effective quantum yield (\( \Phi_{\text{PSI}} \)) - and relative electron transport rates (rETR) of PSII photochemistry. The scalar irradiance used for correcting \( \Phi_{\text{PSI}} \) and rETR at the position of the symbionts was integrated from 630-700nm (Fig. S1; dashed lines).

**Figure S1**: Spectral composition of the fiber-optic halogen lamp (black line) and the red LED ring (red line) used for measurements of gross photosynthesis, effective quantum yield- and relative electron transport rates of PSII photochemistry and the spectral area (630-700nm) (dashed lines) used in the photon scalar irradiance profiles.

**Figure S2**: Field radiance measured in the mouth region of a polyp showing the blue excitation light provided by the USB microscope and the host pigment fluorescence at wavelengths >450 nm.
Fig. S2 shows the distribution and spectral characteristics of host pigment fluorescence measured with a field radiance microprobe when excited with light from a miniature epifluorescence microscope. Field radiance was measured with a field radiance microprobe (tip diameter ~35 µm; acceptance half angle ~30°; Kühl and Jørgensen (1992)), connected to a fiber-optic spectrometer (USB2000+, Ocean Optics, Dunedin, FL, USA) controlled by the manufacturer’s software (Spectrasuite, Ocean Optics, Dunedin, FL, USA). The USB microscope (AM4113FVT Dino-Lite, AnMo Electronics Corporation, Taiwan) provided near-UV light (~390-410 nm) for excitation of coral host pigments (Fig. S2A).

Figure S3: Schematic drawing of the experimental setup. A) a motorized micromanipulator used to move the sensors in vertical steps through the coral tissue, B) microsensors for O₂, scalar irradiance and variable chlorophyll fluorescence, C) LED ring providing external actinic red illumination (used in measurements of O₂ and variable chlorophyll fluorescence), D) fiber optic halogen lamp (used in measurements of scalar irradiance), E) a coral fragment placed in F) the flow chamber ensuring a laminar flow of air-saturated seawater across the coral. O₂ sensor signals were recorded on a strip-chart recorder via a pA meter connected to the microelectrode. The scalar irradiance microprobe was connected to a PC via a fiber-optic spectrometer. The microfiber used to measure variable chlorophyll fluorescence was connected to a PC via the PAM control box, a photo-multiplier tube and a fiber-optic coupler that allowed separation of the excitation light and the detected fluorescence. The red LED ring was controlled on the PC via the PAM control box.
Tables

Table S1. Photosynthetic parameters calculated from the variable chlorophyll fluorescence measurements of effective quantum yield ($\varphi_{\text{PSII}}$) and relative electron transport through PSII (rETR) using rapid light curves (RLC) or steady state light curves (LC). Calculated parameters are, light use efficiency ($\alpha$), maximum rate of electron transport (rETR$_{\text{max}}$), the so-called $E_k$ parameter describing the photon irradiance at the onset of photosynthetic saturation and effective quantum yields of PSII related photochemistry ($\varphi_{\text{PSII}}$). rETR$_{\text{max}}$ and $E_k$ values for the aboral symbiont band were not calculated as saturation of electron transport was not achieved for the aboral layer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Position</th>
<th>RLC</th>
<th>LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>Oral</td>
<td>0.34</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>Aboral</td>
<td>0.27</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>52.4</td>
<td>82.6</td>
</tr>
<tr>
<td>rETR$_{\text{max}}$</td>
<td>Aboral</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>156.0</td>
<td>239.3</td>
</tr>
<tr>
<td>$E_k$</td>
<td>Aboral</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.63</td>
<td>0.59</td>
</tr>
<tr>
<td>$\varphi_{\text{PSII}}(\text{max})$</td>
<td>Aboral</td>
<td>0.58</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>$\varphi_{\text{PSII}}(\text{min})$</td>
<td>Aboral</td>
<td>0.50</td>
<td>0.49</td>
</tr>
</tbody>
</table>

References


Chapter 4

Radiative energy budgets of phototrophic surface-associated microbial communities and their photosynthetic efficiency under diffuse and collimated light

Published in *Frontiers in Microbiology*

by

Mads Lichtenberg, Kasper Elgetti Brodersen and Michael Kühl
Radiative energy budgets of phototrophic surface-associated microbial communities and their photosynthetic efficiency under diffuse and collimated light

Mads Lichtenberg¹, Kasper Elgetti Brodersen¹ and Michael Kühl¹,²
¹Marine Biological Section, University of Copenhagen, Denmark
²Climate Change Cluster (C3), University of Technology Sydney, Australia
¹These authors contributed equally to this work.

Abstract
We investigated the radiative energy budgets of a heterogeneous photosynthetic coral reef sediment and a compact uniform cyanobacterial biofilm on top of coastal sediment. By combining electrochemical, thermocouple and fiber-optic microsensor measurements of O₂, temperature and light, we could calculate the proportion of the absorbed light energy that was either dissipated as heat or conserved by photosynthesis. We show, across a range of different incident light regimes, that such radiative energy budgets are highly dominated by heat dissipation constituting up to 99.5% of the absorbed light energy. Highest photosynthetic energy conservation efficiency was found in the coral sediment under low light conditions and amounted to 18.1% of the absorbed light energy. Additionally, the effect of light directionality, i.e., diffuse or collimated light, on energy conversion efficiency was tested on the two surface-associated systems. The effects of light directionality on the radiative energy budgets of these phototrophic communities were not unanimous but, resulted in local spatial differences in heat-transfer, gross photosynthesis, and light distribution. The light acclimation index, Ek, i.e., the irradiance at the onset of saturation of photosynthesis, was >2 times higher in the coral sediment compared to the biofilm and changed the pattern of photosynthetic energy conservation under light-limiting conditions. At moderate to high incident irradiances, the photosynthetic conservation of absorbed energy was highest in collimated light; a tendency that changed in the biofilm under sub-saturating incident irradiances, where higher photosynthetic efficiencies were observed under diffuse light. The aim was to investigate how the physical structure and light propagation affected energy budgets and light utilization efficiencies in loosely organized vs. compact phototrophic sediment under diffuse and collimated light. Our results suggest that the optical properties and the structural organization of phytoelements are important traits affecting the photosynthetic efficiency of biofilms and sediments.
**Introduction**

Photosynthetic sediments and biofilms are characterized by pronounced vertical stratification of the microbial environment as a result of steep light gradients, high metabolic activity and limitations of heat and solute transport by diffusion (Kühl *et al.*, 1996; Kühl & Fenchel, 2000; Al-Najjar *et al.*, 2012). The radiative energy balance in such phototrophic microbial communities is affected by the incident radiative energy from the sun, of which a fraction is backscattered and thus not absorbed, while absorbed light energy is either photochemically conserved via photosynthesis or dissipated as heat via radiative energy transfer and non-photochemical quenching (Al-Najjar *et al.*, 2010; Brodersen *et al.*, 2014). The quantity and quality of light are the main controlling factors of photosynthesis, and the microscale distribution of light in microphytobenthic systems has been studied intensively over the last decades (Jørgensen & Des Marais, 1988; Lassen *et al.*, 1992b; Kühl & Jørgensen, 1994; Kühl, 2005). A sub-saturating flux of photons will limit the rate of photosynthesis, as the available light is insufficient to support the maximal potential rate of the light reactions but as the photon flux increases the photosynthetic system saturates, whereby O₂ becomes a competitive inhibitor on the binding-site of CO₂ to Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) (Falkowski & Raven, 2007). In addition, when light energy absorption exceeds the capacity for light utilization, excess energy is channelled into heat production via non-photochemical quenching (NPQ) processes to avoid degradation of pigments and other cell constituents e.g. by reactive singlet oxygen produced by the de-excitation of triplet state chlorophyll ("Chl") (Müller *et al.*, 2001).

Photosynthetic organisms deploy different mechanisms to avoid photo-damage, where NPQ is an effective short-term solution to dispose of excess energy (Müller *et al.*, 2001). If a photosynthetic cell experiences high light conditions on a daily basis, long-term regulation can be achieved by regulating light harvesting pigment composition and concentration (Nymark *et al.*, 2009). One strategy is to lower the light harvesting pigment content to decrease the absorption cross section by increasing transmittance, while another strategy involves upregulation of photoprotective pigments such as xanthophylls, that absorb energy-rich blue-green light but quench non-photochemically (Zhu *et al.*, 2010).

Since photosynthetic cells perceive light from all directions, the light field angularity is important for determining the total radiance experienced by a cell (Kühl & Jørgensen, 1994), and it has e.g. been shown that the incident light geometry can influence photosynthetic light use efficiencies and photoinhibition in terrestrial plant canopies (Gu *et al.*, 2002). In sediments, incident light will be spread by multiple scattering and, while the light field will become entirely diffuse with depth (Kühl & Jørgensen,
the response of benthic photosynthetic organisms to incident diffuse light is unknown. Through evaporation, an increase in cloud-cover has been predicted with global warming (Schiermeier, 2006), which will potentially change the direction of light from relatively collimated beams (~85% in clear-sky conditions) to a more isotropic diffuse light field (~100% in cloud covered conditions) (Bird & Riordan, 1986; Brodersen et al., 2008; Gorton et al., 2010). In addition, submerged benthic systems will experience temporal and spatial differences in light field isotropy depending on turbidity, water depth, sun angle and the reflective properties of the surrounding environment (Brakel, 1979; Kirk, 1994; Wangpraseurt & Kühl, 2014).

Increased rates of photosynthesis have been observed in forest communities with an increasing proportion of diffuse light, possibly due to a more even distribution of light in the canopy (Gu et al., 1999; Krakauer & Randerson, 2003; Misson et al., 2005; Urban et al., 2007), whereby light energy is more efficiently harvested from all directions deeper in the canopy. However, at the single leaf scale a 2-3% lower absorptance was found under diffuse light as compared to collimated light at equivalent incident irradiances (Brodersen & Vogelmann, 2007). In corals, it has been observed that gross photosynthesis increased ~2-fold under collimated compared to diffuse light of identical downwelling irradiance (Wangpraseurt & Kühl, 2014) and the directional quality of light may thus elicit different photosynthetic responses and could potentially change the photosynthetic efficiency. A factor that could contribute to differences in photosynthetic activity under diffuse and collimated light is photoinhibition, which occurs when the electron transport chain is fully reduced and the photosystems are light saturated (Murata et al., 2007). Under high collimated light conditions, chloroplasts in leaves move to periclinal walls, and this might lead to decreased photoinhibition due to shading of other chloroplasts (Gorton et al., 1999). Under diffuse light, chloroplast movement to the periclinal walls is not complete (Williams et al., 2003) and thus distributed more randomly, which could lead to less effective self-shading and photoprotection (Brodersen et al., 2008).

The balance between photosynthesis and respiration and as such, light use efficiency in benthic phototrophic systems is also influenced by the thickness of the diffusive and thermal boundary layers (Jørgensen & Des Marais, 1990; Jimenez et al., 2011; Brodersen et al., 2014). The diffusive boundary layer (DBL) is a thin water layer over submerged objects through which molecular diffusion is the dominant transport mechanism controlling the exchange of dissolved gases (e.g. O₂ and CO₂) and solutes with the ambient water (Jørgensen & Des Marais, 1990; Shashar et al., 1996). The DBL can thus impose a major control on respiration and photosynthesis in aquatic environments. Dissipation of absorbed solar radiation as heat drives an increase in surface temperature that is counter-balanced by
heat transfer to the surrounding water via a thermal boundary layer (TBL), where convection dominates the transport of heat and the surface warming increases linearly with the incident irradiance (Jimenez et al., 2008). Heat and mass transfer phenomena through boundary layers are therefore important processes when considering rates of photosynthesis and radiative energy budgets.

![Figure 1. Major pathways of light energy conversion and dissipation in biofilm and coral sediment.](image)

Incident irradiance was either diffuse or collimated (top yellow arrows) and supplied the sediments with the incoming energy flux, $J_{IN}$ (solid yellow arrow). A fraction of the incoming light energy was reflected from the surface and thereby not a part of the absorbed light energy ($J_{ABS}$). Through multiple scattering by biotic and abiotic components in the biofilm/sediment, the light field becomes increasingly diffuse with depth. The absorbed light energy is either photochemically conserved in photosynthesis ($J_{PS}$) in the photic zone or dissipated as heat ($J_{H}$) via radiative energy transfer and non-photochemical quenching leading to local heating in the biofilm/sediment (red line). Gross photosynthesis (blue bars) is dependent on light and is thus higher near the surface which drives a production of $O_2$ (blue line) that exceeds the consumption via respiration and leads to the formation of a diffusive boundary layer (DBL). The surplus of $O_2$, i.e., the net photosynthesis, can be calculated as the difference between the upwards ($J_{O_2(up)}$) and downwards ($J_{O_2(down)}$) flux of $O_2$ from the photic zone. Similarly, the fraction of the absorbed energy that is dissipated as heat can be calculated as the difference between upwards ($J_{H(up)}$) and downwards ($J_{H(down)}$) heat flux through the thermal boundary layer (TBL) into the overlaying water and into the aphotic sediment/biofilm layer, respectively.
In the present study, we present the first radiative energy budget of a heterogeneous coral reef sediment and compare it with the energy budget of a compact photosynthetic biofilm on a coastal sediment, and we investigate how diffuse and collimated light fields with identical levels of incident irradiance affect the radiative energy budget of the two microphytobenthic systems. Our analysis is based on a modified experimental approach first described by Al-Najjar et al. (2010).

**Materials and methods**

**Sample sites and collection**

Coral reef sediment was sampled in April 2012 from a sheltered pseudo-lagoon (‘Shark Bay’ (Werner et al., 2006) on the reef flat surrounding Heron Island (151°55' E, 23°26' S) that is located on the southern boundary of the Great Barrier Reef, Australia. Maximal incident solar photon irradiance at the sediment surface of the shallow reef flat during calm mid-day low tides is ~1500-2000 µmol photons m\(^{-2}\) s\(^{-1}\) (Jimenez et al., 2012; Wangpraseurt et al., 2014b). The coral sediment (CS) was mainly composed of bright, semi-fine grained particles (mostly in the 200–500 µm size fraction) of deposited CaCO\(_3\) from decomposed corals and other calcifying reef organisms. Diatoms, dinoflagellates and cyanobacteria were found as dispersed aggregates in the sediment pore space along with amorphic organic material (detritus) throughout the upper few mm of the sediment (Fig. S1).

The biofilm (BF) originated from a shallow sand bar at Aggersund, Limfjorden (Denmark) experiencing maximum summer photon irradiance of 1000-1500 µmol photons m\(^{-2}\) s\(^{-1}\). The biofilm was comprised of a ~1 mm thick smooth layer of photosynthetically active filamentous cyanobacteria and microalgae (*Microchlorella chtonoplastes, Oscillatoria spp.*, and pennate diatoms) embedded in exopolymers on top of fine-grained (125-250 µm) dark sulfidic sandy sediment (Lassen et al., 1992a; Nielsen et al., 2015). Coral reefs are usually considered oligotrophic but around Heron Island NH\(_3\) and PO\(_4\) concentrations of ~0.3 mg L\(^{-1}\) and ~0.1 mg L\(^{-1}\) (corresponding to ~17 µmol L\(^{-1}\) NH\(_3\) and ~1 µmol L\(^{-1}\) PO\(_4\)) have been found (Smith & Johnson, 1995) which is lower but, in the same order as what is found in Danish waters (see e.g. Figure 16.1 in Henriksen et al. (2001)). In our experiments, we used a recirculating system containing 20L of seawater (see below) which was changed daily. We therefore do not estimate that nutrient concentrations had a large impact on production between the two systems.

The porosity of the coral sediment and biofilm, \(\phi\), was 0.78 and 0.80, respectively, as determined from the weight loss of wet sediment (known initial volume and weight) after drying at 60°C until a constant weight was reached.
\[ \phi = \frac{M_W}{D_W} \left( \frac{M_W}{D_W} + \frac{M_S}{D_S} \right) \]  

where \( M_W \) is the weight of water, \( D_W \) is the density of water, \( M_S \) is the weight of sediment/biofilm, and \( D_S \) is the sediment/biofilm density.

**Coral Sediment samples**

The CS samples were collected with Perspex corers (inner diameter 5.3 cm), and were maintained under a continuous flow of aerated seawater at ambient temperature and salinity (26°C and S=35) under a natural solar light regime for ~24 h prior to further handling at the Heron Island Research Station (HIRS), Australia. Sediment cores were then mounted in a custom-made flow-chamber flushed with aerated seawater (26°C and S=35) for another 24 h prior to measurements. The flow-chamber (interior dimensions: 25 cm × 8 cm × 8 cm) had a honeycomb baffle between the water inlet and the sample, ensuring a stable laminar flow (see more details in Lichtenberg et al. (2016)). During the acclimation time in the flow-chamber, the sediment cores were kept under a downwelling photon irradiance of ~1000 µmol photons m\(^{-2}\) s\(^{-1}\) provided by a fiber-optic tungsten halogen lamp equipped with a collimating lens (KL2500-LCD, Schott GmbH, Germany). Before measurement at each experimental irradiance, the coral sediment core was illuminated for at least 45 minutes to ensure steady state O\(_2\) and temperature conditions; as confirmed from repeated microprofile measurements. Throughout measurements, the flow-chamber was flushed with a stable laminar flow (~0.5 cm s\(^{-1}\)) of filtered aerated seawater over the sediment surface as generated by a Fluval U1 pump submerged in a 20L thermostated aquarium (26°C and S=35) and connected with tubing to the flow-chamber.

**Biofilm samples**

The BF samples were collected and contained in small rectangular plastic trays (7 × 2 × 5 cm) with the upper surface exposed and flush with the upper edge of the tray wall. After collection, the samples were kept humid and under a 12:12 h light-dark regime (~100 µmol photons m\(^{-2}\) s\(^{-1}\)) in a thermostated room (16-18°C). The biofilm surface appeared dark green–brownish due to predominance of dense communities of cyanobacteria and diatoms (Lassen et al., 1992a). Prior to measurements, a sample tray was placed for 2 days in a flow-chamber flushed with 0.2 µm filtered aerated seawater (21°C, S=30) under a downwelling photon irradiance of ~500 µmol photons m\(^{-2}\) s\(^{-1}\). During measurements, a stable laminar flow (~0.5 cm s\(^{-1}\)) over the biofilm surface was maintained by a water pump (Fluval U1, Hagen...
GmbH, Germany) immersed in a 20L aquarium with filtered aerated seawater (21°C, S=30) and connected with tubing to the flow-chamber.

**Experimental setup**

Illumination was provided by a fiber-optic tungsten halogen lamp equipped with a collimating lens (KL-2500 LCD, Schott, Germany) positioned vertically above the flow-chamber. A spectrum of the used halogen lamp can be found in the Suppl. Info. in Lichtenberg et al. (2016) and is compared to typical solar spectrum measured on Heron Island reef flat in the Suppl. Info. in Wangpraseurt et al. (2014a), who found no major spectral effects on gross photosynthesis measurements. The intensity of the lamp could be controlled without spectral distortion by a built-in filter wheel with pinholes of various sizes. The downwelling photon irradiance of photosynthetically active radiation (PAR, 400-700nm), $E_d$(PAR), (see definitions of abbreviations) was measured with a calibrated irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a cosine collector (LI-192S, LiCor, USA). Defined experimental irradiances (0, 50, 100, 200, 500 and 1000 µmol photons m$^{-2}$ s$^{-1}$) were achieved by adjusting the aperture on the fiber-optic lamp. The downwelling spectral irradiance at the above-mentioned levels was also measured in radiometric energy units (in W m$^{-2}$ nm$^{-1}$) with a calibrated spectroradiometer (Jaz, Ocean Optics, USA).

Collimated light was achieved by attaching a collimating lens to the fiber cable of the lamp. Diffuse light was achieved by inserting a TRIMMS diffuser (Transparent Refractive Index Matched Microparticles) (Smith et al., 2003) between the collimator and the sample followed by lamp adjustment to achieve the same absolute levels of downwelling irradiance on the biofilm/sediment surface in collimated and diffuse light treatments.

**Microscale measurements of O$_2$ and temperature.**

Oxygen concentrations were measured with a Clark-type O$_2$ microsensor (tip diameter $\sim$25 µm, OX-25, Unisense AS, Aarhus, Denmark) with a fast response time (<0.5 s) and a low stirring sensitivity (<1-2%) (Revsbech, 1989). The microsensor was connected to a pA-meter (Unisense A/S, Aarhus, Denmark) and was linearly calibrated at experimental temperature and salinity from measurements in the aerated seawater in the free-flowing part of the flow-chamber and in anoxic layers of the sediment. Temperature measurements were performed with a thermocouple microsensor (tip diameter $\sim$50 µm; T50, Unisense A/S, Aarhus, Denmark) connected to a thermocouple meter (Unisense A/S, Aarhus, Denmark). The temperature microsensors were linearly calibrated against readings of a high precision
thermometer (Testo 110, Testo AG, Germany; accuracy ±0.2°C) in seawater at different temperatures. Analogue outputs from the temperature and O₂ microsensor meters were connected to an A/D converter (DCR-16, Pyroscience GmbH, Germany), which was connected to a PC. All microsensors were mounted in a PC-interfaced motorized micromanipulator (MU-1, PyroScience, GmbH, Germany) controlled by dedicated data acquisition and positioning software (ProFix, Pyroscience, Germany). The micromanipulator was oriented in a 45° angle relative to the vertically incident light to avoid self-shading, especially in the light measurements. Depth profiles of temperature and O₂ concentration were measured in vertical steps of 100 µm. Before profiling, the microsensor tips were manually positioned on the sample surface to define the z=0 position, determined from visual detection through a stereo microscope. The precisions of this approach is about ± average grain size of the sediments, i.e., 125-500 µm.

### Definition of abbreviations and parameters

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL</td>
<td>Diffusive boundary layer</td>
<td></td>
</tr>
<tr>
<td>TBL</td>
<td>Thermal boundary layer</td>
<td></td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetically active radiation (420-700nm)</td>
<td></td>
</tr>
<tr>
<td>BF</td>
<td>Biofilm from Limfjorden, Denmark</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>Coral sediment from Heron Island lagoon, Australia</td>
<td></td>
</tr>
<tr>
<td>Jₚₚₚ</td>
<td>Upward O₂ flux from photic zone</td>
<td>nmol O₂ m⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₚₚₚₚ</td>
<td>Downward O₂ flux from photic zone</td>
<td>nmol O₂ m⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₚₚ₂</td>
<td>Total net photosynthesis in photic zone (Jₚₚₚ - Jₚₚₚₚ)</td>
<td>nmol O₂ m⁻² s⁻¹</td>
</tr>
<tr>
<td>ϕ</td>
<td>Sediment porosity</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>PGₙ(λ)</td>
<td>Volume-specific rate of gross photosynthesis</td>
<td>nmol O₂ cm⁻³ s⁻¹</td>
</tr>
<tr>
<td>PGₙ</td>
<td>Area-specific rate of gross photosynthesis</td>
<td>nmol O₂ cm⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₚₙ</td>
<td>Area-specific rate of gross photosynthesis in energy terms</td>
<td>J m⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₜₜ</td>
<td>Upward heat flux from photic zone</td>
<td>J m⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₜₜₜ</td>
<td>Downward heat flux from photic zone</td>
<td>J m⁻² s⁻¹</td>
</tr>
<tr>
<td>Jₜₜₚ</td>
<td>Total heat flux out of photic zone (Jₜₜ + Jₜₜₚ)</td>
<td>J m⁻² s⁻¹</td>
</tr>
<tr>
<td>Eₜ(λ)</td>
<td>Spectral downwelling irradiance</td>
<td>Counts nm⁻¹</td>
</tr>
<tr>
<td>Eₜ(λ)</td>
<td>Spectral scalar irradiance</td>
<td>% of Eₜ(λ)</td>
</tr>
<tr>
<td>Jₚₚ</td>
<td>Absorbed light energy</td>
<td>J m⁻² s⁻¹</td>
</tr>
<tr>
<td>R</td>
<td>Reflection</td>
<td>%</td>
</tr>
<tr>
<td>Kₜ(PAR)</td>
<td>Diffuse attenuation coefficient of PAR scalar irradiance</td>
<td>mm⁻¹</td>
</tr>
<tr>
<td>Kₜ(λ)</td>
<td>Spectral attenuation coefficient of scalar irradiance</td>
<td>mm⁻¹</td>
</tr>
<tr>
<td>Eₜ</td>
<td>Photochemical light acclimation index, i.e. the irradiance at onset of photosynthesis saturation</td>
<td>µmol photons m⁻² s⁻¹</td>
</tr>
</tbody>
</table>
The local volumetric rates of gross photosynthesis \( P_G(z); \) in units of nmol O\(_2\) cm\(^{-3}\) s\(^{-1}\)) were measured with O\(_2\) microsensors using the light-dark shift method (Revsbech & Jørgensen, 1983). Volumetric rates were measured in vertical steps of 100 µm throughout the sediment until no photosynthetic activity in the given depth was detected. The immediate O\(_2\) depletion rate upon brief (2-4 s) darkening equalled the local rate of photosynthesis just prior to darkening; while no response in the O\(_2\) signal upon darkening indicated a zero rate of photosynthesis. Areal rates of gross photosynthesis (in nmol O\(_2\) cm\(^{-2}\) s\(^{-1}\)) were calculated by depth integration over the euphotic zone with respect to the measuring interval used in the depth profile measurement of \( P_G(z)\), similar to Al-Najjar et al. (2010); Al-Najjar et al. (2012):

\[
P_G = \Delta z \cdot \sum P_G(z)
\]  

(2)

**Temperature and O\(_2\) calculations.**

The net upward flux of O\(_2\) from the photic zone of the sediments into the overlaying seawater was calculated (in nmol O\(_2\) cm\(^{-2}\) s\(^{-1}\)) from measured steady-state O\(_2\) concentration profiles using Fick’s first law of diffusion:

\[
J_{NPP↑} = -D_0 \frac{\partial c}{\partial z}
\]  

(3)

where \( D_0 \) is the diffusion coefficient of O\(_2\) in seawater at experimental temperature and salinity and \( \frac{\partial c}{\partial z} \) is the linear O\(_2\) concentration gradient in the DBL.

The downward O\(_2\) flux from the photic zone of the sediments to the aphotic part of the sediment/biofilm was calculated in a similar manner as:

\[
J_{NPP↓} = -\phi D_0 \frac{\partial c}{\partial z}
\]  

(4)

The total flux of O\(_2\) out of the photic zone, i.e., the total net photosynthesis in the photic zone (NPP), was subsequently calculated as the difference between the upward and downward O\(_2\) flux (Kühl et al., 1996).

To calculate the radiative energy conserved via photosynthesis \( J_{PS} \) (in J m\(^{-2}\) s\(^{-1}\)) we multiplied the areal gross photosynthesis, GPP, with the Gibbs free energy formed in the light-dependent reactions, where O\(_2\) is formed by splitting water, which gains (including the formation of ATP) a Gibbs free energy of \( E_G = 482.9 \text{ kJ (mol O}_2\text{)}^{-1} \) (Thauer et al., 1977).

\[
J_{PS} = J_{GPP} E_G
\]  

(5)
The amount of the absorbed light energy that was not photochemically conserved was dissipated as heat resulting in a local increase of the sediment/biofilm temperature relatively to the ambient seawater and thereby leading to the establishment of a thermal boundary layer (TBL). The heat dissipation, i.e., the heat flux (in J m\(^{-2}\) s\(^{-1}\)) from the sediment/biofilm into the water column was calculated by Fourier’s law of conduction:

\[
J_{H1} = k \frac{dT}{dz}
\]  

(6)

where \(k\) is the thermal conductivity in seawater (0.6 W m\(^{-1}\) K\(^{-1}\)) and \(dT/dz\) is the measured linear temperature gradient in the TBL (Jimenez et al., 2008). The heat flux from the photic zone into the aphotic sediment/biofilm, \(J_{H2}\), was calculated as in Eq. 6 but with the thermal conductivity constant of the sediment, \(k(b)\), which was estimated as:

\[
k(b) = k_{s}^{(1-\phi)} k_{f} \phi
\]  

(7)

where \(k_{s}\) is the carbonate thermal conductivity (3.1 W m\(^{-1}\) K\(^{-1}\); (Clauser & Huenges, 1995)), \(k_{f}\) is the seawater thermal conductivity, and \(\phi\) is the porosity of the sediment (Lovell, 1985).

The total heat flux, was used as an estimate of the total heat dissipation in the photic zone and was calculated as

\[J_{H} = J_{H1} - J_{H2}\]

**Microscale light measurements.**

Spectral photon scalar irradiance was measured in units of counts nm\(^{-1}\) with a fiber-optic scalar irradiance microprobe (integrating sphere diameter \(~100\ \mu m\); (Lassen et al., 1992b)) connected to a fiber-optic spectrometer (USB2000, Ocean Optics, Dunedin, FL, USA). A black non-reflective light-well was used to record spectra of the downwelling photon scalar irradiance, \(E_{d}(\lambda)\), (in units of counts nm\(^{-1}\)) with the tip of the scalar irradiance microsensor positioned in the light path at the same distance from the light source as the sediment surface. Using identical light settings, the absolute downwelling irradiance, \(E_{ABS}(\lambda)\) (in W m\(^{-2}\)) was also quantified with a calibrated spectroradiometer (Jaz-ULM, Ocean Optics, Dunedin, Florida, USA).

**Irradiance calculations.**

The spectral scalar irradiance, \(E_{o}(\lambda)\), was measured in vertical steps of 0.1-0.2 mm in the sediment and was calculated as the fraction of the incident downwelling irradiance, i.e., \(E_{o}(\lambda)/E_{d}(\lambda)\), and plotted as transmittance spectra in % of \(E_{o}(\lambda)\). The relative measurements of scalar irradiance in different depths in the biofilm/sediment were converted to absolute scalar irradiance spectra in units of W m\(^{-2}\) nm\(^{-1}\) as
Absolute scalar irradiance spectra were converted to photon scalar irradiance spectra (in units of µmol photons m\(^{-2}\) s\(^{-1}\) nm\(^{-1}\)) by using Planck’s equation:

\[ E_\lambda = h \frac{c}{\lambda} \]

(8)

where \( E_\lambda \) is the energy of a photon with wavelength, \( \lambda \), \( h \) is Planck’s constant \((6.626 \times 10^{-34} \text{ W s}^2)\), and \( c \) is the speed of light in vacuum \((\text{in m s}^{-1})\).

Spectral attenuation coefficients of scalar irradiance, \( K_0(\lambda) \), were calculated as \((\text{Kühl, 2005})\):

\[ K_0(\lambda) = -\ln \left( \frac{E_0(\lambda)_1/E_0(\lambda)_2}{z_2-z_1} \right) \]

(9)

where \( E_0(\lambda)_1 \) and \( E_0(\lambda)_2 \) are the spectral scalar irradiances measured at depth \( z_1 \) and \( z_2 \), respectively.

Light attenuation was also calculated by integrating the spectral quantum irradiance over PAR (420-700 nm) yielding the PAR scalar irradiance \((E_0(PAR))\), in µmol photons m\(^{-2}\) s\(^{-1}\), i.e., the light energy available for oxygenic photosynthesis at each measurement depth. The diffuse attenuation coefficient of \( E_0(PAR) \), \( K_0(PAR) \), was obtained by fitting the measured \( E_0(PAR) \) vs. depth profiles with an exponential model:

\[ E_0(z) = E_0(0)e^{-K_0(PAR)(z-z(0))} \]

(10)

Reflectance measurements.

The PAR irradiance reflectance \((R)\) of the sediment/biofilm surface was calculated as

\[ R(PAR) = \int_{420}^{700} \frac{E_u(\lambda)}{E_d(\lambda)} \, d\lambda \]

(11)

where \( E_u(\lambda) \) is the upwelling irradiance at the sediment surface, here estimated as the diffuse backscattered spectral radiance measured at the sediment surface \((\text{Kühl, 2005})\) and \( E_d(\lambda) \) is the downwelling irradiance estimated as the backscattered spectral radiance measured over a white reflectance standard \((\text{Spectralon; Labsphere, North Sutton, NH, USA})\); both measured with a fiber-optic field radiance microprobe \((\text{Jørgensen & Des Marais, 1988})\). The \( R(PAR) \) measurements assumed that the light backscattered from the sediment/biofilm surface was completely diffused \((\text{Kühl & Jørgensen, 1994})\).

Absorbed light energy.

The absorbed light energy \((J_{ABS})\)\((\text{in W m}^{-2} = J \text{ m}^{-2} \text{ s}^{-1})\) in the sediment/biofilm was estimated by subtracting the downwelling and upwelling irradiance at the surface:

\[ J_{ABS} = \int_{420}^{700} E_d(\lambda)(1 - R(\lambda)) \, d\lambda \]

(12)
where \( E_o(\lambda) \) and \( R(\lambda) \) are the downwelling spectral irradiance and irradiance reflectance, respectively. This parameter is equivalent to the so-called vector irradiance, which is a measure of the net downwelling radiative energy flux.

**Energy budget and photosynthetic efficiency calculations.**

A balanced radiative energy budget of the sediment/biofilm was calculated according to (Al-Najjar et al., 2010) with slight modifications (Fig. 1) as:

\[
J_{\text{ABS}} = J_{\text{H}} + J_{\text{PS}}
\]  

(13)

assuming that autofluorescence from the sediment/biofilm was negligible. Consequently, \( \varepsilon_{\text{PS}} + \varepsilon_{\text{H}} = 1 \), where \( \varepsilon_{\text{PS}} \) and \( \varepsilon_{\text{H}} \) represent the efficiency of photosynthetic energy conservation and heat dissipation, respectively, for a given absorbed light energy \( J_{\text{ABS}} \) in the entire euphotic zone (Al-Najjar et al., 2010):

\[
\varepsilon_{\text{PS}} = \frac{J_{\text{PS}}(J_{\text{ABS}})}{J_{\text{ABS}}} \quad \text{and} \quad \varepsilon_{\text{H}} = \frac{J_{\text{H}}(J_{\text{ABS}})}{J_{\text{ABS}}}
\]  

(14)

Areal gross photosynthesis rates as a function of \( J_{\text{ABS}} \), were fitted with the saturated exponential model (Webb et al., 1974) to estimate the maximum conserved energy flux by photosynthesis \( (J_{\text{PS}})_{\text{max}} \) (in J m\(^{-2}\) s\(^{-1}\)):

\[
J_{\text{PS}}(J_{\text{ABS}}) = J_{\text{PS, max}}(1 - e^{-J_{\text{ABS}}/E_k})
\]  

(15)

This yielded an estimate of the maximum photochemically conserved energy flux \( J_{\text{PS, max}} \). The respective efficiencies under light-limiting conditions, i.e., for \( J_{\text{ABS}} \rightarrow 0 \), were then calculated as:

\[
\varepsilon_{\text{PS, max}} = \frac{J_{\text{PS, max}}}{E_k} \quad \text{and} \quad \varepsilon_{\text{H, min}} = 1 - \varepsilon_{\text{PS, max}}
\]  

(16)

where \( E_k \) is the photochemical light acclimation index, i.e., the irradiance at the onset of photosynthetic saturation, calculated as \( E_k = J_{\text{PS, max}} / \alpha \), where \( \alpha \) is the initial slope of the fitted photosynthesis vs. \( J_{\text{ABS}} \) curve.
Results

Light environment
At all incident irradiances, the photon scalar irradiance, $E_0$(PAR), decreased with increasing sediment depth (Fig. 2). Light attenuation was strongly enhanced around wavelengths 625 nm and 670 nm, corresponding to absorption maxima of phycocyanin and Chl a, respectively (Fig. 3). Surface reflection from the biofilm surface was on average 1.8% and 1.7% of the incident PAR under diffuse and collimated light, respectively, while it was >15 times higher in the coral sediment, i.e., 30.2% and 28.1% for diffuse and collimated light, respectively. Reflection did not change with increasing irradiance (Fig. S2). The profiles of scalar irradiance showed non-uniform attenuation with depth and could be influenced by local enhancement of photon pathlength (Kühl & Jørgensen, 1994; Kühl et al., 1997) in the uppermost layers (Fig. 2). At the highest incident photon irradiances (500 and 1000 µmol photons m$^{-2}$ s$^{-1}$), the exponential attenuation of collimated light within the biofilm was observed below 0.2 mm, whereas diffuse light was attenuated exponentially from the biofilm surface under all investigated irradiance levels (Fig. 2). In the coral sediment, the exponential attenuation occurred deeper (below 0.5-0.7 mm) due to enhanced scattering, redistribution and trapping of photons in the upper sediment layers (Fig. 2). In the biofilm, PAR attenuation was stronger in the top layer than in the bottom layer both for diffuse and collimated light (Fig. 2). Additionally, attenuation of collimated light in the top layer was stronger than for diffuse light at all irradiances except 1000 µmol photons m$^{-2}$ s$^{-1}$, whereas light attenuation in the lower sediment dominated layers was similar for diffuse and collimated incident light. In the coral sediment no distinct differences in light attenuation was observed between top- and bottom layers other than a deeper onset of exponential attenuation (0.5-0.7 mm). The top layer of the biofilm showed ~10 times stronger light attenuation than the coral sediment with average PAR attenuation coefficient of $\alpha = 9.52$ mm$^{-1}$ and $\alpha = 10.54$ mm$^{-1}$ for diffuse and collimated light, respectively, compared to $\alpha = 1.18$ mm$^{-1}$ in the coral sediment (both light types).
In both sediments, attenuation of light corresponded to absorption maxima of Chl a (440 and 670 nm) and phycocyanin (620 nm) (Fig. 3). A third attenuation maximum was observed around 575 nm indicative of phycoerythrin, commonly found in cyanobacteria (Colyer et al., 2005). In the biofilm, attenuation of visible light was strongest in the top 0.3 mm of the biofilm, except under the highest collimated irradiance (1000 µmol photons m^{-2} s^{-1}), where the strongest attenuation occurred over the 0.3-0.6 mm zone (Fig. 3). Below 0.6 mm, the enhanced attenuation around wavelengths 575 nm, 625 nm and 670 nm decreased and the attenuation of light in the PAR region became more uniform in the underlying layers (Fig. 3). Again, attenuation of collimated light was slightly higher than diffuse light.

In the coral sediment, the highest light attenuation was 1-2 mm below the sediment surface (~1.6 mm\(^{-1}\) at 670 nm at all incident irradiances) while the lowest attenuation was found in the upper 0.1 mm, consistent with the scalar irradiance profiles (Fig. 2 and 3).
Temperature and O2 microenvironment
In the biofilm, a ~0.8 mm thick diffusive boundary layer (DBL) developed between the biofilm and the surrounding water (Fig. 1 and 4). In dark, O2 was depleted within the upper 1.5 mm and the areal dark respiration rate was calculated to 0.039 nmol O2 cm^-2 s^-1. The fluxes of O2 increased with irradiance until saturation was reached at a downwelling photon irradiance of ~100 µmol photons m^-2 s^-1, where the top of the biofilm experienced O2 concentrations >450% of air saturation (Fig. 4). The O2 concentration profiles for diffuse and collimated light were similar, although O2 penetrated deeper under diffuse light, especially at the highest photon irradiances (500 and 1000 µmol photons m^-2 s^-1) (Fig. 4). The coral sediment had a ~1-1.4 mm thick DBL; dark respiration was similar to the biofilm (0.037 nmol O2 cm^-2 s^-1), while saturation of photosynthesis was reached at a higher downwelling photon irradiance of ~200 µmol photons m^-2 s^-1 (Fig. 3). The similar dark respiratory O2 uptake in sediment and biofilm indicated that the combined respiration of autotrophic and heterotrophic organisms was of similar magnitude in the two systems. The more variable DBL thickness in the coral sediment varied independently of irradiance and was likely a result of the heterogeneous surface topography (Fig. 4).

Figure 3. Spectral attenuation coefficients, $K_o(\lambda)(\text{PAR})$ of photon scalar irradiance calculated over 300 µm (biofilm; upper panels) and 1000 µm (coral sediment; lower panels) depth intervals. Numbers in panels indicate incident photon irradiance in µmol photons m^-2 s^-1, while the letters C and D denote collimated and diffuse incident light, respectively. Curves represent averages (n=3; S.D. not shown for clarity).
A detailed mapping of the DBL landscape was beyond the scope of this study but, we estimate that the mass transfer between the sediment and overlying water was not influenced by turbulences which would have been evident as non-linear concentration gradients between sediment surface and bulk water (Lichtenberg et al., 2017). At incident irradiances >200 µmol photons m⁻² s⁻¹ the O₂ productive zone was stratified under both diffuse and collimated light, with an O₂ concentration maximum of ~600% air saturation ~1.7 mm below the sediment surface (Fig. 4). Photosynthesis was apparently distributed in two major layers, a ~0.5 mm thick layer at the sediment surface, and a ~1 mm thick layer peaking 2 mm below the sediment surface (Fig. 4 and S3). The O₂ concentration profiles for diffuse and collimated light were similar at low to moderate irradiance, then showed a deeper O₂ penetration depth under diffuse light at incident irradiance >500 µmol photons m⁻² s⁻¹ in comparison to O₂ profiles measured under collimated light (Fig. 4). The O₂ profiles in the coral sediment showed high standard deviations, possibly due to a more patchy distribution of the photosynthetic organisms within the sediment and overall variability in the sediment grain size and surface topography.

Figure 4. Vertical microprofiles of O₂ concentration in biofilm (upper panel) and coral sediment (lower panel). Red and black symbols represent measurements under diffuse and collimated light, respectively, while numbers in panels denote downwelling photon irradiance in µmol photons m⁻² s⁻¹. The line in y=0 indicates the biofilm/sediment surface. Symbols represent mean values, while dashed lines represent ± 1 S.D. (n=3).
In both biofilm and coral sediment, the surface temperature increased relative to the overlaying seawater with increasing irradiance. The local heating was dissipated by heat transfer over a ~3 mm thick thermal boundary layer (TBL) into the overlaying seawater and into deeper sediment layers (Fig. 5 and 6). Robust measurements of biofilm/sediment heating could only be obtained at incident photon irradiances of \( \geq 200 \text{ µmol photons m}^{-2} \text{s}^{-1} \) (\( \geq 500 \text{ µmol photons m}^{-2} \text{s}^{-1} \) for the coral sediment under collimated light).

At the highest irradiance (1000 \( \text{µmol photons m}^{-2} \text{s}^{-1} \)), the biofilm surface was 0.51 ± 0.036°C and 0.41 ± 0.008°C warmer than the overlaying water, while the coral sediment surface was 0.53 ± 0.031°C and 0.48 ± 0.040°C warmer than the surrounding water for diffuse and collimated light, respectively. Similar temperature profiles were observed between collimated and diffuse light, although a slightly enhanced surface heating and thus a higher efflux of heat was observed under diffuse light (Fig. 5). Comparing the slope of the surface warming vs. vector irradiance under diffuse and collimated light, respectively, diffuse light had a greater impact on surface warming by 30% and 27% in the biofilm and in the coral sediment, respectively (Fig. 6).

Figure 5. Vertical depth profiles of temperature change. \( \Delta T \) (in °C) measured in biofilm (upper panels) and coral sediment (lower panel) at downwelling photon irradiances of 0, 200, 500 and 1000 \( \text{µmol photons m}^{-2} \text{s}^{-1} \) under collimated (A, C) and diffuse light (B, D). Symbols represent means, while dashed lines indicate ± 1 S.D. (\( n=3 \)). The dotted line in y=0 indicates the sediment surface, while the dotted line in x=0 indicates a 0°C temperature change.
Maximal volume-specific gross photosynthesis rates of the biofilm ranged between 7.0 nmol O₂ cm⁻³ s⁻¹ and 8.7 nmol O₂ cm⁻³ s⁻¹ (collimated and diffuse light, respectively) under low irradiance (50-200 µmol photons m⁻² s⁻¹), while rates decreased at photon irradiances of >200 µmol photons m⁻² s⁻¹ (Fig. S3A). The thickness of the photic zone generally increased with increasing photon irradiance and varied from 0.4-1.2 mm in the biofilm under diffuse light and from 0.2-0.9 mm under collimated light.

In the coral sediment, the highest volume-specific rates of photosynthesis were measured within the upper 1 mm, with maximal gross photosynthesis rates of 11.97 nmol O₂ cm⁻³ s⁻¹ at the sediment surface under collimated light and 3.05 nmol O₂ cm⁻³ s⁻¹ at a depth of 0.6 mm under diffuse light (Fig. S3B). The photic zone in the coral sediment increased with increasing irradiance and ranged in thickness from 1.5 to 3 mm under diffuse light and from 2 to 3.5 mm under collimated light. The apparent stratification in O₂ concentration found in the coral sediment was confirmed in the profiles of gross photosynthesis with peaks in gross photosynthesis in the upper 1 mm and 1.5-2.5 mm from the surface at photon irradiances >50 µmol photons m⁻² s⁻¹ (Fig. S3B).

Under low photon irradiance <200 µmol photons m⁻² s⁻¹ in the biofilm, the area specific gross photosynthesis rate (PG) was higher under diffusive illumination, while PG under diffuse and collimated illumination were similar at higher irradiances (Fig. 6). In contrast, PG in the coral sediment was generally in the range of 3-4 times lower under diffuse- compared to collimated light (Fig. S3B; Fig. 6B). We note that the gross photosynthesis measurements in the coral sediment under diffuse light were performed at the University of Technology Sydney (UTS) rather than on Heron Island Research Station (HIRS), where the rest of the measurements took place. We speculate that the transport from Heron Island created prolonged anoxic conditions throughout the sediment and this might have caused a change in community composition and structure of the sediment. These measurements were therefore excluded when calculating the light energy budget for diffuse light in the coral sediment.
Figure 6. Energy conversion by photosynthesis, heat dissipation and the sum of photosynthesis and heat dissipation versus downwelling irradiance in biofilm (left panels) and corals sediment (right panels). Red symbols and lines show data for diffuse illumination, while black symbols and lines show data for collimated illumination. A, B) Areal gross photosynthesis rates (in J m⁻² s⁻¹) measured at downwelling photon irradiances of 0, 50, 100, 200, 500 and 1000 µmol photons m⁻² s⁻¹, and then fitted with a saturated exponential model (Webb et al., 1974); CS: $R^2_{\text{diff}} = 0.92$, $R^2_{\text{coll}} = 0.97$; BF: $R^2 = 0.88$ for both diffuse and collimated; $n = 3$). C, D) Temperature gradients (in °C) between the ambient seawater and the sediment surface (flow = 0.3-0.4 cm s⁻¹), measured at vector irradiances of 30, 75 and 149 J m⁻² s⁻¹ or 40, 100 and 200 J m⁻² s⁻¹ for the coral sediment and biofilm, respectively. Data points show means ± SD ($n = 3$); CS: $R^2_{\text{diff}} = 0.99$, $R^2_{\text{coll}} = 0.96$; BF: $R^2 = 0.99$ for both diffuse and collimated light. E, F) The summed energy dissipation of the system (in J m⁻² s⁻¹), i.e., the sum of energy conserved by photosynthesis and energy dissipated as heat, measured at vector irradiances of 30, 75 and 149 J m⁻² s⁻¹ and 40, 100 and 200 J m⁻² s⁻¹ for the coral sediment and biofilm, respectively. The dashed line represents a 1:1 relationship between the incoming and outgoing energy of the system (i.e. the theoretically expected relationship). CS: $R^2_{\text{diff}} = 0.99$, $R^2_{\text{coll}} = 0.96$; BF: $R^2 = 0.99$ for both diffuse and collimated light; ($n = 3$).
Energy budgets

The photosynthesis-irradiance (PE) curve of the coral sediment measured in diffuse light increased with increasing light intensity with an initial slope of 0.05±0.01, until reaching an asymptotic saturation level at $J_{PS,max} = 1.72±0.20 \text{ J m}^{-2} \text{ s}^{-1}$ at a downwelling photon irradiance of ~300 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 6B). In contrast, the PE-curve of the coral sediment in collimated light increased with the with a slope of 0.26±0.04, reaching a maximum saturation value of $J_{PS,max} = 4.24±0.23 \text{ J m}^{-2} \text{ s}^{-1}$, at downwelling photon irradiance ~110 µmol photons m$^{-2}$ s$^{-1}$ (Fig. 6B). In the biofilm, the onset of photosynthesis saturation occurred already at a downwelling photon irradiance of ~50 µmol photons m$^{-2}$ s$^{-1}$, where $J_{PS,max}$ reached an asymptotic saturation level of 0.87 J m$^{-2}$ s$^{-1}$ for both diffuse and collimated light (Fig. 6A).

Figure 7. Light energy budgets for biofilm (A, B) and coral sediment (C, D) in percent of the incident light energy calculated at downwelling photon irradiance (PAR) of 200, 500 and 1000 µmol photons m$^{-2}$ s$^{-1}$, under diffuse (A, C) and collimated (B, D) incident light. The amount of light backscattered from the sediment surface is shown in blue, while the amount of light energy dissipated as heat and via photosynthesis is shown in red and green, respectively. Notice the break on the y-axis. We assumed similar GPP under diffuse and collimated light in the calculations for the coral sediment under diffuse light (see Table S1 and Figure S3).
Sediment surface warming increased linearly with irradiance under both diffuse and collimated light with average slopes of $CS_{a_{\text{diff}}} = 4.33 \times 10^{-3} \, ^\circ C \, (J \, m^{-2} \, s^{-1})^{-1}$ and $CS_{a_{\text{coll}}} = 2.14 \times 10^{-3} \, ^\circ C \, (J \, m^{-2} \, s^{-1})^{-1}$ in the coral sediment, as compared to $BF_{a_{\text{diff}}} = 2.77 \times 10^{-3} \, ^\circ C \, (J \, m^{-2} \, s^{-1})^{-1}$ and $BF_{a_{\text{coll}}} = 2.0 \times 10^{-3} \, ^\circ C \, (J \, m^{-2} \, s^{-1})^{-1}$ in the biofilm (Fig. 6C,D). Surface warming was stronger under diffuse light as compared to collimated light in both sediments (Fig. 5, 6C,D).

The summed flux of energy conserved by photosynthesis and dissipated as heat ($J_{PS} + J_{H}$) serves as a control to determine the potential deviations between absorbed and dissipated energy (Fig. 6E, F). Dissipation of energy from the system increased linearly with increasing vector irradiance with slopes in the coral sediment of $0.89 \pm 0.003$ and $0.89 \pm 0.120$, for diffuse and collimated light respectively, and slopes in the biofilm of $0.93$ and $1.03$, for diffuse and collimated light respectively. When all outgoing/used energy equals the incoming light energy the slope of the used- vs. incoming energy curve would be $=1$, and thus the method used here apparently accounted for the majority of the incident light energy.

About 29% of the incident light energy was back-scattered from the coral sediment surface and thus not absorbed, whereas the surface reflection was only ~2% of the incident irradiance in the biofilm (Fig. 7; Fig. S2). The fraction of energy conserved by photosynthesis decreased with increasing irradiance in both biofilm and sediment (Fig. 7; 8). Over the investigated incident irradiances ($200 \text{ – } 1000 \, \mu mol \, photons \, m^{-2} \, s^{-1}$), photosynthetic energy conservation in the coral sediment illuminated with diffuse light decreased from 6.7% to 2.0% of the incident light energy, favouring heat dissipation (which increased from 63.1% to 67.8%), and from 9.3% to 2.1% of the incident light energy under collimated light (where heat dissipation increased from 62.6% to 69.8%) (Fig. 7; Table S2).

The proportion of incident light energy that was conserved via photosynthesis was much lower in the biofilm where 1.9% and 2.3% (diffuse and collimated light, respectively) of the incident light energy was conserved whereas 96.3% and 96.0% of the incident light energy was dissipated as heat, respectively (Fig. 7; Table S1). At an incident irradiance of $1000 \, \mu mol \, photons \, m^{-2} \, s^{-1}$, only 0.6% and 0.5% of the incident energy was conserved by photosynthesis while 97.6% and 97.8% was dissipated as heat under diffuse and collimated light, respectively (Fig. 7; Table S2).

The maximum photochemical energy conservation in the coral sediment was observed at an incident irradiance of ~100 $\mu mol \, photons \, m^{-2} \, s^{-1}$ (18.1% of the absorbed light energy), whereas the biofilm had maximum energy conservation through photosynthesis (14.7% of the absorbed light energy) at the lowest measured incident irradiance (50 $\mu mol \, photons \, m^{-2} \, s^{-1}$) (Fig. 8). In addition, the biofilm had higher
photosynthetic efficiencies under diffuse light compared to collimated light at low light intensities (<200 µmol photons m\(^{-2}\) s\(^{-1}\)) (Fig. 8).

The photosynthetic efficiencies of biofilm and coral sediment under light-limiting conditions (\(J_{\text{ABS}} \rightarrow 0\)), \(\varepsilon_{\text{PS,max}}\), were calculated from the initial slope of the areal PG vs. vector irradiance curve to 26.2% of the absorbed light energy (CS, collimated light) compared to 16% and 9.0% of the absorbed light energy (BF, diffuse and collimated light, respectively).

**Discussion**

We present a closed radiative energy budget of a heterogeneous coral reef sediment and compare it to the radiative energy budget of a flat dense biofilm (Fig. 6 and S4). The closed light energy budgets were measured under both diffuse and collimated illumination to test potential effects of the directionality of light on the photosynthetic efficiencies of the phototrophs. We found that a higher fraction of the absorbed light energy was conserved by photosynthesis in the heterogenous loosely organized coral sediment, while the radiative energy budgets of both sediment types were highly dominated by dissipation of heat.
Light

The thin highly pigmented cyanobacterial biofilm was growing on the surface of a fine-grained (125-250 µm) dark sandy sediment, whereas the photosynthetic microorganisms exhibited a more patchy distribution within the large-grained (100-500 µm) bright and highly scattering coral sediment. This structural difference between the two systems led to a ~15 times higher surface reflection and a decreased energy absorption in the coral sediment compared to the biofilm that displayed >8 times higher light attenuation coefficients. As previously shown (Lassen et al., 1992a; Kühl & Fenchel, 2000) the scalar irradiance at, or immediately below, the surface increased, and the spectral composition was altered relative to the incident irradiance (Fig. 2 and 3). Such increase in scalar irradiance in the near surface layer is due to intense multiple scattering by particles (biotic and abiotic) causing a local photon path-length increase and thus a prolonged residence time of scattered photons in the surface layers that also receive a continuous supply of incident photons from the light source (Kühl & Ørjesen, 1994). This effect can be further enhanced in the presence of exopolymers with a slightly higher refractive index than the surrounding seawater leading to photon trapping effects (Kühl & Ørjesen, 1994; Decho et al., 2003). Furthermore, the structural difference between the loosely organized CaCO₃ particles compared to the flat biofilm could possibly result in differences in the reflection characteristics from the uppermost layers. In the biofilm, the flat homogeneous surface reflects light relatively uniformly, with some ratio between specular vs. diffuse reflection. However, in the heterogeneous coral sediment a higher degree of forward scattering will most likely be present as the angle of reflection will be more complex due to the roughness of the surface, resulting in a deeper penetration of light in the coral sediment.

Temperature

We directly measured both the upward and downward heat dissipation of radiative energy (Fig. 5). Previous studies of energy budgets ignored the downward heat flux (Al-Najjar et al., 2010; Al-Najjar et al., 2012), and although Jimenez et al. (2008) estimated the downward heat dissipation from a theoretical model considering the physical properties of heat transfer in coral skeleton, this study presents energy budgets of phototrophic systems for which the complete heat balance was directly measured. Over a range of incident irradiances, the downward heat flux was the same order of magnitude as the upward heat flux in both biofilm and coral sediment and thus is an important parameter when compiling light energy budgets for the photic zone in benthic systems (Fig. 5).
The majority of the absorbed light energy was dissipated as heat (Fig. 7; Table S1) with a linear relationship between increasing incident irradiance and heat dissipation under both diffuse and collimated light, albeit with a ~30% enhanced surface warming under diffuse light as compared to collimated light (Fig. 5 and 6). Apparently, diffuse light was absorbed more efficiently in the uppermost layers, increasing the local photon density and residence time in these layers resulting in increased energy deposition and surface temperatures. This was supported by a higher heat flux into the water column under diffuse light, and a higher heat flux into the sediment under collimated light (data not shown). At increasing irradiances the surface temperature of the sediments exceeded the surrounding water temperature and convective heat transport occurred over the TBL (Fig. 5) (Jimenez et al., 2011). While we cannot dissect the observed heat dissipation into particular mechanisms and their relative magnitude, part of such dissipation in optically dense biofilms and sediments involves non-photochemical quenching (NPQ) processes that protect the photosynthetic apparatus under high irradiance by channelling excess light energy into heat dissipation (Falkowski & Raven, 2007; Al-Najjar et al., 2012). The heat fluxes from the photic zone were generally higher in the biofilm when compared to the coral sediment, due to the lower reflection and thus higher absorption in the biofilm (Fig. S4). However, when normalizing the heat fluxes to the absorbed light energy (which was 33% higher in the biofilm than in the coral sediment) the heat dissipation was of similar magnitude, and variations in heat flux values between the sediment and biofilm became <15%. The degree of heat dissipation therefore seems tightly correlated to the quantity of absorbed energy.

**Photosynthesis**

The overall photosynthetic efficiency of the biofilm and coral sediment decreased with increasing incident irradiance, with photosynthesis exhibiting saturation at higher irradiance under both diffuse and collimated light (Fig. 6). The highest energy storage efficiency of the coral sediment was observed under light-limiting conditions (<200 µmol photons m⁻² s⁻¹) (Fig. 7, 8), and the coral sediment generally exhibited high light use efficiencies that were comparable to those observed in corals at equivalent incident photon irradiances (Brodersen et al., 2014). The photosynthetic activity in the coral sediment was stratified at incident irradiances >50 µmol photons m⁻² s⁻¹ under both diffuse and collimated light (Fig. 4). This stratification could be a result of different factors influencing the photosynthetic activity such as steep light attenuation over depth, locally high volume-specific rates of metabolic activity, higher local biomass of phototrophs and diffusion limitation of metabolic products and substrates (Kühl et al., 1996; Kühl & Fenchel, 2000; Al-Najjar et al., 2012). Such vertical stratification has also been associated
with phototactic responses to light (Whale & Walsby, 1984; Lassen et al., 1992a), where motile photosynthetic organisms migrate to an optimal depth for photosynthesis at a given irradiance, where the available light is neither limiting nor inhibiting the rate of photosynthesis (Al-Najjar et al., 2012). These migration patterns are well documented both as photo- and aero-tactic responses and to escape from e.g. toxic levels of sulphide (Kühl et al., 1994; Bebout & Garcia-Pichel, 1995). The two photosynthetic active layers were situated at the sediment surface and ~2 mm below (~0.5 mm and 1 mm thick layers, respectively; Fig. 3).

The area-specific rates of gross photosynthesis of the coral sediment were ~4 times higher than in the biofilm, due to a ~3 times deeper euphotic zone and slightly higher volume-specific photosynthesis rates in the coral sediment than in the biofilm (Fig. 6, 7 and Fig. S3). Consequently, the coral sediment reached an asymptotic maximum in PG in terms of energy dissipation via photosynthesis of ~4.2 J m\(^{-2}\) s\(^{-1}\) as compared to only ~0.9 J m\(^{-2}\) s\(^{-1}\) in the biofilm (Fig. 6). The E\(_s\) value, i.e., the irradiance at the onset of photosynthesis saturation, was >2 higher in the coral sediment compared to the Danish biofilm, which reflects the different in situ light conditions experienced by the two systems in their respective geographic locations (Denmark: 55°N, Heron Island: 23°S). Thus, the dense biofilm appeared acclimated to low irradiances as previously shown (Kühl et al., 1996; Kühl & Fenchel, 2000; Al-Najjar et al., 2012) where highly reduced quantum efficiencies are seen at increasing irradiance due to the employment of e.g. NPQ processes. Accordingly, the coral sediment maintained higher photosynthetic efficiencies, even at high irradiance. This could in part be explained by the high scattering in the sediment particles that creates a more even spread of the light field over the sediment matrix and a more dispersed photic zone; a factor that have been speculated to be responsible for the high photosynthesis in coral tissues (Brodersen et al., 2014; Wangpraseurt et al., 2014a). A more homogenous distribution of light would create a larger region where light is neither limiting nor inhibiting photosynthesis. Thus, the loosely organized more heterogenous coral sediment apparently exhibit a more open, canopy-like organization compared to the dense biofilm.

Community photosynthesis is generally higher than that of individual phytoelements (Binzer & Sand-Jensen, 2002; Binzer & Middelboe, 2005; Binzer et al., 2006) and in addition, higher community photosynthesis has been found under diffuse illumination in open canopy systems which was explained by a more even light field inside the canopy (Gu et al., 2002; Brodersen et al., 2008). In spite of this difference in overall acclimation to light, a decrease in the surface layer photosynthesis was seen in the coral sediment at an incident irradiance of 500 µmol photons m\(^{-2}\) s\(^{-1}\), which could either reflect the heterogeneity and patchiness of the phototrophs found in the sediment, or could point to a possible
migration of motile phototrophic organisms. Migration as a phototactic response is recognized as an effective mechanism for controlling photon absorption across different systems such as terrestrial plants (Wada et al., 2003) and microphytobenthic assemblages (Serodio et al., 2006; Cartaxana et al., 2016a; Cartaxana et al., 2016b), and similar phototactic response has been shown in coral tissues where the in hospite light environment can be modulated by host tissue movement (Wangpraseurt et al., 2014a; Wangpraseurt et al., 2016). Downward migration at high irradiances is probably correlated with increasing photic stress e.g. by the formation of reactive oxygen species that can damage photosystem II by preventing the synthesis of the D1 protein in these layers (Hihara et al., 2001; Nishiyama et al., 2001; Aarti et al., 2007; Latifi et al., 2009; Al-Najjar et al., 2010). Several ways to counter such photic stress exists. One of the most effective short-term responses to photic stress is to employ non-photochemical quenching (NPQ) in which photons are emitted as heat when cells experience over-saturating photon fluxes. Another strategy to avoid photo-damage is to upregulate the expression of sun-protective pigments such as β-carotenes (Zhu et al., 2010), which were found in significant amounts by HPLC analysis of the coral sediment (Fig. S1).

Photosynthetic energy conservation was higher under collimated light as compared to illumination with diffuse light at moderate irradiance (200 µmol photons m⁻² s⁻¹) (Fig. 6). This finding correlates with previous studies of individual terrestrial leaves reporting 10-15% higher energy conservation via photosynthesis under collimated- relative to diffuse light (Brodersen et al., 2008) and in corals it has been shown that gross photosynthesis was 2-fold higher under direct vs. diffuse light (Wangpraseurt & Kühl, 2014). In terrestrial leaves, the more efficient utilization of collimated light has been ascribed to specialized tissue structures such as columnar palisade cells (Vogelmann & Martin, 1993), that increase the absorptance of direct light over diffuse light (Brodersen & Vogelmann, 2007). Furthermore, light-induced chloroplast movement has been shown to be less effective under diffuse illumination (Gorton et al., 1999; Williams et al., 2003). In corals the higher photosynthesis at the tissue surface was explained by optical properties enhancing the scalar irradiance near the surface under direct illumination (Wangpraseurt & Kühl, 2014). This tendency changed dramatically in the dense photosynthetic biofilm at light-limiting conditions (≤100 µmol photons m⁻² s⁻¹) favouring effective light utilization under diffuse light (Fig. 7). Thus, the optical properties and the structural organization of phytoelements seem tightly linked to the photosynthetic quantum efficiencies across different systems and light angularity may therefore elicit differential photosynthetic responses depending on the system and on the scale at which it is studied.
Conclusion

Our results show that a higher fraction of the absorbed light energy was conserved by photosynthesis in the heterogeneous coral sediment due to a deeper photic zone and slower saturation of photosynthesis with increasing irradiance as compared to the flat and highly absorbing biofilm. The balanced radiative energy budget of biofilm and coral sediment was highly dominated by heat dissipation and the efficiency of photosynthetic energy conservation decreased with increasing irradiance. Although the two systems exhibited similar heat dissipation, the photic zones wherein such dissipation took place was very different e.g. by a three times deeper photic zone in the coral sediment than in the biofilm. In addition, several variances were found between illumination with diffuse or collimated light: i) diffuse light enhanced dissipation of heat (~30%) in the upper sediment layers as compared to collimated light; ii) at low incident irradiance (200 µmol photons m⁻² s⁻¹) photosynthetic energy conservation was higher (3-4% of the absorbed light energy) in collimated light as compared to diffuse light; a tendency that dramatically changed in the photosynthetic biofilm at low and light-limiting incident irradiances (≤100 µmol photons m⁻² s⁻¹) favouring effective light utilization under diffuse light (up to a ~2-fold increase). However, cyanobacterial and diatom dominated mats have been shown to migrate vertically employing photo- and/or chemo-tactic responses (Richardson & Castenholz, 1987; Bhaya, 2004; Serodio et al., 2006; Coelho et al., 2011; Cartaxana et al., 2016a) and the motility of the phototrophs was not considered here. Thus, there is a need to further explore how vertical migration affects the radiative energy balance and thereby the light use efficiency in microbenthic systems such as sediments and biofilms.

Acknowledgements

We thank P. J. Ralph, P. Brooks, M. Zbinden and other colleagues at University of Technology Sydney (C3, UTS) for access to laboratory facilities, technical support and help with HPLC analysis of the coral sediment. We thank the staff at Heron Island Research Station for technical assistance during the field work. V. Schrameyer, D. Wangpraseurt and D. A. Nielsen are thanked for thoughtful discussions. The research was conducted under research permits for field work on the Great Barrier Reef, Australia (G11/34670.1 and G09/31733.1) and was funded by the Danish Council for Independent Research | Natural Sciences (MK), the Knud Høgaard's Fund, the Oticon Foundation, Thorsons Travel Grant and Københavns Universitets Fælleslegat (ML, KEB).
References


Cartaxana P, Cruz S, Gameiro C, Kühl M. 2016a. Regulation of intertidal microphytobenthos photosynthesis over a diel emersion period is strongly affected by diatom migration patterns. *Frontiers in Microbiology* 7: 872


Supplementary information

Pigment analysis

The upper 6 mm of the coral sediment, i.e., the entire photic zone in the sediment was analysed for pigment-content by high-pressure-liquid-chromatography (HPLC). From each core (5.3 cm in diameter), smaller sub-cores (1.2 cm in diameter) were taken and sliced in 2 mm sections until 6 mm depth, yielding three depth samples: 0-2 mm, 2-4 mm and 4-6 mm. All samples were taken in four replicates. Prior to analysis, the samples were stored at –80°C. To extract the pigments, the samples were shaken for 5 min and sonicated for 15 min in 3 mL 100% acetone in an ultrasound ice bath. After extraction, samples were left for 24 hours at -20°C. Before analysis, the extracts were diluted with 200 μL MiliQ water to a final concentration of 94% acetone. The extractions were then filtered through sterile syringe filters (Advantec HP020AN 13 mm, porosity = 0.2 μm, Advantech MFS Inc., Japan) and kept at –80°C in the dark until analysis. Photopigment extraction of the coral sediment was performed using the method by Van Heukelem and Thomas (2001) with slight modifications. A Waters reversed phase high-performance liquid chromatography (HPLC) system was used to separate pigments, which was achieved by using Eclipse XDB C8 HPLC 4.6 mm x 150mm column and guard column (Agilent Technologies, Australia) using a liner elution gradient from 5-95% of solvent B (100% Methanol, HPLC grade, Lomb Scientific, Australia, Pty Ltd., Part no. C2517-4L). Photopigment peaks were identified and quantified by comparison to known pigment standards (DHI Waters and Environment, Denmark). The pigment analysis revealed spatial heterogeneities in pigment composition in the upper 6 mm of the sediment (Fig. S1). The microphytobenthos was generally dominated by diatoms, dinoflagellates and cyanobacteria, and results also indicated small amounts of flagellate protozoa. Overall there was a pattern of decreasing concentrations of photopigments with depth, with decreases in the Chl a content from 1.16–0.65 μg cm⁻³ and fucoxanthin from 0.37-0.18 μg cm⁻³ within the upper 6 mm of the sediment. The photosynthetic community within the coral sediment was largely dominated by diatoms as indicated by a fucoxanthin:peridin ratio of ~10:1 in all three layers. With depth the relative amount of cyanobacteria increased compared to diatoms, visible from the fucoxanthin:zeaxanthin ratio that changed in the favour of zeaxanthin with depth, i.e., from 0.13 – 0.20 μg cm⁻³ at 0-2 mm and 4-6 mm depth, respectively.
Figure S1. Depth distribution of major photopigments in the coral sediment (in depth intervals of 2 mm). The top panel shows the amount of fucoxanthin, peridinin, zeaxanthin and antheraxanthin within the sediment over depth (in µg cm$^{-3}$). The bottom panel shows the chlorophyll a, b, and c$_2$ content within the coral sediment over depth (in µg cm$^{-3}$). Fucoxanthin is indicative of diatoms, peridinin is found in dinoflagellates, zeaxanthin is found in cyanobacteria, and antheraxanthin in protozoan flagellates of the genus Euglenozoa. Bars represent means ± 1 S.D. ($n = 3$).
**Surface reflection**

In the biofilm sample, surface reflection (Fig. S2) measured in the PAR range was on average 1.8% and 1.7% of the incident photon irradiance under diffuse and collimated light, respectively; while it was more than 15 times higher in the coral sediment, i.e., 30.2% and 28.1% for diffuse and collimated light, respectively.

**Photosynthesis distribution**

The photosynthetic activity increased with incident photon irradiance in both sediments (Fig. S3A,B). At the highest photon irradiance we observed the highest rates of photosynthesis within the first mm of the coral sediment, with an average gross photosynthesis of 11.97 nmol O₂ cm⁻³ s⁻¹ at the sediment surface under collimated light, and 3.05 nmol O₂ cm⁻³ s⁻¹ at a depth of 0.6 mm under diffuse light (Fig. S3B). In the biofilm the maximum gross photosynthesis rates were found in the surface layer in the low-light treatments (i.e. 50-200 µmol photons m⁻² s⁻¹) and ranged between 7.01 nmol O₂ cm⁻³ s⁻¹ and 8.7 nmol O₂ cm⁻³ s⁻¹ (collimated and diffuse light, respectively) (Fig. S3A). Besides the peak in photosynthesis found ~0-1 mm below the sediment surface in the coral sediment, there was a tendency to a second
peak 1.5-2.5 mm below the coral sediment surface at irradiances >50 µmol photons m\(^{-2}\) s\(^{-1}\) (Fig. S3B). The gross photosynthesis rates measured under diffuse and collimated light in the coral sediment were in the order of 3-4 times lower under diffuse light compared to collimated light. The gross photosynthesis rates of the coral sediment under diffuse light were measured at University of Technology Sydney (UTS) rather than on Heron Island, Australia, where the rest of the measurements took place. This apparently resulted in a change in the microbial community of the coral sediment probably from prolonged anoxic conditions in the sample during the transport from Heron Island.

![Figure S3A. Depth profiles of volume-specific gross photosynthesis rates in photosynthetic biofilm (in nmol O\(_2\) cm\(^{-3}\) s\(^{-1}\)) measured under diffuse (left) and collimated (right) light at downwelling photon irradiances of 50, 100, 200, 500 and 1000 µmol photons m\(^{-2}\) s\(^{-1}\). Bars represent means ± 1 S.D. (n = 3).]
Figure S3B. Depth profiles of volume-specific gross photosynthesis rates in coral sediment (in nmol O₂ cm⁻³ s⁻¹) measured under diffuse (left) and collimated (right) light at downwelling photon irradiances of 50, 100, 200, 500 and 1000 µmol photons m⁻² s⁻¹. Bars represent means ± 1 S.D. (n = 3).
Absorbed energy under diffuse and collimated light

The linear relationship between the vector irradiance and the downwelling photon irradiance under both diffuse and collimated light in both sediments (Fig. S4) confirmed that the same amount of energy was absorbed under diffuse and collimated light in the two investigated surface-associated photosynthetic systems.

Table S1: Calculated fluxes of O$_2$, heat and absorbed light energy at different downwelling photon irradiances and light fields: diffuse (D) and collimated (C) light. Absorbed light energy ($J_{ABS}$), areal gross photosynthesis rates ($J_{PS, GPP}$) and energy dissipated as heat ($J_H$) (in J m$^{-2}$ s$^{-1}$).

<table>
<thead>
<tr>
<th>Downwelling photon irradiance</th>
<th>Incident light field</th>
<th>$J_{ABS}$</th>
<th>$J_{PS, GPP}$</th>
<th>$J_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol photons m$^{-2}$ s$^{-1}$</td>
<td>Diffuse (D) /Collimated (C)</td>
<td>J m$^{-2}$ s$^{-1}$</td>
<td>J m$^{-2}$ s$^{-1}$</td>
<td>J m$^{-2}$ s$^{-1}$</td>
</tr>
<tr>
<td>200</td>
<td>D</td>
<td>29.47</td>
<td>40.15 (3.81)</td>
<td>0.89</td>
</tr>
<tr>
<td>200</td>
<td>C</td>
<td>31.00</td>
<td>40.41 (3.81)</td>
<td>0.97</td>
</tr>
<tr>
<td>500</td>
<td>D</td>
<td>73.85</td>
<td>99.71 (4.25)</td>
<td>0.69</td>
</tr>
<tr>
<td>500</td>
<td>C</td>
<td>76.54</td>
<td>99.80 (4.25)</td>
<td>0.70</td>
</tr>
<tr>
<td>1000</td>
<td>D</td>
<td>149.31</td>
<td>199.59 (4.06)</td>
<td>1.03</td>
</tr>
<tr>
<td>1000</td>
<td>C</td>
<td>147.92</td>
<td>199.20 (4.06)</td>
<td>0.95</td>
</tr>
</tbody>
</table>
Table S2: Calculated proportions of the incident irradiance either reflected from the surface, conserved by photosynthesis or dissipated as heat for the coral sediment (CS) and the biofilm (BF) under collimated (C) and diffuse (D) incident irradiance.

<table>
<thead>
<tr>
<th>Downwelling photon irradiance</th>
<th>Incident light field</th>
<th>Reflected</th>
<th>Conserved by photosynthesis</th>
<th>Dissipated as heat</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol photons m(^{-2}) s(^{-1})</td>
<td>Diffuse (D) /Collimated (C)</td>
<td>% of incident irradiance</td>
<td>% of incident irradiance</td>
<td>% of incident irradiance</td>
</tr>
<tr>
<td>200</td>
<td>D</td>
<td>30.2</td>
<td>1.8</td>
<td>6.7</td>
</tr>
<tr>
<td>200</td>
<td>C</td>
<td>28.1</td>
<td>1.7</td>
<td>9.3</td>
</tr>
<tr>
<td>500</td>
<td>D</td>
<td>30.2</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>500</td>
<td>C</td>
<td>28.1</td>
<td>1.7</td>
<td>3.7</td>
</tr>
<tr>
<td>1000</td>
<td>D</td>
<td>30.2</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>1000</td>
<td>C</td>
<td>28.1</td>
<td>1.7</td>
<td>2.1</td>
</tr>
</tbody>
</table>

References

Chapter 5

Pronounced gradients of light, photosynthesis and \( \text{O}_2 \) consumption in the tissue of the brown alga *Fucus serratus*

Published in *New Phytologist*

by

Mads Lichtenberg and Michael Kühl
Pronounced gradients of light, photosynthesis and O$_2$ consumption in the tissue of the brown alga *Fucus serratus*

Mads Lichtenberg$^1$ and Michael Kühl$^{1,2}$

$^1$Marine Biological Section, University of Copenhagen, Denmark
$^2$Plant Functional Biology and Climate Change Cluster, University of Technology Sydney, Australia

Abstract
Macroalgae live in an ever-changing light environment affected by wave-motion, self-shading and light-scattering effects and on the thallus scale, gradients of light- and chemical parameters influence algal photosynthesis. However, the thallus microenvironment and internal gradients remain underexplored. In this study, microsensors were used to quantify gradients of light, O$_2$ concentration, variable chlorophyll fluorescence, photosynthesis and O$_2$ consumption as function of irradiance in the cortex and medulla layers of *Fucus serratus* L.

The two cortex layers showed more efficient light utilization compared to the medulla, calculated both from electron transport rates through photosystem II and from photosynthesis-irradiance curves. At moderate irradiance, the upper cortex exhibited onset of photosynthetic saturation, while lower thallus-layers exhibited net O$_2$ consumption. O$_2$ consumption rates in light varied with depth and irradiance and were $>2$-fold higher than dark respiration.

We show that the thallus microenvironment of *F. serratus* exhibits a highly stratified balance of production and consumption of O$_2$, and when the frond was held in a fixed position, high incident irradiance levels on the upper cortex, did not saturate photosynthesis in the lower thallus layers. We discuss possible photoadaptive responses and consequences for optimizing photosynthetic activity on the basis of vertical differences in light attenuation coefficients.
Introduction

Brown macroalgae in the genus *Fucus* are predominant primary producers in intertidal and subtidal communities of Arctic and temperate coastal regions of the North Atlantic and North Pacific (Riget *et al.*, 1997; Coyer *et al.*, 2006; Coyer *et al.*, 2011), where they are found both in brackish and full saline waters (Svahn *et al.*, 2012). Dense *Fucus* beds also serve as important spawning and breeding grounds that provide shelter for many macrofauna, epifauna and fish species (Schramm & Nienhuis, 1996).

Macroalgal stands including *Fucus* spp. exhibit a structural organization resembling canopies of terrestrial systems (Binzer & Sand-Jensen, 2002). In terrestrial canopies, the upper leaves are typically oriented more vertically and often have thicker tissues with well-developed palisade layers that propagates light deeper into the leaf, while leaves in the lower canopy are more horizontally oriented and have thinner leaves adapted to low light (Mc Millen & Mc Clendon, 1979; Vogelmann, 1993; Vogelmann & Martin, 1993; Poorter *et al.*, 2006; Brodersen *et al.*, 2008). *Fucus* stands also exhibit a structured spatial organization and a consequence of such canopy organization is that even maximal summer midday irradiances do not saturate photosynthesis at the level of the whole macrophyte (Sand-Jensen *et al.*, 2007). However, sessile aquatic macrophytes have developed flexible/elastic tissues to withstand strong drag and shear forces created by currents and wave action (Denny, 2006; Mach *et al.*, 2007; Rosman *et al.*, 2010) and this may constrain the optimal organization of photosynthetic elements in regards to light harvesting (Binzer and Sand-Jensen (2002).

Terrestrial plants can modulate their photon absorption, e.g. by changing leaf inclination to enhance light-interception from low angles (Mc Millen & Mc Clendon, 1979; Falster & Westoby, 2003) or by changing tissue thickness (Vogelmann, 1993; Terashima *et al.*, 2011). Such adaptations counteract changes in light quality, quantity and direction throughout the canopy, and they have been studied intensively in terrestrial system both on the ecosystem level (Gu *et al.*, 2002; Farquhar & Roderick, 2003), leaf level (Johnson *et al.*, 2005; Brodersen *et al.*, 2008) and the level of individual cells in leaf tissue (Brodersen & Vogelmann, 2007).

Elegant studies by Sand-Jensen and co-workers (Binzer & Sand-Jensen, 2002; Middelboe & Binzer, 2004; Binzer *et al.*, 2006) have shown strong evidence for canopy effects of thallus organization optimizing community gross photosynthesis in *Fucus* stands, but the optical properties and chemical microenvironment of macroalgal tissue and their role in light harvesting and photosynthesis remain underexplored and only a few papers have investigated the internal physical and chemical microenvironment of macroalgae (Lassen *et al.*, 1994; De Beer & Larkum, 2001; Spilling *et al.*, 2010; Larkum *et al.*, 2011).
The thallus of *F. serratus* is divided into morphologically different meristoderm, cortex and medulla layers, but lack specialized cell types known from terrestrial systems such as epidermal cells that can focus light (Vogelmann *et al.*, 1996) or palisade cells that facilitate CO₂ exchange (Vogelmann, 1993) and light funnelling (Vogelmann, 1989; Vogelmann & Martin, 1993). In many macroalgae, densely pigmented meristoderm and cortex layers (henceforth referred to as cortex) surround the more transparent medulla (Garbary & Kim, 2005), which has been speculated to have light guiding properties (Ramus, 1978) in addition to a role in translocation of e.g. photo assimilates (Hellebust & Haug, 1972; Lobban & Wynne, 1981; Raven, 2003).

The thallus of fucoid macroalgae display plastid differentiation with well-developed chloroplasts lining the outer cortex layers (Garbary & Kim, 2005) while the medulla layer displays plastids with reduced thylakoid content (Moss, 1983).

It is currently unknown, whether photosynthetic tissue of aquatic macrophytes display interactions between physical structure, light climate and photosynthetic performance that resemble canopy interactions known from terrestrial leaves.

Ecophysiological studies of macroalgal photosynthesis as a function of light, often rely on measurements of incident irradiance (Beer *et al.*, 1998) and whole plant or tissue disk O₂ dynamics by gas exchange methods (Carpenter, 1985; Longstaff *et al.*, 2002). More advanced methods allow precise point measurements of photosynthetic capacity e.g. by surface measurements of variable chlorophyll fluorescence (Ralph & Gademann, 2005; Ulstrup *et al.*, 2006) or via the net efflux of O₂ measured with microsensors (Borum *et al.*, 2002; Larkum *et al.*, 2003). Common for these methods is that the information retrieved is limited to the surface of the measured photosynthetic system. However, microsensors have also proven useful tools to examine the internal microenvironment and quantify photosynthesis and respiration within biofilms, sediments, corals and macroalgae (Kühl & Jørgensen, 1994; Kühl *et al.*, 1998; Spilling *et al.*, 2010; Brodersen *et al.*, 2014).

The assessment of photosynthesis with variable chlorophyll fluorescence techniques has become increasingly popular since the development of sensitive and user friendly fluorescence detector systems (Schreiber, 1986). However, only the microfiber PAM system (Schreiber *et al.*, 1996) allows intra tissue measurements of chlorophyll fluorescence yields under natural light gradients in intact photosynthetic tissues, and it is thus a powerful tool to describe internal differences in photosynthetic activity under natural light conditions (Terashima *et al.*, 2009; Oguchi *et al.*, 2011).

In the present study, we used microsensors to measure internal gradients of scalar irradiance, O₂ concentration and consumption, gross photosynthesis and PSII derived electron transport rates in the
densely pigmented thallus of *Fucus serratus*. Our data demonstrate that the microenvironment and metabolic activity is highly dynamic and stratified in the thallus on sub millimetre scales.

**Materials and methods**

Specimens of *Fucus serratus L.* were collected at a water depth of 0.5-1.5 m from various coastal locations around Helsingør (Denmark) and were kept in 30 L aquaria, continuously flushed with 0.2 µm filtered aerated seawater (flow=28 L h⁻¹; salinity=32; 10°C). Samples were kept under a photon irradiance (PAR, 400-700 nm) of ~100 µmol photons m⁻² s⁻¹ from a fluorescent tube (Philips Master TL-D90, 18W, Philips, Netherlands) on a 14:10 h light:dark cycle.

**Experimental setup**

Vertical micro profiles of O₂ concentration, gross photosynthesis (GPP), photon scalar irradiance, and variable chlorophyll fluorescence were measured in vertical steps of 100 µm through macroalgal thallus pieces comprised of three distinct zones: the upper cortex (UC), the medulla (M), and the lower cortex (LC) (Fig. 1c). In addition, O₂ concentrations were measured through the upper- and lower diffusive boundary layer (DBL) surrounding the thallus. The thickness of the thallus varied between 0.6 - 1.2 mm. Prior to measurements, a ~3 cm long and ~1.5 cm wide apical thallus fragment was mounted in a custom made sample holder that ensured a steady sample during microsensor measurements (Fig. S1). The sample holder was designed to fit the interior dimensions of a flow chamber (25 x 8 x 5 cm), and was made of two thin (2 mm) acrylate plates with a slit, between which the sample could be fixed. A 3 mm hole was made through the acrylate plates to allow for water-flow across both upper- and lower side the thallus fragment, and through which the microsensor measurements were made. The sample holder was positioned in the middle of the flow chamber, so that a water-height of approximately 2.5 cm was achieved both below and on top of the thallus. The flow chamber was positioned on top of a 25 L aquarium continuously aerated with atmospheric air by an air pump (Sera air 110 plus, Sera GmbH, Germany), and was connected to a submersible aquarium pump (Fluval U1, Rolf C. Hagen Ltd., England) maintaining a seawater flow of ~1 cm s⁻¹ through a built in honey comb grid to obtain laminar flow. No additional inorganic carbon was added to the seawater.
Assuming a photosynthetic quotient of 1.0, the thallus surface \( \text{O}_2 \) increase (~400 \( \mu \text{mol L}^{-1} \)) at the highest irradiance (Fig. 3, bottom left panel) and the subsequent drawdown of inorganic carbon from the air equilibrated seawater would only change the DIC concentration from about 2000 to 1600 \( \mu \text{mol L}^{-1} \). Surface positioning of the microsensors was done visually using a PC-interfaced USB microscope (AM7013MZT Dino-Lite, AnMo Electronics Corporation, Taiwan). Prior to measurements the sample was allowed to dark acclimate for at least 15 min.

**Measurements**

**Light.** Spectral photon scalar irradiance was measured in vertical steps of 100 \( \mu \text{m} \) through the thallus with a scalar irradiance microprobe (Lassen et al., 1992) mounted in a manually operated micromanipulator (MM33, Märzhäuser, Germany) and connected to a fiber-optic spectrometer (USB2000+, Ocean Optics, USA) that was interfaced to a PC running spectral acquisition software (Spectra Suite, Ocean Optics, USA). Because of the toughness of the outer cortex layer, a small incision was made with the tip of a hypodermic needle prior to measurements of photon scalar irradiance. Incident light was provided by a blue LED ring light and light was quantified as the downwelling photon scalar irradiance from the LED ring with the fiber-optic microprobe positioned over a black non-reflective light-well at a distance similar to the position of the thallus surface during measurements (Lassen et al., 1992). Similarly, absolute incident photon irradiance (in \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \)) were

---

**Figure 1.** a) Conceptual drawing of a *F. serratus* thallus cross-section showing the different thallus layers (upper cortex, medulla and lower cortex) and the surrounding diffusive boundary layer (DBL). b) Overview of the apical part of the thallus with hyaline hairs (white dots) protruding from the tissue. c) Cross-section of an apical thallus fragment showing the densely pigmented cortex layers and the loose medulla.
measured with a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a planar cosine collector (LI-192S, LiCor, USA) positioned under the LED ring at the same distance as the thallus surface.

O₂ concentrations and photosynthesis. Vertical profiles of O₂ concentration and gross photosynthesis were recorded through the thallus of F. serratus using a Clark-type O₂ sensor (OX25, Unisense, Denmark; (Revsbech, 1989)) with a tip diameter of <25 µm, low stirring sensitivity (<1-2 %) and a fast response time (t₉₀<0.5 s), mounted on a motorized micromanipulator (MU-1, Pyro Science GmbH, Germany). The microsensor was connected to a pA-meter (Unisense, Denmark) and signals were recorded via an A/D converter (DCR-16, Pyro Science GmbH, Germany) through dedicated PC-controlled data acquisition software (Profix, Pyro Science GmbH), and additionally on a Flatbed Recorder (BD 12E, Kipp & Zonen B.V., Netherlands). The electrode was linearly calibrated from signal readings in air saturated- and in O₂ free seawater (by addition of sodium dithionite) at experimental temperature and salinity.

Variable chlorophyll fluorescence. Microscale measurements of variable chlorophyll fluorescence with the saturation pulse method were done with a sensitive fiber-optic fluorometer (Microfiber PAM, Waltz GmbH, Germany) (Schreiber et al., 1996; Ulstrup et al., 2006). The fiber-optic fluorescence microprobe consisted of a single strand graded index multimode fiber cable (Radiall Inc., France) mounted in a syringe and needle with a tapered and rounded measuring tip (~40 µm) at the light collecting end (Kühl, 2005) and connected to a sensitive pulse-amplitude modulated detector system at the other end via a fiber-optic beam splitter/coupler. On the system side of the coupler, one fiber branch was connected to a LED light source, while the other was connected to a sensitive PMT detector equipped with a long-pass filter to screen out the LED excitation light and only detect chlorophyll fluorescence. On the measuring side of the coupler one branch was connected to the fluorescence microprobe. To ensure good optical throughput, a small droplet of microscope immersion oil was added in the fiber-fiber connections. Fiber connections used the ST-connector standard.

The fluorescence microprobe was mounted on a motorized micromanipulator (MU-1, Pyro Science GmbH) for precise positioning of the fiber tip. The PAM control unit was connected to a LED ring (Ulstrup et al., 2006) providing known irradiance levels of blue actinic light (32, 47, 70, 104, 147, 204, 335 and 495 µmol photons m⁻² s⁻¹; peak emission: 470nm).

Measurements were done in three distinct vertical zones: the upper cortex, the medulla and the lower cortex. In each zone, rapid light curves (RLC’s; Ralph and Gademann (2005)) and steady state light curves (LC’s) were recorded under the above mentioned 8 actinic irradiance levels. The experimental
procedure was as follows: First the sample was dark acclimated for at least 15 min. A RLC was then measured (RLC1) using 10 s acclimation to each of the increasing irradiance steps (Ralph & Gademann, 2005). During these measurements, the thallus photosynthesis is thus not in a steady state (Schreiber et al., 1997), and the RLC depicts the current ability of the algal photosynthetic apparatus to handle light rather than a traditional photosynthesis vs. irradiance curve, where longer illumination periods allow establishment of steady-state conditions. After the RLC, a steady state light curve (LC) was recorded using 10 min acclimation to each irradiance level; we found that 10 min was sufficient to obtain steady-state fluorescence levels in accordance with other studies (Ulstrup et al., 2006). Directly after completion of the steady state light curve, another RLC was recorded (RLC2). The light curves were constructed in this order to assess the response of the photosynthetic apparatus to increasing irradiance and the recovery of photosynthesis after potential photoinhibition during the steady-state light curve measurement. Data were collected using a PC controlled data acquisition software (Win Control v. 2.08, Walz GmbH) that controlled the Microfiber PAM system.

Calculations

Photon scalar irradiance. To quantify the light available for photosynthesis in each vertical depth, we integrated the acquired photon scalar irradiance spectra over the PAR region (400-700 nm) and calculated the available light at each measuring depth as fractions of the downwelling photon scalar irradiance. By multiplying with the known, absolute incident photon irradiance measured at the upper thallus surface, we could thus calculate absolute PAR levels of blue light at each measuring depth in the thallus. The photon scalar irradiance attenuation coefficient, \( K_0 \) (mm\(^{-1}\)) was calculated as the slope of the natural logarithm transformed photon scalar irradiance plotted as a function of depth (Kühl, 2005).

Photosynthesis and respiration. Net photosynthesis (NPP) was calculated from steady state O\(_2\) profiles using the linear efflux of O\(_2\) through the diffuse boundary layer (DBL) surrounding the thallus using Fick’s first law of diffusion:

\[
J_{O_2} = -D_0 \frac{dC}{dz}
\]

where \( D_0 \) is the molar diffusion coefficient at experimental salinity and temperature (2.3·10\(^{-5}\) cm\(^2\) s\(^{-1}\); Unisense.com) and \( dC/dz \) is the linear concentration gradient in the DBL. As the thallus is bifacial, the efflux was calculated as the total flux of O\(_2\) from the upper and lower thallus surface. In darkness, the negative NPP was equal to the areal O\(_2\) respiration of the thallus. The term ‘areal’ will henceforth be used to describe the calculated production/consumption integrated over depth through the whole thallus, i.e. the total influx/efflux of O\(_2\). The compensation irradiance, \( E_c \), i.e. the irradiance above which
the thallus shifted from net O₂ consumption to net O₂ production, was determined when NPP became >0.

Volumetric gross photosynthesis (GPP) rates (in nmol O₂ cm⁻³ s⁻¹) were calculated for each 100 µm interval from the initial O₂ depletion rate measured immediately after a brief darkening (Revsbech & Jørgensen, 1983). Simple depth integration over the thallus zones yielded areal GPP rates, P (in nmol O₂ cm⁻² s⁻¹). To estimate the light acclimation index, E_k (µmol photons m⁻² s⁻¹), and the maximal photosynthesis rate, P_max, we fitted the areal GPP values, P(E), as a function of the incident photon irradiance, E, with an exponential model (Webb et al., 1974):

\[ P(E) = P_{\text{max}} \left(1 - \exp^{-\frac{aE}{P_{\text{max}}}}\right) \]

From this we could estimate the irradiance at onset of photosynthetic saturation as \( E_k = P_{\text{max}}/a \).

Local net O₂ conversion rates (in nmol O₂ cm⁻³ s⁻¹) were calculated from the O₂ concentration profiles (De Beer & Stoodley, 2013):

\[ r_b = D \frac{C_a - 2C_b + C_c}{\Delta z^2} \]

where \( r_b \) is the conversion rate in point b, D is the diffusion coefficient of O₂ (here we assume that D in the tissue is the same as in the surrounding water), C_a, C_b, C_c are the concentrations of O₂ in the points a, b, c respectively, and \( \Delta z \) is the depth difference between points.

These local net conversion rates allowed us to calculate local rates of O₂ consumption in the light, i.e. the sum of mitochondrial respiration and photorespiration, R(z) (in nmol O₂ cm⁻³ s⁻¹) as:

\[ R(z) = GPP(z) - r_b(z) \]

Depth integration of R(z) across the whole thallus, yielded an estimate of the total areal O₂ consumption in light. We fitted these data with equation 2 with an extra term, R, to account for the O₂ consumption (Spilling et al., 2010):

\[ P(E) = P_{\text{max}} \left(1 - \exp^{-\frac{aE}{P_{\text{max}}}}\right) + R \]

In addition we also calculated the total areal O₂ consumption (in nmol O₂ cm⁻² s⁻¹) from the areal gross photosynthesis rates, P(E), and the areal net photosynthesis rate.

rETR. Rates of electron transport through PSII can be calculated from the effective quantum yield of photosystem II (PSII), \( \phi_{\text{PSII}} \),

\[ \phi_{\text{PSII}} = (F'_m - F')/F'_m \]
where $F'_m$ is the local maximum fluorescence yield measured at the fiber tip during a saturating pulse, and $F'$ is the fluorescence yield when exposed to ambient light (Baker, 2008). ETR can be calculated as (Ralph et al., 2002):

$$\text{ETR} = \phi_{\text{PSII}} \times E_0 \times 0.5 \times 0.84$$

where $E_0$ is the scalar irradiance measured in the relevant depth, 0.5 is a conversion factor assuming half the photons are absorbed via PSII, and 0.84 is an empirically determined average absorption cross-section from a variety of vascular plants (Demmig & Björkman, 1987). The absorption cross-section cannot be directly determined with the saturation pulse method (Kühl et al., 2001), but can be determined using other techniques e.g. the pump and probe technique (Kolber & Falkowski, 1993) or a combination of scalar irradiance profiles and use of the Multi-Color-PAM system (Szabó et al., 2014). In this study we did not measure the appropriate parameters to estimate the absorption cross-section, but previously it was estimated from the thallus absorptance to ~0.9 (Lüning & Dring, 1985). However, the absorptance is likely different in the distinct thallus layers, so instead we calculated relative ETR rates ($r\text{ETR}$) as $r\text{ETR} = \phi_{\text{PSII}} \times E_0$, under the assumption that the absorption cross-section remained constant during measurements (Hofstraat et al., 1994).

**Results**

*Light microenvironment.*

Light in the outermost 0.2 mm of the cortex exhibited no or very weak attenuation, while a strong exponential attenuation of light was observed >0.2 mm below the thallus surface with a scalar irradiance attenuation coefficient, $K_0$, of 6.8 mm$^{-1}$ in the cortex and 3.4 mm$^{-1}$ in the medulla, respectively, as calculated from the average of the two replicates (Fig. 2). Only ~10% of the incident irradiance remained ~0.7 mm from the thallus surface. Scalar irradiance measurements deeper than 0.7 mm were not possible as the microprobe was unable to penetrate the tough lower cortex.

*Steady state O$_2$ concentrations and photosynthesis.*

The steep light gradient across the thallus affected the extent of the photic zone and the balance between O$_2$ production and consumption in the macroalgal tissue under different irradiances. The O$_2$ concentration profiles showed the presence of an upper and lower DBL of ~0.6 mm and ~0.4 mm thickness, respectively (Fig. 3). The presence of the DBL coupled with high O$_2$ consumption in the dark, resulted in anoxic conditions in thallus layers >0.1 mm from the surface.
Under increasing unilateral irradiance, increasing photosynthetic activity first resulted in a net efflux of O₂ from the upper thallus surface, while the lower thallus still consumed O₂. At irradiances >147 µmol photons m⁻² s⁻¹, photosynthetic O₂ production exceeded consumption throughout the thallus and a net efflux of O₂ occurred from both the upper- and lower thallus surface.

Gross photosynthesis in the thallus increased with irradiance and the most productive zone was located 0.1 – 0.2 mm below the upper thallus surface, with the highest local rate of 27 nmol O₂ cm⁻³ s⁻¹ observed at 104 µmol photons m⁻² s⁻¹ (Fig. 3). Under increasing irradiance, the gross photosynthesis rates in the upper thallus saturated, while rates in the lower thallus continued to increase as light penetrated deeper. Curve fitting of areal GPP vs. irradiance data (Fig. 4) showed a maximal GPP of $P_{\text{max}} = 0.59$ nmol O₂ cm⁻² s⁻¹. The light acclimation index, i.e., the irradiance at the onset of saturation was $E_k = 75$ µmol photons m⁻² s⁻¹.
The compensation irradiance, Ec, i.e. the irradiance at which photosynthesis equalled O\textsubscript{2} consumption on whole thallus scale was 70 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} and in spite of the high GPP rates in the upper cortex there was still a net consumption under this irradiance. An incident irradiance of >147 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} was necessary before the lower thallus layers became net O\textsubscript{2} producing.

Figure 3. Vertical O\textsubscript{2} concentration profiles (open circles) measured through the thallus of *F. serratus* and the surrounding water, and gross photosynthesis (black bars) plotted as function of incident photon irradiance. Symbols and error bars represent means ± 1 S.D. (n=3). The shaded area indicates the thallus and the white area above indicates the surrounding water.

The compensation irradiance, Ec, i.e. the irradiance at which photosynthesis equalled O\textsubscript{2} consumption on whole thallus scale was 70 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} and in spite of the high GPP rates in the upper cortex there was still a net consumption under this irradiance. An incident irradiance of >147 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} was necessary before the lower thallus layers became net O\textsubscript{2} producing.
Volumetric rates of gross photosynthesis revealed different zones of activity in the upper cortex (UC), the medulla (M) and the lower cortex (LC) when plotted against the scalar irradiance in the respective layers (Fig. 5). The initial slope of gross photosynthesis vs. photon scalar irradiance in the cortex layers was similar (UC = 0.29; LC = 0.22) while the medulla layer exhibited a >2 times less steep initial slope of the gross photosynthesis vs. photon scalar irradiance curve (M = 0.11).

The GPP of the upper cortex peaked at 104 µmol photons m⁻² s⁻¹, and at higher irradiances the rates declined towards a stable level indicative of the onset of moderate photoinhibition. Gross photosynthesis in the medulla was about 2 times lower than in the upper cortex and showed saturation without inhibition at high incident irradiance. Because of the strong attenuation of light, the lower cortex
did not experience irradiances high enough to become saturated and gross photosynthesis in this layer exhibited a linear increase with irradiance.

**Dark respiration and O₂ consumption in light.**

Areal O₂ consumption in the illuminated thallus increased with irradiance and reached an asymptotic maximum value of 0.24 nmol O₂ cm⁻² s⁻¹ at 31 µmol photons m⁻² s⁻¹. Apparent O₂ uptake rates in the light was thus twice the dark O₂ respiration (0.12 nmol O₂ cm⁻² s⁻¹). The total thallus O₂ consumption was on average 17% higher when calculated from the areal net photosynthesis values and the areal gross photosynthesis as compared to the depth integrated volumetric rates (Fig. S2). This underestimation can partly be explained as the volumetric rates of O₂ consumption were calculated from flux changes through a point (equation 3). The estimation of the rate in depth z = 0 thus relies on the flux of O₂ from the water into the thallus and is thereby influenced by other factors than the O₂ consumption of the thallus, e.g. the difference in diffusion coefficient, and the point z = 0 was therefore discharged in the calculation.

Local O₂ uptake rates in the _Fucus_ thallus increased with irradiance and revealed an increased consumption in the cortex layers (0.2 mm and 0.4-0.5 mm from the upper surface) relative to the medulla. The highest O₂ consumption was found in the upper cortex under high irradiance, with a 4.5
times higher rate as compared to the medulla (Fig. 6). The dark respiration was limited to the outermost cortex layers by the diffusive supply through the DBL and the centre of the thallus reached anoxia.

The balance between O₂ production and consumption shifted with irradiance and depth in the thallus (Fig. 7). Under low irradiance, a negative net production was observed, i.e. the respiratory demand exceeded the O₂ supplied from photosynthesis. A positive net production from all three tissue layers was only observed at an incident irradiance of >147 µmol photons m⁻² s⁻¹. Saturation of photosynthesis was only reached in the upper cortex while production in the lower layers increased under the investigated light intensities. The upper cortex exhibited the highest O₂ consumption rates across all investigated irradiances, while the lower cortex had the lowest rates except at the two highest irradiances (335 and 495 µmol photons m⁻² s⁻¹), where it exceeded the O₂ consumption in the medulla.

Light curves of photosynthetic electron transport.

Relative electron transport rates (rETR) were calculated for the upper cortex, medulla and lower cortex as a function of the scalar irradiance in the respective zones. In all light treatments (RLC1, LC, and RLC2), rETR in the upper cortex showed saturation with increasing irradiance, while the deeper layers did not experience enough light to become saturated. During the first RLC, photosynthesis in the upper cortex reached saturation but was not inhibited. However, this was the case when a longer acclimation time was used in the steady state LC measurements. In the RLC2 higher irradiance was necessary to saturate the upper cortex.
The light utilization efficiency under subsaturating irradiance levels, i.e. the slope on the linear part of the light curve ($\alpha$), was reduced in all depths in the RLC2 that was measured just after the steady state LC. Highest light utilization under subsaturating irradiance was found in the upper cortex when the thallus was allowed to acclimate to light for 10 minutes (LC). However, the rETR$_{\text{max}}$ value for the upper cortex was reduced when the thallus was exposed to 10 min acclimation to each experimental irradiance as compared to both the RLC1 and RLC2. In all light treatments, we found a more efficient use of subsaturating light fluxes in the two cortex layers relative to the medulla. The diminished rETR$_{\text{max}}$ values in the steady state LC's, compared to the value measured in RLC1, quickly returned to the previous level during the measurements of the RLC2. However, the efficient light use of the upper cortex was impeded during the RLC2 measurement compared to the medulla and lower cortex layer which maintained relatively stable light use efficiencies independent of light treatment history (Table 1).

**Discussion**

Our study presents strong evidence for a pronounced irradiance-dependent functional stratification in the thallus of macroalgae that in part followed the structural layering of the tissue. While the present study presents the hitherto most detailed insight to light propagation, photosynthesis and $O_2$ consumption in the thallus microenvironment, we note that the experiments were conducted using...
blue actinic light. The effects of different light types have been shown to significantly change physiological parameters in e.g. terrestrial vascular plants and corals such as gradients of CO$_2$ fixation (Sun et al., 1998), light absorption (Terashima et al., 2009; Szabó et al., 2014) and photoinhibition (Oguchi et al., 2011) and further studies with other spectral ranges of light are thus necessary for a more robust extrapolation of our results to natural conditions. In addition the measurements were done with the thallus fixed in a sample-holder to allow fine-scale microsensor positioning. The in situ position of the thallus sides will be dynamic and constantly changing which might affect photo acclimation.

The vertical distribution of O$_2$ production and consumption showed a pronounced light-driven stratification within the thallus. Light was only weakly attenuated in the uppermost 0.2 mm of the thallus, which can be either caused by an enhanced photon path-length caused by multiple scattering leading to increased scalar irradiance (Kühl & Jørgensen, 1994), or by a measuring artefact caused by the micro-incision done to penetrate the thallus. Below 0.2 mm, we found a two times higher attenuation coefficient in the upper cortex as compared to light attenuation in the medulla. Spilling et al. (2010) showed an almost 10-times higher attenuation of light in the cortex layer relative to the medulla. Lassen et al. (1994) also found a decreased light attenuation in the medulla of the green alga Codium fragile. It has been speculated that the loosely arranged medulla layers of macroalgae have light guiding properties, and can increase the backscattered light reaching the light-absorbing cortex layers (Ramus, 1978). At the thallus level, the highest photosynthetic production will occur when the largest area possible is irradiated. In situ, the position of algal stands of F. serratus relative to the incident light is dynamic and strongly dependent on water-motion created by waves and currents (Denny, 2006; Mach et al., 2007; Rosman et al., 2010) and this suboptimal positioning is alleviated by having a densely pigmented outer layer. Here we only attempt to comment on the tissue organisation relative to light-acquisition; for a complete review on resource acquisition and growth of aquatic macrophytes in a turbulent environment see Hurd (2000). This considered, a relatively transparent medulla layer increases the amount of light reaching the shaded side of the thallus thereby increasing the potential photosynthetic capacity, simply by ensuring a larger light-exposed area. Other structural adaptations to changing light conditions have been observed in the green alga Codium fragile, where reflection increased significantly in the presence of a gas-filled medulla due to build-up of O$_2$ from photosynthesis (Lassen et al., 1994). However, fucoid algae do not have gas-filled medulla which decreases the basis for backscattering. Medulla tissue can also serve other functions such as translocation of inorganic/organic compounds and photoassimilates (Hellebust & Haug, 1972; Lobban & Wynne, 1981; Raven, 2003) as well as facilitating nutrient supply from older tissue regions towards fast-growing regions (Lobban & Harrison, 1994). Therefore, more
thorough investigations of the optical properties of such tissues are necessary to be able to make any conclusions on the possible role of structural adaptations for light-capture in macroalgae (Vogelmann, 1993; Kühl & Jørgensen, 1994).

The max net efflux of O\(_2\) (NPP) in *F. serratus* was 149 mmol O\(_2\) m\(^{-2}\) d\(^{-1}\), i.e. about 3 times higher than the NPP found by Spilling *et al.* (2010) in *F. vesiculosus*. This large difference in NPP is partly due to calculation differences, as Spilling *et al.* (2010) only considered the upward flux of O\(_2\) from the thallus, whereas we considered the total O\(_2\) efflux across the DBL on both thallus sides. Under increasing irradiance, the lowermost side of the thallus contributed increasingly to the O\(_2\) efflux, as light penetrated deeper and stimulated photosynthesis in the lower cortex; at the highest investigated irradiance the lower side of thallus thus contributed 40% of the total net O\(_2\) efflux. In this study we used the molecular diffusion coefficient of O\(_2\) in seawater to describe the flux of O\(_2\) across the thallus-water interface. However, the diffusion coefficient of O\(_2\) in the thallus is most likely different from the surrounding water and the calculations of NPP will therefore only be estimates of the true flux. Numerical procedures for interpretation of measured O\(_2\) concentration profiles in biofilms and sediment pore water, have been developed (Berg *et al.*, 1998), but such modelling was not attempted here.

The photosynthetic activity in the thin thallus of *F. serratus* showed marked differences over very small spatial scales. At moderate irradiance (<100 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) effective light absorption resulted in supersaturated O\(_2\) conditions in the upper cortex, while the lower cortex (<1 mm away) showed net O\(_2\) consumption and hypoxia. Such stratification was also evident from the apparently similar compensation irradiance, \(E_c\) (70 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) and light acclimation index, \(E_k\) (75 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) considered on whole-thallus scale. This was possible because the upper light exposed part of the thallus produced O\(_2\), while the lowermost shaded part of the thallus exhibited net O\(_2\) consumption. A positive areal net-production of O\(_2\) on whole-thallus scale thus occurred only at the same irradiance where the upper layer began to saturate.

Strong attenuation of light limited photosynthesis in the lower thallus layers of *F. serratus*. However, when GPP rates were plotted vs. the actual scalar irradiance in the different thallus layers (Fig. 5) it was evident that both the upper and lower cortex utilized low light more efficiently and exhibited higher maximum photosynthetic rates as compared to the medulla. This pattern was confirmed in the microfiber PAM measurements of rETR rates (Fig. 8), where the light utilization was always more efficient in the cortex layers relative to the medulla under subsaturating light fluxes. Garbary and Kim (2005) showed higher maximum quantum efficiencies of PSII in the outer- relative to the inner cortex, however
by a much more invasive method, and the vertical microdistribution of chlorophyll in _F. vesiculosus_ (Spilling et al., 2010) and _Laminaria digitata_ (Hellebust & Haug, 1972), showed >3 times higher values in outer layers compared to the medulla indicating a higher photon absorption in the cortex.

The onset of saturation of photosynthesis, $E_k$, was $\sim 75 \, \mu \text{mol photons m}^{-2} \text{s}^{-1}$ when calculated from the areal GPP rates and $56 \, \mu \text{mol photons m}^{-2} \text{s}^{-1}$ when calculated from the GPP rates in the upper cortex. The thallus of _F. serratus_ thus exhibited effective light harvesting at low irradiance. When the $E_k$ parameter was calculated from the rETR rates of the upper cortex, we found a similar value from the steady state light curves of $86 \, \mu \text{mol photons m}^{-2} \text{s}^{-1}$. Interestingly, the $E_k$ calculated from the first RLC was >2 times higher ($178 \, \mu \text{mol photons m}^{-2} \text{s}^{-1}$) compared to the steady state light curve. As shown by Ralph and Gademann (2005), RLC's assess the photosynthetic activity in a fluctuating light environment and thus do not show the optimal photosynthetic capacity but the actual state, which is a function of the light exposure just prior to the RLC measurement. Because of the short acclimation time (10 s) used in RLC's, no up- or down regulation of the photosynthetic apparatus is possible; e.g. by the xanthophyll cycle which takes 5-30 min to activate (Ralph et al., 1999). Measured with longer light incubation times, the steady-state LC's will reflect the potential for short-term light adaptation, where sufficient time is given to upregulate photoprotective mechanisms. This was also evident from the >30% higher rETR$_{\text{max}}$ in the RLC relative to the LC (Table 1) which indicates a capacity for short-term photoacclimation in _F. serratus_.

The microfiber PAM is a useful tool to gain insight into the distribution of photosynthetic rates, which, so far, apart from a few studies done on the surface of corals (Ralph et al., 2002; Ulstrup et al., 2006; Ralph et al., 2007), mainly have been used in terrestrial systems (Schreiber et al., 1996; Terashima et al., 2009). Here we provided the first local ETR rates from the three distinct microzones within the thallus of _F. serratus_.

The large standard deviations found in the calculations of rETR could be explained by the high spatial sensitivity of the method. The microfiber was tapered to a diameter of $\sim 40 \, \mu \text{m}$ and was rounded to avoid cell-puncture and chlorophyll leaking (Schreiber et al., 1996). Rounding of the fiber measuring tip broadens the light acceptance angle of the fluorescence microprobe and brings the region of most efficient light collection down to approximately the fiber tip diameter or less (Kühl, 2005). This results in a spatial resolution of <40 µm, and since the chloroplasts of _F. serratus_ were 10-20 µm in diameter (data not shown) the measurement heterogeneity may largely reflect heterogeneity in chloroplast distribution.
The increase in areal O$_2$ consumption of the thallus observed across the low light regime (<100 µmol photons m$^{-2}$ s$^{-1}$) correlated with a gradual extension of the oxic zone within the thallus. At irradiances >100 µmol photons m$^{-2}$ s$^{-1}$, the areal O$_2$ consumption approached an asymptotic maximum value but the activity did not show a uniform depth distribution and volumetric O$_2$ consumption rates increased with irradiance throughout the thallus. Enhanced O$_2$ consumption under high prevailing O$_2$ concentrations have previously been ascribed to photorespiration in many marine macroalgae (Dromgoole, 1978) as O$_2$ acts as a competitive inhibitor for CO$_2$ at the binding site of RuBisCO in the Calvin-Benson cycle (Bowes & Ogren, 1972) under high O$_2$/CO$_2$ ratios. The likelihood of photorespiration in F. serratus is reduced by its CO$_2$ concentrating mechanism, that can alleviate the competitive inhibition by O$_2$ (Surif & Raven, 1989; Surif & Raven, 1990), but the hyperbolic relationship between the measured O$_2$ consumption rates and increasing irradiance indicates a link to photosynthetic activity. Previously light enhanced respiration, has been shown to increase O$_2$ consumption >6 times in corals (Kühl et al., 1995; Schrameyer et al., 2014) and in the macrophyte Ulva lactuca (Beer et al., 2000). However, from the present data it is not possible to partition the measured O$_2$ consumption between photorespiratory and mitochondrial O$_2$ uptake and more work is thus needed to determine their relative significance on the total O$_2$ consumption in light.

In conclusion, this study has demonstrated that the thallus of F. serratus exhibits a highly stratified balance of production and consumption of O$_2$, where not even high irradiance (~500 µmol photons m$^{-2}$ s$^{-1}$) saturated photosynthesis in the lower thallus layers. The cortex and medulla of F. serratus show distinct optical properties that allows light, that is not absorbed in the upper photosynthetic layers, to propagate efficiently from the upper to the lowermost cortex layers facilitating a more even illumination across the thallus. There is now a need for a more detailed investigation of the optical properties of F. serratus tissues to unravel how tissue organisation may enhance light propagation and photosynthesis e.g. by light guiding through the medulla (Ramus, 1978).

Acknowledgments

This study was supported by grants from the Danish Council for Independent Research | Natural Sciences and the Villum Foundation. We thank Lars F. Rickelt for manufacturing scalar irradiance microsensors, Erik Trampe for help with microscopy images, and Thomas C. Vogelmann for valuable discussions and advice on the work presented in this study.
References


Supplementary information

Figure S1: Experimental setup for microenvironmental analysis of *F. serratus*. O₂ microelectrodes (B) were PC interfaced (L) through a pA-meter (E) and an A/D converter (F). The optic fiber (B) used for fluorescence was connected to the UNIVERSAL-PAM control box (G) through a fiber-optic coupler (J) and the photomultiplier-tube (I) and then PC interfaced (L). The scalar irradiance microprobe (B) was connected to the PC (L) via the spectrometer (K). The LED ring light source (C) was controlled from the PC (L) via the UNIVERSAL-PAM control box (G) and a LED driver unit (H).
Figure S2: Comparison of the 2 methods used to estimate the total O₂ consumption rate of F. serratus. The areal based method (open circles) was calculated as the areal net photosynthesis rates, calculated from Fick’s 1st law of diffusion (equation 1 in Materials and Methods), subtracted from the areal gross photosynthesis rates. The volumetric based method (closed circles) was calculated as the local conversion rates (equation 3 in Materials and Methods) subtracted from the local gross photosynthesis rates. The shaded bars represent the difference (%) between the methods of calculation.
Chapter 6

Vertical migration optimizes photosynthetic efficiency of motile cyanobacteria in a coastal microbial mat

Intended for submission to *Environmental Microbiology*

by

Mads Lichtenberg, Paulo Cartaxana and Michael Kühl
Vertical migration optimizes photosynthetic efficiency of motile cyanobacteria in a coastal microbial mat

Mads Lichtenberg¹, Paulo Cartaxana¹ and Michael Küh¹²
¹Marine Biological Section, University of Copenhagen, Denmark
²Climate Change Cluster (C3), University of Technology Sydney, Australia

Abstract

Dense microbial mat systems are characterized by steep light, temperature and chemical gradients and a highly diverse community structure. The high optical density of such systems creates a competition for light among the phototrophic algae and bacteria residing in the uppermost layers of the mat. Hence, organisms display various strategies to counter this resource limitation, including metabolic investment in a broader range of protective and light harvesting pigments thereby exploiting separate niches in terms of irradiance and spectral composition or investment in motility to enable migration to an optimal light microenvironment. In this study, we used microsensor measurements of light, temperature and gross oxygenic photosynthesis in a coastal microbial mats dominated by motile cyanobacteria to study how migration affected the radiative energy budget and photosynthetic efficiency in the mats. The optical density of the microbial mat was extremely high and >99% of incident irradiance was attenuated <0.4 mm below the surface. The energy conservation efficiency did not change dramatically between different light treatments, but vertical profiles of photosynthetic efficiency showed a shift in the position of maximum efficiency of ~0.2 mm, depending on light treatment. Vertical migration over short distances in microbial mats thus enable cyanobacteria to track zones in the mat with optimal light exposure thereby efficiently counteracting detrimental effects of excessive light at the surface and insufficient light deeper in the mat.
Introduction

Light-exposed coastal sediments in shallow waters are often colonized by benthic microalgae and cyanobacteria, which under environmental extremes such as desiccation, high sulphide or salinity can form complex stratified microbial biofilm communities, i.e., microbial mats (Stal, 1995). Composed of different groups of microalgae and bacteria, these communities are densely populated and highly compacted. A pronounced vertical stratification of the microenvironmental conditions exists in the form of steep physical and chemical gradients that create a conspicuous vertically stratified microbial community (Kühl et al., 1996; Al-Najjar et al., 2012; De Beer & Stoodley, 2013). The upper thin top layer of the microbial mat is typically composed of diatoms covering a dense green layer of motile filamentous cyanobacteria, often dominated by Microcoleus chthonoplastes and various oscillarians (Wieland et al. 2003). Usually, purple sulfur bacteria are found below the cyanobacteria followed by a reduced black layer of precipitated iron sulfide (Jørgensen, 1982). Unlike microalgae and cyanobacteria, purple sulfur bacteria use hydrogen sulfide instead of water as their reducing agent in their anoxygenic photosynthesis. Besides light-driven sulphide oxidation by anoxygenic phototrophs, sulphide can also be oxidized efficiently by colorless sulfur bacteria such as filamentous Beggiatoa spp. (Nelson & Castenholz, 1981) that are motile and produce white patches in the microbial mat at the oxygen-sulfide interface (Jørgensen & Revsbech, 1983).

Light is the primary energy source in photosynthetic microbial mats. Due to the high density of photopigments, organic matter, and sediment particles, light is subject to intense scattering and absorption within microbial mats (Kühl & Jørgensen, 1994; Kühl et al., 1994). This leads to an extremely narrow photic zone (Kühl et al., 1997) and a rapid change in spectral composition with depth (Lassen et al., 1992; Cartaxana et al., 2016b). Ploug et al. (1993) related changes in light quality to the vertical zonation of a population of diatoms over a dense filamentous cyanobacteria layer that largely sustained their oxygenic photosynthesis via phycobiliproteins with absorption characteristics complementary to chlorophylls. Similarly, complementary use of visible and near-infrared radiation by chlorophylls/phycobilins vs. bacteriochlorophylls enables coexistence of dense populations of oxygenic phototrophs on top of anoxygenic phototrophs (Kühl & Fenchel, 2000). Apart from light, other parameters such as nutrient availability or the presence of toxic compounds such as sulfide may vertically limit photosynthesis in microbial mats (Kühl et al., 1996).

The ecological success of benthic microbes in optically dense and vertically stratified communities has recurrently been linked to cell motility allowing individual microbes to search for optimal environmental conditions regarding crucial parameters such as light, temperature, O$_2$ or nutrient availability (Whale &
Walsby, 1984; Bebout & Garcia-Pichel, 1995; Bhaya, 2004; Serôdio et al., 2006). Complex migratory rhythms determined by day/night cycles, tidal regimes, UV exposure and changes in irradiance levels have been described for both diatom and cyanobacteria dominated phototrophic microbial communities (Bebout & Garcia-Pichel, 1995; Serôdio et al., 2006; Coelho et al., 2011). Similar strategies to optimize photon capture are well known in terrestrial plants, where the position of chloroplast in palisade and mesophyll layers in leaves can change depending on light levels and light field directionality, i.e., diffuse versus collimated light (Vogelmann, 1993; Gorton et al., 1999; Wada et al., 2003). Raphidic diatoms and filamentous cyanobacteria are able to glide within an extracellular polymeric matrix at speeds that can reach several hundred micrometers per minute (Gupta & Agrawal, 2007; Tamulonis et al., 2011). Because of the steep light gradient, migration and the resultant vertical redistribution of the productive biomass have important consequences for both the photobiology of the phototrophs and the net primary productivity of the microbial mat ecosystem (Bebout & Garcia-Pichel, 1995; Cartaxana et al., 2016b).

Recent studies have focused on the efficiency with which light is utilized and converted to chemical energy in cyanobacterial mats and cyanobacteria–diatom mixed biofilms (Al-Najjar et al., 2010; Al-Najjar et al., 2012). In these studies, relatively low photosynthetic efficiencies were estimated for microbial mats compared with ecosystems with a more open canopy-like organization such as coarse sediments (Lichtenberg et al., 2017), macroalgal stands (Sand-Jensen et al., 2007), corals (Brodersen et al., 2014) or terrestrial forest communities (Terashima et al., 2016), where light propagation is not hindered to the same extent by photosynthetic inactive components. However, how the photosynthetic efficiency of biofilms and microbial mats is modulated by the migration of motile phototrophic populations remains to be studied in detail. In this study we used fiberoptic scalar irradiance and field radiance microprobes in combination with O2 and temperature microsensors to investigate the radiative energy budgets for the euphotic zone in a coastal cyanobacterial mat dominated by motile cyanobacteria and colorless sulfur bacteria to reveal how photosynthetic efficiency in the microbial mat was affected by changes in vertical biomass distribution imposed by different light regimes.
Materials and Methods

Sample collection and preparation

Microbial mats were collected from a periodically desiccated sand flat in Aggersund, Limfjorden, Denmark. The water level at the sample site is mainly determined by wind and local current patterns, and the mats can experience desiccation for extended periods. The sampled mats were dark green/black in appearance and were dominated by the cyanobacteria Microcoleus spp. and beneath the cyanobacterial band a population of the sulfur bacteria Beggiatoa spp. was found (see also Nielsen et al. (2015)).

Mat samples were collected using small plastic trays (7 x 2 x 5 cm) and were brought back to a field laboratory (Rønbjerg Marine Research Station, Aarhus University, Denmark), where they were incubated submerged in seawater (20°C; Salinity=27) under a low photon irradiance (~75 μmol photons m⁻² s⁻¹) of photosynthetically active radiation (PAR, 400-700 nm) provided by a halogen light source. Within few hours of incubation, the mat turned dark green and extensive bubble formation from oxygenic photosynthesis appeared on the surface of the mat.

When measuring, the mats were transferred to a flow chamber (25 x 8 x 8 cm) that provided a stable laminar flow (~2 cm s⁻¹) of aerated seawater (22°C; Salinity=27) (see also Brodersen et al. (2014); Lichtenberg et al. (2017)). Light was provided vertically from above by a white LED lamp (KL-2500 LED, Schott, Germany) equipped with a collimating lens. The incident irradiances from the lamp could be regulated electronically without spectral distortion. The downwelling photon irradiance was measured with a calibrated photon irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a factory-calibrated photon irradiance detector (LI-192S, LiCor, USA). Incident spectral irradiance was also measured in radiometric units (W m⁻² nm⁻¹) with a calibrated spectroradiometer (Jaz ULM, Ocean Optics, USA). All sensors were mounted in a 45° angle on a motorized micromanipulator (MU-1, PyroScience, Germany), which could be controlled by the manufacturers software (Profix, PyroScience, Germany). All measurements were performed under an incident collimated photon irradiance of 1000 μmol photons m⁻² s⁻¹ provided by the white LED lamp. Prior to measurements, a biofilm sample was incubated for at least 3 h in darkness, low light (~75 μmol photons m⁻² s⁻¹) or high light (1000 μmol photons m⁻² s⁻¹), respectively.
Table 1: Definition of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBL</td>
<td>Diffusive boundary layer</td>
<td></td>
</tr>
<tr>
<td>TBL</td>
<td>Thermal boundary layer</td>
<td></td>
</tr>
<tr>
<td>PAR</td>
<td>Photosynthetic active radiation ((420-700\text{nm}))</td>
<td></td>
</tr>
<tr>
<td>(J_{\text{NPP}(up)})</td>
<td>Upwards (\text{O}_2) flux</td>
<td>(\text{nmol \text{O}_2 \text{m}^{-2} \text{s}^{-1}})</td>
</tr>
<tr>
<td>(\phi)</td>
<td>Sediment porosity</td>
<td>Dimensionless</td>
</tr>
<tr>
<td>PS((z))</td>
<td>Volumetric rates of gross photosynthesis</td>
<td>(\text{nmol \text{O}_2 \text{cm}^{-3} \text{s}^{-1}})</td>
</tr>
<tr>
<td>PS((a))</td>
<td>Areal rates of gross photosynthesis</td>
<td>(\text{nmol \text{O}_2 \text{cm}^{-2} \text{s}^{-1}})</td>
</tr>
<tr>
<td>(J_{\text{PS}})</td>
<td>Areal rates of gross photosynthesis in energy terms</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(J_{\text{H}\uparrow})</td>
<td>Upwards heat flux</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(J_{\text{H}\downarrow})</td>
<td>Downwards heat flux</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(J_{\text{H}})</td>
<td>(J_{\text{H}\uparrow} - J_{\text{H}\downarrow})</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(E_d(\lambda))</td>
<td>Downwelling quantum irradiance</td>
<td>Counts \text{nm}^{-1}</td>
</tr>
<tr>
<td>(E_0(\lambda))</td>
<td>Spectral scalar irradiance</td>
<td>% of (E_d(\lambda))</td>
</tr>
<tr>
<td>(E_0(z))</td>
<td>Local light availability</td>
<td>(\mu\text{mol photons m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(J_{\text{IN}})</td>
<td>Incident light energy</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(J_{\text{ABS}})</td>
<td>Absorbed light energy</td>
<td>(\text{J m}^{-2} \text{s}^{-1})</td>
</tr>
<tr>
<td>(R)</td>
<td>Irradiance reflectance</td>
<td></td>
</tr>
<tr>
<td>(K_0(\lambda))</td>
<td>Diffuse attenuation coefficient of PAR</td>
<td>(\text{mm}^{-1})</td>
</tr>
<tr>
<td>(K_0(\lambda))</td>
<td>Spectral attenuation coefficient</td>
<td>(\text{mm}^{-1})</td>
</tr>
<tr>
<td>(E_k)</td>
<td>Photochemical light acclimation index</td>
<td>(\mu\text{mol photons m}^{-2} \text{s}^{-1})</td>
</tr>
</tbody>
</table>

Light measurements

Spectral scalar irradiance was measured with a fiber-optic scalar irradiance microprobe (spherical tip diameter \(\sim 70\mu\text{m}\)) (Rickelt et al., 2016) connected to a fiber-optic USB spectrometer (USB2000+, Ocean Optics, USA). Spectral downwelling irradiance, \(E_d(\lambda)\), was measured with the microprobe tip positioned in a black, non-reflective, light-well at the same distance from the light source as the mat surface. In the mat samples, spectral scalar irradiance, \(E_0(\lambda)\) was measured in vertical increments of 0.1 mm. These measurements where then corrected for different exposure time and normalized to similarly corrected downwelling irradiance spectra yielding scalar irradiance transmittance spectra in different mat layers in \% of \(E_d(\lambda)\), i.e., \(E_0(\lambda)/E_d(\lambda) \cdot 100\).

Spectral attenuation coefficients of scalar irradiance, \(K_0(\lambda)\) were calculated for specific depth intervals in the microbial mats as (Kühl, 2005):

\[
K_0(\lambda) = -\ln \left( \frac{E_0(\lambda)_1/E_0(\lambda)_2}{z_2 - z_1} \right)
\]

where \(E_0(\lambda)_1\) and \(E_0(\lambda)_2\) are the spectral scalar irradiances measured at depth \(z_1\) and \(z_2\), respectively.
Similarly, attenuation coefficients of PAR, $K_0$, were calculated from integrated values over the spectral region of interest. Due to mismatch in the measurements step size between the spectroradiometer and the fiber-optic spectrometer, integrations were carried out from 420-700 nm (henceforth mentioned as PAR). In deeper layers of the mat, integration of the measurements encompassed regions in the blue part of the spectrum exhibiting very noisy signals and an increasing contribution from stray light in the spectrometer. These noisy signals were therefore excluded.

Reflectance of the microbial mat surface was measured with a fiber-optic field radiance miniprobe (tip diameter = 1 mm) connected to the same fiber-optic spectrometer used for scalar irradiance measurements. The PAR reflectance ($R_{PAR}$) was calculated from the upwelling irradiance ($E_u(\lambda)$), here measured as the backscattered spectral radiance assuming fully Lambertian (diffuse) backscatter from the mat surface (Kühl & Jorgensen, 1994), and the downwelling irradiance ($E_d(\lambda)$) measured as the backscattered spectral radiance measured over a white Lambertian reflectance standard (99%; Spectralon, Labsphere, USA) as (Kühl, 2005):

$$R_{PAR} = \int_{420}^{700} \frac{E_u(\lambda)}{E_d(\lambda)} d\lambda$$

The acceptance angle of light collection through the fiber depends on the numerical aperture (NA) of the fiber and the refractive index of the medium. Since $E_d(\lambda)$ was estimated in air, and $E_u(\lambda)$ was measured in water we corrected for the acceptance angle differences by the relation:

$$\Theta_a = \sin^{-1}\left(\frac{NA}{RI_w}\right)$$

where $\Theta_a$ is the acceptance angle, NA is the numerical aperture of the fiber (0.22) and RI$_w$ is the refractive index of water (1.33).

Microsensor measurements of $O_2$ concentration and gross photosynthesis

Vertical depth profiles of $O_2$ concentration were measured using Clark-type $O_2$ microelectrodes (tip diameter=25µm, OX-25, Unisense A/S, Aarhus, Denmark) with fast response time (<0.5 s) and low stirring sensitivity (<1-2%) (Revsbech, 1989), connected to a pA-meter (Unisense A/S, Aarhus, Denmark) and interfaced through an A/D converter (DCR-16, PyroScience, Germany) to data acquisition software (Profix, PyroScience, Germany). The $O_2$ signals were also recorded on a strip-chart recorder (BD 12E; Kipp & Zonen BV, Netherlands) connected to the pA-meter. Sensor signals were linearly calibrated at experimental temperature and salinity from measurements in the aerated free flowing water in the flow-chamber and in anoxic zones in the sediment. Depth profiles of $O_2$
concentration were measured in 0.1 mm increments relative to the mat surface position determined by placing the sensor tip at the mat surface (z = 0 mm), as observed through a USB microscope (AM7013MZT Dino-Lite, AnMo Electronics Corporation, Taiwan). The sensor tip was then moved to 1.5 mm above the mat surface (z = -1.5 mm) and from here profiles were measured in steps of 100 µm until reaching anoxic mat layers (z~1.5 mm). The flux of O₂ from the mat into the water, i.e. net areal photosynthesis (J_{NPP(up)}) was calculated from Fick’s 1st law of diffusion using the linear O₂ concentration gradient in the diffusive boundary layer (DBL) and the diffusion coefficient of O₂ in seawater (D₀) at experimental temperature and salinity:

\[ J_{NPP(\text{up})} = -D_0 \frac{\partial C}{\partial z} \]

The volume-specific rate of gross photosynthesis (PS(z) in nmol O₂ cm⁻³ s⁻¹) was measured using the light/dark shift method (Revsbech & Jørgensen, 1983) in vertical steps of 0.1 mm starting from just above the microbial mat surface until the depth where no more production could be measured, i.e. the depth where no immediate O₂ signal changes were observed upon darkening. Areal rates of gross photosynthesis (PS(a) in nmol O₂ cm⁻² s⁻¹) were calculated by integrating the volumetric rates over depth.

**Temperature microsensor measurements**

Temperature profiles were measured using thermocouple microsensors (tip diameter=50µm, TP-50, Unisense A/S, Aarhus, Denmark) connected to a thermocouple meter (Unisense A/S, Aarhus, Denmark) and interfaced through an A/D converter (DCR-16, PyroScience, Germany) to data acquisition software (Profix, PyroScience, Germany). Signals were linearly calibrated against a high precision digital thermometer (±0.2°C; Testo 110, Testo AG, Germany) in seawater of different temperatures. The areal heat dissipation from the microbial mat was calculated using Fourier’s law of conduction using the linear temperature gradient in the thermal boundary layer (TBL) and the thermal conductivity of seawater (k = 0.6 W m⁻¹ K⁻¹):

\[ J_H = k \frac{\partial T}{\partial z} \]

The downward heat dissipation could not be directly calculated from the measured temperature profiles, and were estimated from the principle of energy conservation as the difference between the absorbed light energy and the sum of photosynthesis and upward heat flux (both in energy units).
Energy calculations

To obtain absolute scalar irradiance spectra, we multiplied the measured transmittance spectra for each depth with the measured incident radiometric spectra (in W m⁻²). By using Planck’s equation:

\[ E_\lambda = h \frac{c}{\lambda} \]

where \( E_\lambda \) is the energy of a photon with a wavelength \( \lambda \), \( h \) is Planck’s constant \((6.626 \cdot 10^{-34} \text{ W s}^2)\) and \( c \) is the speed of light in vacuum \((\text{in m s}^{-1})\), we then converted absolute scalar irradiance spectra to absolute spectra of photon scalar irradiance \( E_0(z) \) in \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \text{nm}^{-1} \).

The total absorbed light energy in the mat \( (J_{ABS} \text{ in J m}^{-2} \text{s}^{-1}) \), i.e., the vector irradiance, was calculated from the downwelling spectral irradiance \( E_d(\lambda) \) and the irradiance reflectance as:

\[ J_{ABS} = \int_{420}^{700} E_d(\lambda)(1 - R(\lambda)) d\lambda \]

The amount of energy dissipated via photosynthesis was calculated by multiplying the areal photosynthesis rates \( (PS(a)) \) with the Gibbs free energy from the light reactions, where \( \text{O}_2 \) is produced by the photolysis of water. Including the formation of ATP this yields 482.9 kJ (mol \( \text{O}_2 \))⁻¹ (Thauer et al., 1977).

Energy budgets for the entire photic zone were calculated under the assumption that the total energy stored in photosynthesis \( (J_{PS}) \), and dissipated via heat \( (J_H) \) and reflection \( (R) \) balanced the incoming radiative energy \( (J_{IN}) \):

\[ J_{IN} = J_H + J_{PS} + R \quad \text{and} \quad J_{IN} - R = J_{ABS} = J_H + J_{PS} \]

To investigate how photosynthetic quantum efficiency varied with depth in the microbial mat, we calculated photosynthetic efficiencies \( (\text{in mol O}_2 \ (\text{mol photons})^{-1} \text{ mm}^{-1}) \) by dividing the photosynthetic rates in a specific depth layer with the photon scalar irradiance just above that layer (Lassen et al., 1992).

We note that the calculated efficiency parameter does not reflect the true photosynthetic efficiency, where photosynthesis is related the amount of absorbed photons in the particular depth layer (see discussion).

HPLC pigment analysis

Sediment samples of the 0–0.5 mm surface layer were collected using the “crème brûlée” sampler described by Laviale et al. (2015) and stored at -80°C. Approximately 100 mg of sampled mat material were ground with a micro pestle and extracted in a mixture of acetone and methanol (7:2). Samples were sonicated (S-4000, Branson Ultrasonic Corporation, USA) for 20 s to improve pigment extraction.
and were then centrifuged for 60 s at 13,400 rpm. Supernatants were filtered through 0.45 μm PTFE-membranes and immediately injected in a HPLC (1260 Infinity, Agilent Technologies, USA). 15 μL of 1 M ammonium acetate was added to each HPLC vial prior to injection as a resolution-improving agent. The solvent gradient followed Frigaard et al. (1997) with a 69 min elution program, a flow rate of 1.0 mL min⁻¹ and an injection volume of 100 μL. Chromatographic separation was carried out using a C18 Ascentis® column for reverse phase chromatography (5 μm particle size; L x I.D: 25 cm x 4.6 mm). Pigments were identified from retention times and absorbance spectra.

Figure 1: Conceptual drawing of the biomass distribution during different light acclimation scenarios. The surface layers of the microbial mat were dominated by dense populations of motile cyanobacteria (*Microcoleus* sp. and other filamentous species) and filamentous sulfide oxidizing bacteria (*Beggiatoa* spp.). Beneath the narrow photic zone, a dark FeS-containing sediment layer was present. In darkness, the entire mat gets anoxic and the *Beggiatoa* moved to the mat surface forming a white layer on top of the cyanobacteria, to avoid the high levels of H₂S and remain at the O₂-H₂S interface (Preisler *et al.*, 2007), whereas the cyanobacteria remained in a dense layer underneath. In low light, the upper layers became oxidized driving the O₂-H₂S interface and thus *Beggiatoa* deeper into the mat, while the motile cyanobacteria moved towards the mat surface under non-inhibitory light levels. In high light, a larger part of the biofilm is oxidized, *Beggiatoa* moved further down, while motile cyanobacteria in the top layers started to move downwards to avoid high photoinhibitory light levels at the microbial mat surface.
Results

Light, temperature and photosynthesis

Microprofiles of scalar irradiance in the dense biofilm were measured after incubation in dark, low light, and high light, respectively, which yielded a different organization of motile microbes in the mat (Fig. 1). In low light-acclimated state, a cyanobacterial band formed near the surface while in the high light-acclimated state the cyanobacteria were found in deeper layers. In the dark-acclimated biofilm, a dense band of colorless Beggiatoa spp. formed at the surface with the cyanobacteria located below the colorless sulfur bacteria (Fig. 1).

The attenuation of light through the biofilm did not follow a mono-exponential decay, but attenuated with varying degree in different layers depending on light acclimation (Fig. 2 A-C). The measurements were done in a low light-acclimated (left panels), high light-acclimated (middle panels), and dark-acclimated biofilms (right panels). PAR attenuation coefficients, $K_0$, were estimated in the upper- and lower part of the biofilm (0 – 0.2 mm; 0.3 – 0.5 mm) from the slope of linear regressions on natural logarithm transformed data.

The attenuation of light through the biofilm did not follow a mono-exponential decay, but attenuated with varying degree in different layers depending on light acclimation (Fig. 2 A-C). In the low light acclimated state, the steepest attenuation was found in the top 0.2 mm ($K_0 = 13.9 \text{ mm}^{-1}$), whereas the
underlying part of the microbial mat (0.3 – 0.5 mm) showed a slightly lower light attenuation (K₀=10.3 mm⁻¹). In the high light-acclimated state this trend was reversed, where the attenuation in the top layer (K₀=10.6 mm⁻¹) was lower than in deeper layers of the microbial mat (K₀=16.5 mm⁻¹). In the dark-acclimated state, a lower light attenuation was found in the top 0.2 mm (K₀=5.8 mm⁻¹) of the microbial mats followed by a very steep attenuation from 0.2 – 0.4 mm depth (K₀=22.2 mm⁻¹).

Spectral attenuation was clearly enhanced around absorption maxima of most abundant photopigments commonly found in cyanobacterial mats, e.g. Chl a (440 nm; 675 nm), phycocyanin (620 nm) and phycoerythrin (565 nm) (Fig. 2 D-F). HPLC analysis revealed the presence of other cyanobacterial pigments such as myxoxanthophyll, zeaxanthin, oscillaxanthin and β,ε-carotene in the upper 0.5 mm of the mat. Furthermore, bacteriochlorophyll a was also observed (Fig. 3). Due to the very high attenuation of light in the mat, <1% of PAR surface scalar irradiance remained just 0.4 mm below the biofilm surface (Fig. 2).

The very high light-attenuation of PAR (400-700 nm) resulted in low surface reflection (Fig. 4). In the low-light acclimated state, only 0.75% of the incident light was reflected, whereas the reflection from the mat surface in the high light-acclimated state was twice as high (1.6%). In the dark-acclimated state,
where the surface of the microbial mat was covered by a layer of motile, filamentous colorless sulfur bacteria (*Beggiatoa*, spp.), the reflection increased ~10-fold to 9.2% of the incident irradiance. Most of the incident light in the PAR region was thus absorbed in the mat. Most of the absorbed light was dissipated as heat as quantified from the heat flux over a ~1.2 mm thick thermal boundary layer (TBL) (Fig. 5). The surface heating differed depending on light acclimation, where the surface temperature of the high light-acclimated biofilm increased by 0.3°C as compared to the low light-acclimated mat showing a mat surface temperature increase of 0.24°C. The lowest surface heating was measured when illuminating the dark-acclimated mat reaching a surface temperature increase of 0.2°C.

Due to the high light-attenuation, the euphotic zone was restricted to the uppermost 0.6 – 0.8 mm of the microbial mat (Fig. 6). The depth distribution of gross photosynthesis rates varied with light acclimation, and very high rates were observed in all treatments (up to 30 nmol O₂ cm⁻³ s⁻¹). In the low light-acclimated state, photosynthetic peaks were found at the mat surface and 0.3 mm beneath the surface. In the high light-acclimated state, the photosynthetic rates near the surface were diminished, while a stronger sub-surface peak was found around 0.2 mm below the mat surface. The dark-acclimated mat showed a small photosynthesis peak near the mat surface and a sub-surface peak at 0.3-0.4 mm depth (Fig. 6).

Figure 5: Vertical microprofiles of temperature increase (ΔT in °C) relative to the free flowing water temperature. The dashed line indicates the microbial mat surface. Black, red and blue symbols represent measurements in a low light-, high light-, and dark-adapted mats, respectively. The straight black, red and blue lines indicate the linear regressions used to estimate the areal heat dissipation from the microbial mat. Data points are means (n=3) ± 1 SD.
Energy budgets

The vector irradiance (i.e. the net downwelling energy flux) was very similar between light acclimations and amounted to 218.0 ± 5.6 W m⁻². However, the heat dissipation from the mat to the water differed between light incubations with the dark-acclimated mat exhibiting the lowest upward heat dissipation of 140 W m⁻², while the heat dissipation in the low light-acclimated mat was 144 W m⁻². The highest heat dissipation was found in the high light-acclimated mat and amounted to 179 W m⁻² (Fig. 7).

Areal photosynthesis only accounted for a small part of the absorbed light energy. The highest amount of energy stored via photosynthesis was found in the low light-acclimated microbial mat and amounted to...
to 5.4 ± 0.7 W m⁻² (Fig. 7), while the high light- and the dark-acclimated mat conserved 4.06 ± 1.6 W m⁻² and 4.8 ± 1.1 W m⁻², respectively.

Relative to the incident energy, the photosynthetic energy conservation efficiency for the entire photic zone only accounted for ~2% (2.3%, 2.4% and 1.8% for the dark-, low light-, and high light- acclimated biofilm, respectively), while the majority of incident light energy was dissipated as heat (Fig. 8).

![Closed radiative energy budgets](image)

**Figure 8: Closed radiative energy budgets** of the entire photic zone in a low light-, high light-, and dark-acclimated microbial mat as a function of the incident light energy. Blue bars depict the energy reflected from the surface, red bars are the amount of energy that was dissipated as heat and the green bars are the amount of light energy photochemically conserved via photosynthesis. Note the break in the y-axis. (n=3).

**Photosynthetic efficiencies**

Photosynthetic efficiencies, calculated as the volumetric gross photosynthesis rates divided by the scalar irradiance incident to that depth, showed sub-surface peaks in efficiencies in all light acclimation states of the microbial mat (Fig. 9). In the low light-acclimated mat, a peak in efficiency was located closest to the surface at 0.3 mm depth. The dark-acclimated mat exhibited highest photosynthetic efficiencies 0.4 mm beneath the surface, while the highest photosynthetic efficiencies in the high light acclimated mat were found near the lower boundary of the photic zone at 0.5 mm below the mat surface.
Discussion

Radiative energy budgets in microbenthic systems have previously been studied in cyanobacterial mats and biofilms (Al-Najjar et al., 2010; Al-Najjar et al., 2012), sediments (Lichtenberg et al. 2017), and corals (Brodersen et al., 2014), however, under the assumption of a homogenous depth distribution of biomass. In the studied microbial mat, dense populations of phototrophic microalgae and especially cyanobacteria resided in a steep gradient of resource stratification as shaped by communities of phototrophic, chemo-, and heterotrophic microorganisms creating a steep redox gradient (Braker et al., 2001). Consequently, the physical and chemical landscape in such microbial mats can change, within less than one mm, from intense sun exposure (>1000 µmol photons m⁻² s⁻¹) and O₂ supersaturation (up to 2 mM), to complete darkness and a reduced sediment high in H₂S (Jørgensen, 1982).

The studied microbial mat exhibited an extremely high optical density and attenuation of PAR, where >99% of the surface irradiance was effectively absorbed <0.4 mm below the microbial mat surface. Light was thus the primary limiting resource for microbial phototrophic organisms in the mat. One strategy to remain competitive under light limitation is to complement light absorption by Chl a, the main light harvesting pigment in photosynthesis, by metabolic investment in producing a range of accessory pigments absorbing a broader part of the available light spectrum and channeling it to the photosynthetic reaction centers (Şener et al., 2011). HPLC pigment analysis of the upper 0.5 mm of the mat.

Figure 9: Photosynthetic efficiencies (in mol O₂ (mol photons)⁻¹ mm⁻¹) calculated from volume-specific gross photosynthesis rates and the photon scalar irradiance incident to the corresponding depth in a low light-, high light-, and dark-acclimated biofilm. (n=3).
microbial mat revealed that very efficient light absorption was achieved by a complex cassette of light harvesting pigments, able to absorb light from the far-UV well into the NIR region (Fig. 3). Consequently, low surface reflection was observed in the low and high light-acclimated microbial mats. In the low light-acclimated mat, the cyanobacterial population was located near the surface and only 0.75% of the incident irradiance was backscattered, while in the high light-acclimated state, the motile cyanobacteria migrated downward changing the surface biomass composition leading to higher reflectance. During dark-acclimation, the surface of the biofilm became anoxic and a layer of the motile, filamentous colorless sulfur bacteria *Beggiatoa* formed on the mat surface. *Beggiatoa* spp. are sulfide oxidizing bacteria known to store granules of elemental sulfur (Nelson & Castenholz, 1981), which make the filaments appear white due to strong light scattering, which in our measurements increased the surface reflectance to 9.2% of the incident irradiance. Light-dependent migration patterns of cyanobacteria and *Beggiatoa* thus clearly modulated the light energy input to the microbial mat.

The strong light absorption in the uppermost mat layers, resulted in very high local photosynthetic rates reaching >30 nmol O$_2$ cm$^{-3}$ s$^{-1}$. This is about 2-5 fold higher than rates measured in other cyanobacteria-dominated microbial mats (Al-Najjar et al., 2012), but e.g. comparable to a highly stratified intertidal mudflat community dominated by motile diatoms (Cartaxana et al., 2016b). While a multitude of studies have investigated photosynthesis under increasing irradiances (Webb et al., 1974; Al-Najjar et al., 2010; Lichtenberg & Kühl, 2015; Lichtenberg et al., 2017), we investigated photosynthesis under high light exposure of microbial mats exhibiting different distributions of motile microbes in response to different light history. Investigations of photosynthesis under increasing irradiances in motile microbial systems are complex as migrating cells under changing light can change the spatial characteristics of the biofilm/mat and thereby potentially affect light exposure and distribution in different mat layers (Kühl et al., 1997). In the present study, the main aim was thus to investigate how the photosynthetic efficiency was modulated by migration of motile cyanobacteria under different light exposure inducing differences in the spatial organisation of phototrophs in in the microbial mat.

Areal rates of photosynthesis, integrated across the entire euphotic zone, did not differ markedly between different light acclimations (0.99, 1.11 and 0.84 nmol O$_2$ cm$^{-2}$ s$^{-1}$ for dark-, low light- and high light-acclimated samples, respectively). However, the spatial distribution of gross photosynthesis varied with light acclimation, probably as a result of light-induced migrations of the cyanobacteria to avoid exposure to high, potentially damaging, light levels. One of the most important short-term regulatory mechanisms to avoid photo damage is non-photochemical quenching (NPQ), where excess energy decays from singlet excited chlorophyll (’Chl*$^*$) into heat dissipation (Müller et al., 2001). This mechanism
significantly lowers the effective quantum yield of photosynthesis but avoids the formation of reactive singlet oxygen (\(^1\text{O}_2\)) by the triplet state of chlorophyll (\(^3\text{Chl}^*\)) (Müller et al., 2001), which can have long term detrimental effects on the photosystems by degradation of the D1 protein; an important component in photosystem II (Nymark et al., 2009). Photophic responses or phototaxis enabling movement along a light gradient (Jekely, 2009), are alternative strategies of photoprotection enabling motile phototrophs to align their position at optimum irradiance in light gradients depending on the status of the electron transport chain and time of the day (Burns & Rosa, 1980).

From our measured depth profiles of gross photosynthesis, it appears that the distance of migration was limited to only 0.2-0.3 mm vertically. However, due to the steep light gradients in the investigated microbial mat, the changes in the light field over such short distances are dramatic, where cyanobacteria migrating 200 µm deeper into the mat experienced a >84-fold decrease in scalar irradiance (Fig. 2C; 0.2 – 0.4 mm) from >350 µmol photons m\(^{-2}\) s\(^{-1}\) to <10 µmol photons m\(^{-2}\) s\(^{-1}\). Such strong changes in light exposure upon migration distances of a few 100 µm have previously been demonstrated in hypersaline cyanobacterial mats (Kühl et al., 1997).

Compared to earlier studies of heat dissipation in corals and microbial mats (Jimenez et al., 2008; Al-Najjar et al., 2010; Brodersen et al., 2014), we found a relatively small heat dissipation from the biofilm surface into the water column and the surface temperature of the microbial mat increased by only ~0.3°C. However, the downward heat dissipation was larger than expected. The zone of maximum temperature increase was expected to be found in the same zone as the largest energy deposition, i.e., in the upper few hundred micrometers of the biofilm, where >90% of the incident light was attenuated. However, the heat dissipation below 0.5 mm was in the same order of magnitude as the upward heat dissipation (Fig. 5). We note that below the dense photic zone, a black layer of precipitated iron sulfide was found and absorption of light energy in this sediment layer apparently contributed significantly to the heating of the lower zones in the sediment. While we did not measure temperature deep enough in the sediment to be able to calculate the downward transport of heat, we estimated from the principle of energy conservation that the downward heat flux contributed with 20-50% of the total heat dissipation.

The amount of photochemically conserved energy did not change markedly between light acclimation, despite the higher reflection and lower heat dissipation observed in the dark-acclimated biofilms. Consequently, the photosynthetic energy conservation was 2.4%, 2.4% and 1.8% of the incident energy, for dark-, low light- and high light-acclimated biofilms, respectively. This indicates that by migration, the
phototrophic community can apparently sustain similar energy conservation efficiencies, while avoiding the detrimental effects of excessive light when situated near the surface during peak daylight. The vertical stratification of photosynthetic efficiencies (Fig. 9) can either be ascribed to i) a higher density of biomass contributing with photosynthesis, or ii) an overall higher efficiency caused by higher cell specific pigment content and/or higher absorption cross section, in that area (Iglesias-Prieto & Trench, 1994; Iglesias-Prieto & Trench, 1997; Falkowski & Raven, 2007; Al-Najjar et al., 2010). However, in our measurements we found differences in the vertical positions of the peaks of photosynthetic efficiency depending on light acclimation. At the cellular level, regulation of the pigment density and the absorption cross section of antennae pigments is on a longer time scale than the acclimation time used in these experiments, although the formation of zeaxanthin from \( \beta \)-carotene is a short term photoprotective mechanism (Falkowski & Raven, 2007). Given the time scale, the difference in vertical position of the peaks in photosynthetic efficiencies, can be ascribed to migration. In a fluctuating light environment, a photosynthetic cell can employ light handling strategies either by regulating pigment densities or by moving to a different light environment. For this mechanism to be energetically successful, the cost of motility must present an advantage compared to employment of regulation of light harvesting or photoprotective pigments. However, in a dense microbial system, where oxygenic phototrophs not only have to cope with alternating light environments but also steep chemical gradients of e.g. cytotoxic compounds such as \( \text{H}_2\text{S} \), motility presents an added advantage to escape anoxic zones and high concentrations of \( \text{H}_2\text{S} \). Furthermore, migration can to a certain degree mitigate effects of continuous sedimentation and overgrowth by other microbes. Previously photosynthetic efficiencies have been calculated by applying a model that estimates the local density of absorbed light and then correlate that to the local rates of gross photosynthesis (Al-Najjar et al., 2010). However, this model assumes a homogenous distribution of light absorption (i.e. biomass), which makes such a model invalid in a motile community exhibiting phototaxis. Calculations using the model of Al-Najjar et al. (2010) on our data showed apparent photosynthetic quantum efficiencies, about 2-fold higher than the theoretical maximum of 0.125 (i.e. 8 photons needed to produce 1 \( \text{O}_2 \) molecule). We speculate that the black ferrous sulfide layer below the biofilm also interfered with such estimations of local density of absorbed light. However, the distribution of sand particles, organic matter and photosynthetic biomass was not further quantified, and a correction for this possible artefact was not attempted. Therefore, the relative quantum efficiencies were estimated by relating the depth specific gross photosynthesis rates to the scalar irradiance incident to that point (Lassen et al., 1992). Lassen et al. (1992) likewise found that the position of maximum quantum efficiencies changed with light exposure.
However, the relative quantum efficiencies from this study were up to three times higher than what was found by Lassen et al. (1992), but depends on photo-pigmentation and other biotic/abiotic substances contributing to light absorption. Thus, the canopy structure will have an influence on the light availability and the energy balance of the system, where more open systems will display larger photic zones and different responses to changes in light environment compared to compact systems (Lichtenberg et al., 2017).

From the present study, we conclude that the cyanobacterial community sustained high photosynthetic efficiencies by vertically migrating to a light environment optimizing the cumulative capacity of light absorption and the status of the electron transport chain. This was evident from the overall photosynthetic conservation efficiency that did not change with light acclimation but showed vertical differences in the zones of maximum photosynthetic efficiency. Further studies could investigate how distribution of gross photosynthesis change in a changing light environment but with vertically fixed populations in different depth horizons, e.g. by applying a motility inhibitor (Cartaxana et al., 2016a).

Acknowledgements.

This study was supported by a Sapere-Aude Advanced research grant from Danish Council for Independent Research Council | Natural Sciences (FNU) (MK), and the Carlsberg Foundation (MK). We thank Lars Rickelt for construction of scalar irradiance microprobes, and Sofie Jakobsen for excellent technical assistance. The members of the Microenvironmental Research Group provided useful feedback, help and suggestions during the experimental and analysis part of this study.
References


Lichtenberg M, Brodersen KE, Kühl M. 2017. Radiative energy budgets of phototrophic surface-associated microbial communities and their photosynthetic efficiency under diffuse and collimated light. Frontiers in Microbiology 8: 452


Chapter 7

Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial $O_2$ loss from the below-ground tissue

Published in *Frontiers in Marine Science*

by

Kasper Elgetti Brodersen, Mads Lichtenberg, Laura-Carlota Paz and Michael Kühl
Epiphyte-cover on seagrass (*Zostera marina* L.) leaves impedes plant performance and radial O$_2$ loss from the below-ground tissue

Kasper Elgetti Brodersen$^{1,a}$, Mads Lichtenberg$^{2,a}$, Laura-Carlota Paz$^3$ and Michael Kühl$^{1,2}$

$^1$Plant Functional Biology and Climate Change Cluster, University of Technology Sydney, Australia  
$^2$Marine Biological Section, University of Copenhagen, Denmark  
$^3$Department of Bioscience – Microbiology, Aarhus University, Denmark  

$a$These authors contributed equally to this work

Abstract

The O$_2$ budget of seagrasses is regulated by a complex interaction between several sources and sinks, which is strongly regulated by light availability and mass transfer over the diffusive boundary layer (DBL) surrounding the plant. Epiphyte growth on leaves may thus strongly affect the O$_2$ availability of the seagrass plant and its capability to aerate its rhizosphere as a defense against plant toxins. We used electrochemical and fiber-optic microsensors to quantify the O$_2$ flux, DBL and light microclimate around leaves with and without filamentous algal epiphytes. We also quantified the below-ground radial O$_2$ loss from roots (~1 mm from the root-apex) to elucidate how this below-ground oxic microzone was affected by the presence of epiphytes. Epiphyte-cover on seagrass leaves (~21% areal cover) resulted in reduced light quality and quantity for photosynthesis, thus leading to reduced plant fitness. A ~4 times thicker diffusive boundary layer around leaves with epiphyte-cover impeded gas (and nutrient) exchange with the surrounding water-column and thus the amount of O$_2$ passively diffusing down to the below-ground tissue through the aerenchyma in darkness. During light exposure of the leaves, radial oxygen loss from the below-ground tissue was ~2 times higher from plants without epiphyte-cover. In contrast, no O$_2$ was detectable at the surface of the root-cap tissue of plants with epiphyte-cover during darkness, leaving the plants more susceptible to sulphide intrusion. Epiphyte growth on seagrass leaves thus has a negative effect on the light climate during daytime and O$_2$ supply in darkness, hampering the plants performance and thereby reducing the oxidation capability of its below-ground tissue.
Introduction

Seagrasses are angiosperms that form coastal habitats of prime importance for marine biodiversity and carbon sequestration (Duarte, 2001; Duarte et al., 2005). Over the past century, seagrasses have faced an alarming global decline, owing to both direct and indirect human interference (Robblee et al., 1991; Zieman et al., 1999; Seddon et al., 2000; Plus et al., 2003; Orth et al., 2006). Seagrasses inhabit organic rich, reduced sediments and the exposure of their below-ground biomass to sediment-derived hydrogen sulphide (H$_2$S), as a result of inadequate internal aeration due to low water-column O$_2$ levels during darkness, has been identified as a key factor in seagrass die-back events (Greve et al., 2003; Borum et al., 2005; Brodersen et al., 2015). Hydrogen sulphide is produced in reduced sediment through bacterial sulphate reduction, which is considered the quantitatively most important anaerobic degradation process in coastal marine sediment (Jørgensen, 1982). H$_2$S is a phytotoxin that leads to chemical asphyxiation, due to a strong chemical binding with cytochrome c in the mitochondrial electron transport chain (Eghbal et al., 2004; Perez-Perez et al., 2012; Lamers et al., 2013). If H$_2$S reaches the root tissue surface it may enter the lacunar system of the seagrass plant via lipid-solution permeation of the plasmalemma (Raven and Scrimgeour, 1997). Such H$_2$S intrusion into the below-ground tissue of seagrasses has mainly been related to inadequate internal aeration during night-time, as a result of a low water-column O$_2$ content and thus a decrease in the diffusive O$_2$ supply from the surrounding water-column (Pedersen et al., 2004; Borum et al., 2005). The amount of O$_2$ passively diffusing into the leaves from the water-column during darkness, is thus highly dependent on the water-column O$_2$ content, but is also strongly affected by other factors such as the diffusive boundary layer (DBL) thickness (Binzer et al., 2005; Borum et al., 2006) and the leaf surface area. The DBL surrounds all aquatic surfaces, such as seagrass leaves, and functions as a diffusive barrier to the exchange of gasses and nutrients with the surrounding water-column by impeding water motions towards the leaf tissue surface (Jørgensen and Revsbech, 1985). The width and thus the mass transfer impedance of the DBL depends on factors such as the surface topography and the flow velocity, where e.g. relative low flow rates and uneven surfaces increases the thickness of the DBL (Jørgensen and Des Marais, 1990); both parameters are highly affected by epiphyte growth on the leaf surface.

Light availability is the key environmental factor regulating photosynthesis and thus the O$_2$ supply during day-time, and small decreases in irradiance can cause significant declines in the growth and distribution of seagrasses (Burkholder et al., 2007; Ralph et al., 2007). In eutrophic coastal waters, light can be attenuated up to 100-fold in the upper 1-4 m of the water column, often with dramatic changes in the spectral composition (Sand-Jensen & Borum, 1991). Therefore, rooted macrophytes are often spatially
limited to biotopes with sufficient light exposure, i.e., water depths experiencing a minimum of 10% of surface irradiance for temperate seagrasses (Borum, 1983; Duarte, 1991). Eutrophication can stimulate epiphyte colonization on seagrass leaves (Richardson, 2006) potentially affecting the light availability for the plant. Epiphytes may thus have a major impact on the photosynthetic $O_2$ evolution of rooted macrophytes, such as seagrasses (Sand-Jensen, 1977).

The $O_2$ budget of seagrass plants is regulated by a complex interaction between several sources and sinks. Sources encompass photosynthetic $O_2$ evolution in leaves during day-time and passive diffusion of $O_2$ into the leaves from the water-column in darkness. Sinks encompass the total $O_2$ demand of the surrounding sediment, including bacterial respiration and chemical reactions with reduced compounds, as well as the plants own respiratory needs. The amount of $O_2$ produced or passively diffusing into the leaves is affected by external physical factors such as the light availability for underwater photosynthesis, the flow-dependent thickness of the DBL and the water-column $O_2$ content, whereas the sinks are highly affected by elevated seawater temperatures and the quantity of accessible organic matter in the rhizosphere (Pedersen et al., 2004; Binzer et al., 2005; Borum et al., 2006; Raun and Borum, 2013).

The $O_2$ is transported from the above-ground tissue to the below-ground tissue through the aerenchyma, i.e., an internal gas-filled lacunar system, whereby the plants supports aerobic metabolism in their root-system and provide protection against reduced toxic compounds such as $H_2S$ and $Fe^{2+}$ (Armstrong, 1979; Borum et al., 2006). Some of the transported $O_2$ is leaked to the rhizosphere as the so-called radial oxygen loss (ROL), especially at the basal meristems, root-shoot junctions and root-caps (Koren et al., 2015). During non-stressful environmental conditions, ROL maintains a ~0.5 mm wide oxic microzone around the leaking areas that continuously oxidizes the surrounding sediment and thus alters the immediate sediment biogeochemistry in the seagrass rhizosphere (Pedersen et al., 1998; Jensen et al., 2005; Brodersen et al., 2015). This chemical defense mechanism is, however, negatively affected by over-night water-column hypoxia (Brodersen et al., 2015).

Seagrass morphology is an important controlling factor affecting the likelihood of $H_2S$ intrusion into seagrasses, where a higher above- to below-ground biomass ratio positively affects the seagrasses oxidation capacity and reduces the risk of $H_2S$ intrusion (Frederiksen et al., 2006). Seagrass roots possess structural barriers to ROL in mature root tissue regions such as Casparian band-like structures of suberin in the hypodermis (Barnabas, 1996). Such barriers to ROL in the basal-parts of seagrass roots increases the intra-plant $O_2$ transport to the active apical root meristem and therefore are very important for seagrass root metabolism.
In this study we used electrochemical and fiber-optic microsensors to investigate effects of epiphyte-cover on seagrass leaves on the below-ground aeration of the rhizosphere of the seagrass *Zostera marina* kept in a custom-made split flow-chamber with natural sediment. This microenvironmental approach allowed us to i) analyze the DBL and light microclimate around seagrass leaves with- and without epiphytes, and ii) correlate changes in these above-ground microenvironmental parameters with changes in the ROL from the root-caps, and thereby, the oxidation capacity of the below-ground tissue.

**Materials and methods**

*Seagrass and sediment sampling*

Marine sediment and *Zostera marina* specimens with and without leaf epiphyte-cover were collected from shallow coastal waters (<2 m depth) at Aggersund, Limfjorden, Denmark. After sampling, plants and sediment were transported to a nearby field station (Rønbjerg Marine Biological Station, Aarhus University, Denmark), where they were kept in constantly aerated and water flushed water reservoirs prior to experiments. Seagrass specimens with similar above- and below-ground biomass ratios were selected from the reservoirs and gently washed free of adhering sediment before transferred to the experimental split flow-chamber (see below; Brodersen et al., 2014). In the following, seagrasses with epiphyte-cover refer to plants with ~21% areal cover with filamentous algal epiphytes on leaves in contrast to seagrasses without visible leaf epiphyte-cover. The above- to below-ground biomass ratio was 1.0 and 0.8 of selected plants with and without leaf epiphyte-cover, respectively, based on g DW values obtained after drying the plants in an oven at 60°C until a constant weight was reached.

*Experimental setup*

Plants were horizontally positioned in the flow-chamber (one plant at a time) with the leaf canopy in the free flowing water phase compartment and the below-ground biomass transplanted in homogenized sediment from the sampling site in the adjoining “sediment” compartment (Fig. 1). An anoxic water column (~2 cm depth) functioned as a liquid-phase diffusion barrier to O2 intrusion over the sediment compartment of the flow chamber, as preliminary studies had shown a constant loss/efflux of reduced compounds such as H2S from the sediment during cultivation. Illumination of the leaf canopy was provided by a fiber-optic tungsten halogen lamp (KL-2500LCD, Schott GmbH, Germany). The downwelling photon irradiance (PAR, 400-700 nm) at the leaf surface was measured with a spherical quantum sensor (US-SQS/L, Walz GmbH, Germany) connected to a calibrated quantum irradiance
meter (ULM-500, Walz GmbH, Germany). A constant flow-rate (~0.5 cm s⁻¹) of aerated seawater (~22°C, Salinity=30) was maintained in the seawater compartment of the flow chamber by means of a pump submersed in an aerated seawater reservoir (Fig. 1).

**Light and O₂ measurements**

We used scalar irradiance microprobes (sphere diameter 50 µm; manufactured by a modified procedure of Lassen et al., 1992; Rickelt et al., submitted) to quantify the light microenvironment around leaves of *Z. marina* with- and without epiphyte cover under two different irradiance levels (50 and 200 µmol photons m⁻² s⁻¹; Fig. 1a). The scalar irradiance microprobe was connected to a fiber-optic spectrometer (USB 2000+, Ocean Optics, USA), interfaced to a PC running spectral acquisition software (SpectraSuite, Ocean Optics, USA). We measured vertical profiles of spectral scalar irradiance in 0.1 mm steps from the leaf surface to 1 mm above the leaf surface, and in 1 mm steps from 1-10 mm from the leaf surface. To quantify the downwelling irradiance, we recorded spectra of the vertically incident light with the scalar irradiance microprobe tip positioned over a black non-reflective light well at the same position and distance in the light beam as the seagrass tissue surface; in a collimated light field the downwelling- and scalar irradiance are identical (Kühl and Jørgensen, 1994).

Clark-type O₂ microsensors (OX-10 and OX-50, Unisense A/S, Aarhus, Denmark; Revsbech, 1989) with a fast response time (<0.5 s) and low stirring sensitivity (<2-3 %) were used to measure i) the
radial O₂ loss from the below-ground biomass of *Z. marina* (~1 mm from the root-apex; Fig. 1b), and ii) the O₂ concentration at and towards the leaf surface (Fig. 1a). The O₂ microsensors were linearly calibrated from signal readings in 100% air saturated seawater and anoxic seawater (by addition of ascorbate) at experimental temperature and salinity; prior to calibrations and measurements in natural sediment, the microsensors were pre-contaminated with sulphide, i.e., they were pre-polarized in a Na₂S solution, to avoid drifting calibrations during experiments.

Microsensors were mounted on a motorized micromanipulator (Unisense A/S, Denmark) and connected to a PC-interfaced microsensor multimeter (Unisense A/S, Denmark); both were controlled by dedicated data acquisition and positioning software (SensorTrace Pro, Unisense A/S, Denmark). Microsensors and microprobes were carefully positioned at the tissue surface (defined as 0 µm) by manual operation of the micromanipulator, while observing the microsensor tip and tissue surface.
through a USB microscope (AD7013MZT, DinoLite, AnMo Electronics Corp., Taiwan). When positioning the O₂ microsensors at the below-ground tissue surface, a root from the first root-bundle was first gently un-covered from sediment before manually moving the microsensor to the surface of the root-cap, where after the root was gently covered again with sediment. Steady state O₂ levels at the below-ground tissue surface were re-established after ~3h (data not shown). Microprofiles of O₂ concentration were measured in depth increments of 50 µm.

**Light calculations**

To quantify PAR, we integrated the measured scalar irradiance spectra over 400-700 nm and calculated the fractions of incident PAR irradiance for each measured depth position. By multiplying with the known incident quantum irradiance (in µmol photons m⁻² s⁻¹), measured with a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany) equipped with a spherical quantum sensor (US-SQS/L, Walz GmbH, Germany), absolute levels of light in each depth could be calculated as:

\[ E(\text{PAR})_z = \left( \frac{A_z}{A_D} \right) E_d \]

where \( E(\text{PAR})_z \) is the PAR photon irradiance in depth \( z \), \( A_z \) is the wavelength integrated signal in depth \( z \), \( A_D \) is the wavelength integrated downwelling irradiance, and \( E_d \) is the downwelling photon irradiance (in µmol photons m⁻² s⁻¹).

Since the leaves of *Z. marina* were ~50 µm thick, it was not possible to measure internal light gradients in the leaves with microprobes. Instead we measured the spectral attenuation of light through leaves with and without epiphyte cover. A leaf, with- or without epiphytes, was positioned in a transparent acrylate chamber illuminated from below and with the incident irradiance determined as above (Fig. 1c). Concomitantly, the microprobe was positioned at the abaxial surface of the leaf and the transmitted spectra were recorded on leaves with- and without epiphytes.

**Flux calculations**

The O₂ flux between the leaf surface and the surrounding seawater was calculated using Fick’s first law of diffusion:

\[ J_{O_2} = -D_0 \frac{\partial C}{\partial Z} \]
where $D_0$ is the molecular diffusion coefficient of O$_2$ in seawater at experimental temperature and salinity ($2.0845 \times 10^{-5}$ cm$^2$ s$^{-1}$; tabulated values available at www.unisense.com), and $\frac{\partial c}{\partial z}$ is the slope of the linear O$_2$ concentration gradient within the diffusive boundary layer.

A cylindrical version of Fick's first law of diffusion, described by Steen-Knudsen (2002), was used to calculate the radial O$_2$ loss from the below-ground tissue surface (assuming a homogenous and cylinder-shaped O$_2$ loss from the roots):

$$J(r)_{\text{root-cap}} = \frac{\phi D_0 (C_1 - C_2)}{r} \ln\left(\frac{r_1}{r_2}\right)$$

where $\phi$ is the porosity of the sediment and $\phi D_0$ estimates the diffusivity of O$_2$ within the sediment at experimental temperature and salinity, $r$ is the radius of the root, and $C_1$ and $C_2$ are the O$_2$ concentrations measured at the radial distances $r_1$ and $r_2$, respectively. Porosity was determined from the weight loss of wet sediment from the sampling site (known initial volume and weight) after drying at 60°C until a constant weight was reached (Porosity=0.51).

**Statistical procedures**

Data were tested for normality (Shapiro-Wilk) and equal variance prior to statistical analysis. Student's $t$-tests were used to compare treatments (with- or without leaf epiphytes) on data that met the above-mentioned assumptions. Mann-Whitney Rank Sum tests were used on data lacking normality and/or equal variance. A two-way ANOVA was performed to examine the influence of leaf epiphyte-cover and incident irradiance on O$_2$ fluxes across the leaf tissue surface (Table S1). Analysis of covariance (ANCOVA) was used to examine the effect of leaf epiphytes on scalar irradiance with distance from the leaf surface as a covariant. The significance level was set to $p < 0.05$. Statistical tests were performed in SigmaPlot and SPSS.

**Results**

**Light climate**

Our observations on the light microclimate around the leaves of *Z. marina* revealed that epiphyte cover affect the quantity and quality of light reaching the seagrass leaf. In the presence of epiphytes, scalar irradiance (PAR, 400-700 nm) on the surface of seagrass leaves was reduced by 54% and 92% under a downwelling irradiance of 50 and 200 µmol photons m$^{-2}$ s$^{-1}$ respectively (Fig. 2). Without
Figure 2. Profiles of photon scalar irradiance measured at two different downwelling photon irradiances (50- and 200 µmol photons m$^{-2}$ s$^{-1}$) on Z. marina leaves with- and without epiphyte cover. Left panels show the scalar irradiance 0-10 mm from the leaf surface measured in 1 mm steps. Right panels show the scalar irradiance 0-1 mm from the leaf surface measured in 0.1 mm steps (enlarged plots of the scalar irradiance showed in the left panels). Data points represents means ± S.D. n=3; leaf level replicates.
epiphytes, we observed a 3% and 4% increase in scalar irradiance at incident irradiance levels of 50 and 200 µmol photons m⁻² s⁻¹, respectively. Analysis of covariance (ANCOVA) confirmed significant difference in the scalar irradiance at the leaf tissue surface of plants with leaf epiphyte cover as compared to plants without leaf epiphyte cover (p < 0.01), as well as between scalar irradiance measured at z = 10 mm and z = 0 mm for plants with leaf epiphyte cover (p < 0.01). No significant difference was found between scalar irradiance measured at z = 10 mm and z = 0 mm for plants without leaf epiphyte cover (p > 0.05).

The decrease in scalar irradiance in the upper canopy (1-10 mm above the leaf surface) was uniform across wavelengths in the PAR region while a spectral shift became evident in the lower canopy (0-1 mm above the leaf surface) with blue light and light around 675 nm being absorbed preferentially (Fig. 3). However, approaching the surface of the seagrass leaf we also observed an enhanced absorption around 625 nm indicative of phycocyanin found in cyanobacteria. This was further clarified in the seagrass light transmission spectra (Fig. 4) where, in the absence of epiphytes, mainly actinic light and light around

![Figure 3. Spectral scalar irradiance measured over Z. marina leaves under an incident irradiance of 50 and 200 µmol photons m⁻² s⁻¹ with- (right panels) and without epiphytes (left panels). Coloured lines represents spectra collected at the given depths in mm above the leaf surface expressed as % of incident irradiance on a log-scale. n=3; leaf level replicates.](image_url)
675 nm were absorbed, corresponding to the absorption spectrum of Chl a. In the presence of epiphytes there was a profound decrease in all wavelengths in the PAR region leading to a reduction in the transmitted light with 71 and 88% (downwelling irradiance of 50 and 200 µmol photons m\(^{-2}\) s\(^{-1}\) respectively). Students t-tests performed at 425nm, 560nm and 675nm (except at 425nm under an incident irradiance of 50 µmol photons m\(^{-2}\) s\(^{-1}\), where a Mann-Whitney test was preformed due to data lacking normality; p < 0.05) confirmed significant difference in the transmitted light spectra between plants with leaf epiphyte cover and plants without leaf epiphyte cover (p < 0.01). In addition there was a relatively larger absorption of green light in the presence of epiphytes, evident from a change in the ratio of wavelengths 560nm:675nm from 6 without epiphytes to 3 with epiphytes suggesting absorption from accessory epiphyte pigments.

Diffusive boundary layer and photosynthesis

The \(O_2\) concentration microprofiles at the \(Z.\ marina\) leaf tissue surface revealed a ~4 times thicker diffusive boundary layer (DBL) around leaves with epiphyte-cover as compared to leaves without epiphyte-cover, i.e., an increase in the DBL thickness from ~350 to 1400 µm (Fig. 5). During darkness, passive diffusion of \(O_2\) from the surrounding water-column resulted in a constant influx of \(O_2\) into leaves both with and without epiphyte-cover, supporting the below-ground tissue with \(O_2\) (Fig. 5; Table 1). However, the thick DBL around leaves with epiphyte-cover impeded the diffusive \(O_2\) supply in darkness as compared to plants without epiphyte-cover (seen as a reduction in the seagrass leaf surface \(O_2\) concentration from ~198 to 51 µmol L\(^{-1}\) (Student’s t-test, p < 0.001); Fig. 5), leaving these plants more vulnerable to low water-column \(O_2\) contents at night-time.

Figure 4. Transmission spectra of photon scalar irradiance through \(Z.\ marina\) leaves with- (red line) and without (black line) epiphyte cover and at two different downwelling irradiances (50- and 200 µmol photons m\(^{-2}\) s\(^{-1}\)). Dashed lines represents ± S.D. n=4; leaf level replicates.
Net O$_2$ production increased with increasing irradiance, as a result of enhanced shoot photosynthesis (Fig. 5). The lower light availability for plants with epiphyte-cover resulted in relatively lower net photosynthesis rates, and the compensation irradiance increased from $\sim$12 to 27 µmol photons m$^{-2}$ s$^{-1}$ for plants with epiphyte-cover (Fig. 6; Table 1). Despite the lower net photosynthesis in plants with leaf epiphyte-cover, there was a higher build-up of O$_2$ in the tissue surface under moderate irradiances (100 µmol photons m$^{-2}$ s$^{-1}$) as compared to plants without leaf epiphyte-cover, owing to limited gas exchange with the surrounding water-column as a result of the enhanced DBL thickness.
Radial O₂ loss

We used the measured steady state O₂ microprofiles around the root-cap of *Z. marina* with and without leaf epiphyte-cover (Fig. 7), to calculate the radial O₂ flux into the surrounding sediment. In light, we calculated the radial O₂ loss (ROL) from the root-cap to be 65.7 nmol O₂ cm⁻² h⁻¹ from plants with leaf epiphyte-cover as compared to 152.7 nmol O₂ cm⁻² h⁻¹ from plants without leaf epiphyte-cover (Table 1). The ROL maintained a ~300 µm thick oxic microzone around the root-cap of *Z. marina* (Fig. 7). In darkness, the ROL from the root-cap dramatically decreased to 0 nmol O₂ cm⁻² h⁻¹ in plants with leaf epiphyte-cover (i.e. no O₂ was detectable at the root surface during darkness; Fig. 7), and 0.8 nmol O₂ cm⁻² h⁻¹ in plants without leaf epiphyte-cover (Table 1). Epiphyte-covered plants did thus lose their oxic microshield against H₂S intrusion in darkness.

Table 1. O₂ fluxes across the leaf surface and radial O₂ loss from the root-cap (~1 mm from the root-apex). (-) indicate no data points. Negative values denote net O₂ uptake. Rates are mean±S.D. n = 3-5; leaf/root level replicates. a,b indicates significant difference between seagrasses with leaf epiphyte cover as compared to seagrasses without leaf epiphyte cover (control plants) (* Two-way ANOVA, F₁₂ (PAR) = 2931.2, F₁,3 (epiphytes) = 3555.1, p < 0.01; b Mann-Whitney test, p < 0.05).

<table>
<thead>
<tr>
<th>Downwelling irradiance (µmol photons m⁻² s⁻¹)</th>
<th>Leaves (+ Epiphytes)</th>
<th>Leaves (- Epiphytes)</th>
<th>Root-cap (+ Epiphytes)</th>
<th>Root-cap (- Epiphytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-106.4 ± 1.8a</td>
<td>-107.1 ± 1.0</td>
<td>0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>50</td>
<td>60.6 ± 4.0a</td>
<td>271.9 ± 3.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>164.7 ± 4.9a</td>
<td>520.7 ± 12.8</td>
<td>65.7 ± 21.0a</td>
<td>152.7 ± 7.5</td>
</tr>
<tr>
<td>200</td>
<td>221.2 ± 27.8a</td>
<td>800.9 ± 14.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

Our results provide clear experimental evidence that epiphyte growth on *Z. marina* leaves reduces both light quantity and quality reaching the seagrass leaf, thereby impeding the overall plant performance during day-time. Furthermore, leaf epiphyte-cover lead to an enhanced thickness of the diffusive boundary layers (DBL) surrounding the leaves, thus impeding the exchange of gasses and essential nutrients with the ambient water-column. In darkness, this resulted in a negative effect on the intra-plant O$_2$ status that subsequently reduced the oxidation capability of the below-ground tissue, thereby rendering plants more vulnerable to sediment-produced reduced phytotoxic compounds, such as H$_2$S.

Light microenvironment and shoot photosynthesis

Light availability on the surface of the leaves of *Z. marina* covered by epiphytes was dramatically decreased compared to leaves without epiphytes in agreement with previous studies (Drake et al., 2003; Pedersen et al., 2014). Effectively, this means that higher downwelling irradiances is needed to meet the compensation irradiance for the epiphyte covered leaf (Fig. 6). We expected a larger change in the spectral quality of light reaching the leaf surface through the epiphyte canopy, but as the generation time of unicellular and filamentous algae colonizing the seagrass are short relative to the seagrass leaves, there might have been a large proportion of dead epiphytes thus acting as particulate organic matter with a more uniform light attenuation (Fig. 3, upper right). However, in the lower canopy (0-1mm above the seagrass surface) there was a non-uniform attenuation of light leading to a strong reduction in blue light reaching the seagrass surface (Fig. 3, lower right). In the transmittance spectra, we saw a disproportionate large amount of green light being attenuated in the presence of epiphytes indicating
the presence of a community possessing accessory pigments able to utilize green light, such as red algal or cyanobacterial phycobiliproteins.

Although a large proportion of the green light was attenuated by epiphytes, blue and red light were almost completely removed, leaving the plant in a light environment with predominately green light which is less effectively absorbed by Chl a. Thus, both quality and especially the quantity of light were diminished in the presence of epiphytes thereby leaving the plant for longer periods near the minimal light requirement for growth, which is high in Z. marina (~20% of surface irradiance; Dennison et al., 1993). A recent study showed ~90% reduction in biomass under prolonged diminished light conditions, comparable to the decrease in light shown here (Kim et al., 2015). It has been speculated that the high minimum light requirement for growth reflects that seagrasses often grow in anoxic, sulphide-rich sediments (Ralph et al., 2007). The presence of sulphide results in decreased photosynthesis and increased O2 consumption in the dark (Goodman et al., 1995; Holmer and Bondgaard, 2001), which means that more light is needed to drive a sufficient photosynthetic O2 supply to maintain positive growth. Diminished light conditions due to epiphyte cover can thus reduce the fitness of the plant.

The ~4 times enhanced DBL thickness around leaves with epiphyte-cover adversely affected the internal O2 supply to the below-ground tissue at night-time. In addition, it lead to a build-up of O2 at the leaves surface under high incident photon irradiance (≥100 µmol photons m−2 s−1; Fig. 5), which potentially could lead to enhanced photorespiration (as surplus internal O2 molecules may bind competitively to RuBisCO instead of CO2 resulting in decreased CO2 fixation and reduced photosynthetic efficiency) and/or internal oxidative stress (Maberly, 2014). At low photon irradiance (50 µmol photons m−2 s−1), the reduced light availability and lower photosynthetic activity, seemed to counter-balance this internal O2 build-up caused by the insulating DBL (Fig. 5). Furthermore, the epiphytes themselves, i.e., filamentous algal epiphytes and most probably leaf- and filamentous algal epiphyte-associated bacterial communities, contribute with oxygenic photosynthesis and respiration, thereby further enhancing the O2 consumption at the leaf surface during night-time. Correspondingly, we found a ~2 times higher compensation irradiance of plants with leaf epiphyte-cover, as compared to plants without epiphyte-cover (Fig. 6). This may be a very important factor during prolonged events of poor light conditions, such as during dredging operations and eutrophication, making plants with leaf epiphyte-cover more prone to sulphide invasion as a result of inadequate internal aeration (Pedersen et al., 2004; Borum et al., 2005). The generally reduced net photosynthesis rates of plants with epiphyte-cover (Fig. 6), was most likely a combined result of the poor light conditions and a limited influx of CO2 from the surrounding water-column. Such DBL-induced limited gas exchange with the ambient water-column can
lead to inorganic carbon limitation enhancing photorespiration (e.g. Maberly, 2014) thereby impeding shoot photosynthesis.

*Light-driven O₂ microdynamics in the rhizosphere*

Photosynthetic O₂ evolution resulted in the establishment of a ~300 µm wide oxic microzone around the root-cap of *Zostera marina* at the approximate position of the apical root meristem (Fig. 7). Plants with epiphyte-cover exhibited a negative effect on the below-ground tissue oxidation capacity with ~2 times lower radial O₂ loss (ROL) from the root-apex during light stimulation of the leaf canopy, as compared to plants without leaf epiphyte-cover. Although the ROL in light from the root-cap of plants with and without epiphyte-cover were of similar magnitude to fluxes previously reported by Jensen et al. (2005; Table 1), a lower oxidation capability of the below-ground tissue will almost certainly have a negative effect on the overall plants performance. ROL has been shown to improve the chemical conditions in the immediate rhizosphere of seagrasses due to enhanced sulphide reoxidation (Brodersen et al., 2015). The oxic microshield at the root-cap surface can thus protect the apical root meristem from reduced phytotoxic compounds, such as H₂S, through chemical re-oxidation with O₂.

*Dark O₂ microdynamics in the rhizosphere*

During darkness, no O₂ was detected at the root-cap surface of plants with leaf epiphyte-cover, indicative of inadequate internal aeration in contrast to plants without leaf epiphyte-cover, where low levels of O₂ were detectable at the root-cap surface during darkness (Fig. 7; Table 1). Such breakdown of the oxic microshield in presence of epiphytes on seagrass leaves can be of great importance, as a shift to anaerobic metabolism in the root-system results in a much less efficient energy utilization than with aerobic metabolism, as anaerobic conditions inhibit the translocation of carbohydrates supporting plant metabolism (Greve et al., 2003; Zimmerman and Alberte, 1996). Previous studies of *Z. marina* have shown that the ROL from the root-apex persists during darkness at a much higher flux rate (up to 16.2 nmol O₂ cm⁻² h⁻¹ measured 2 mm behind the root-apex) than reported in this study (Jensen et al., 2005; Frederiksen and Glud, 2006). This apparent discrepancy may be explained by bacterial colonization of the root-cap surface consuming the small amounts of leaked O₂ through microbial respiration and/or by ferrous sulphide (FeS) and iron plaques. Sulphate reducing bacteria have thus previously been isolated from surface-sterilized roots of *Zostera marina* (Nielsen et al., 1999; Finster et al., 2001).
Interestingly, the root-cap mediated O$_2$ leakage to the rhizosphere may also be important for plant-beneficial root-associated microbial processes, such as H$_2$S re-oxidation, in addition to simply detoxifying reduced substances in the immediate rhizosphere through spontaneous chemical reactions. Bacterially-mediated H$_2$S oxidation is about 10,000-100,000 times faster than the chemical reaction alone (Jørgensen and Postgate, 1982) and therefore has potential to be of high value for the plants. It has been suggested that H$_2$S oxidation also takes place inside the plant (Holmer et al., 2005; Holmer and Hasler-Sheetal, 2014), as seagrass exposed to high sediment H$_2$S levels showed internal accumulation of elemental sulphur that is an intermediate in the sulphide oxidation. This process is, however, driven by simple chemical reactions between H$_2$S and O$_2$ and is not mediated by intra-plant enzymes or bacteria (Pedersen et al., 2004) as seen in some marine invertebrates (Grieshaber and Völkel, 1998).

The lower light availability for photosynthesis of plants with filamentous algal epiphyte-cover seemed to be the key factor behind the lower ROL from the root-cap (Fig. 7), as a result of the relative lower net photosynthesis rates and thereby lower O$_2$ production in leaves, as compared to plants without epiphyte-cover (Fig. 6). This might seem obvious, but the DBL-induced impedance of O$_2$ exchange with the water-column of plants with epiphyte-cover, could also have resulted in an enhancement in the aerenchymal O$_2$ level (seen as the build-up in the surface O$_2$ concentration on Fig. 5) and thereby a concomitant higher ROL from the root-apex, but this effect was apparently overruled by lower seagrass photosynthesis due to epiphyte shading and/or inorganic carbon limitation due to increased DBL thickness.

Burnell et al. (2014) recently demonstrated that high incident irradiance (~200 µmol photons m$^{-2}$ s$^{-1}$) in combination with elevated water-column CO$_2$ concentrations (up to 900 µl L$^{-1}$, representing high future predictions of water-column CO$_2$ levels) had a negative effect on seagrass biomass and leaf growth, as compared to low light conditions. The observed negative growth response to combined high CO$_2$ and light conditions appeared to be closely related to overgrowth of seagrass leaves with filamentous algal epiphytes. This finding supports our microsensor measurements demonstrating the negative effects of leaf epiphyte-cover on the intra-plant O$_2$ status and the below-ground tissue oxidation capacity. Epiphyte-induced low O$_2$ evolution in seagrass leaves causing reduced internal aeration and increased H$_2$S intrusion may result in enhanced seagrass mortality if unfavorable light conditions persist for longer periods of time. This emphasizes the importance of minimizing nutrient loading into seagrass inhabited marine coastal waters, as eutrophication often leads to poor light conditions, low water quality, algal blooms and enhanced night-time O$_2$ consumption in the water column.
In conclusion, the present study shows that epiphyte-cover of seagrass leaves leads to reduced oxidation capability of the below-ground tissue, due to a combined result of lower light availability and thicker diffusive boundary layers around leaves, impeding seagrass photosynthesis. This synergetic negative effect on the plants performance, resulted in a ~2 times higher compensation irradiance in *Z. marina* leaving epiphyte-covered seagrasses more vulnerable to H₂S invasion during prolonged events of poor light conditions in the surrounding water-column. Seagrasses with leaf epiphyte-cover are thus more prone to anthropogenic impacts and activity in coastal environments, as leaf epiphytes reduce their resilience towards environmental disturbances.

**Acknowledgements**

We thank Unisense A/S, the microbiology group at Aarhus University and Johan F. Kraft for providing the microsensor equipment used in this study. We thank Lars F. Rickelt for manufacturing the scalar irradiance microsensors and Peter Ralph (UTS) for financial support of KEB. The research was funded by grants from the *Augustinus Foundation, P. A. Fiskers Fund* and *Jorck and Wife’s Fund* (KEB), the *Danish Council for Independent Research | Natural Sciences* (MK), and the *Australian Research Council (ARC LP 110200454)* (MK).
References


Supplementary information

Results of statistical analysis

Table S1: Two-way ANOVA for O₂ evolution (nmol O₂ cm⁻² h⁻¹). TMT = Experimental treatments, i.e., with or without leaf epiphytes; PAR = incident irradiance (0, 50, 100 and 200 µmol photons m⁻² s⁻¹).

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig. (P-Value)</th>
<th>Noncent. Parameter</th>
<th>Observed Powerᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Model</td>
<td>2043159.8ᵃ</td>
<td>7</td>
<td>291880.0</td>
<td>2040.1</td>
<td>.000</td>
<td>14280.5</td>
<td>1.000</td>
</tr>
<tr>
<td>Intercept</td>
<td>1291479.2</td>
<td>1</td>
<td>1291479.2</td>
<td>9026.7</td>
<td>.000</td>
<td>9026.7</td>
<td>1.000</td>
</tr>
<tr>
<td>TMT</td>
<td>508645.6</td>
<td>1</td>
<td>508645.6</td>
<td>3555.1</td>
<td>.000</td>
<td>3555.1</td>
<td>1.000</td>
</tr>
<tr>
<td>PAR</td>
<td>1258149.6</td>
<td>3</td>
<td>419383.2</td>
<td>2931.2</td>
<td>.000</td>
<td>8793.7</td>
<td>1.000</td>
</tr>
<tr>
<td>TMT * PAR</td>
<td>269624.4</td>
<td>3</td>
<td>89874.8</td>
<td>628.2</td>
<td>.000</td>
<td>1884.5</td>
<td>1.000</td>
</tr>
</tbody>
</table>

ᵃ. R² = .999 (Adjusted R² = .998)
ᵇ. Computed using alpha = .01

Model: Intercept + tmt + PAR + tmt * PAR
Chapter 8

Diffusion or advection? Mass transfer and complex boundary layer landscapes of the brown alga *Fucus vesiculosus*

Published in *Journal of the Royal Society Interface*

by

Mads Lichtenberg, Rasmus D. Nørregaard and Michael Kühl
Diffusion or advection? Mass transfer and complex boundary layer landscapes of the brown alga *Fucus vesiculosus*

Mads Lichtenberg¹,², Rasmus D. Nørregaard²,³ and Michael Kühl¹,³

¹Marine Biological Section, University of Copenhagen, Denmark
²Arctic Research Center, Department of Bioscience, Aarhus University, Denmark.
³Climate Change Cluster (C3), University of Technology Sydney, Australia

Abstract

The role of hyaline hairs on the thallus of brown algae in the genus *Fucus* is long debated and several functions have been proposed. We used a novel motorized setup for two-dimensional and three-dimensional mapping with O₂-microsensors to investigate the spatial heterogeneity of the diffusive boundary layer (DBL) and O₂ flux around single and multiple tufts of hyaline hairs on the thallus of *Fucus vesiculosus*. Flow was a major determinant of DBL thickness, where higher flow decreased DBL thickness and increased O₂ flux between the algal thallus and the surrounding seawater. However, the topography of the DBL varied and did not directly follow the contour of the underlying thallus. Areas around single tufts of hyaline hairs exhibited a more complex mass-transfer boundary layer, showing both increased and decreased thickness as compared to areas over smooth thallus surfaces. Over thallus areas with several hyaline hair tufts, the overall effect was an apparent increase in the boundary layer thickness. We also found indications for advective O₂ transport driven by pressure gradients or vortex shedding downstream from dense tufts of hyaline hairs that could alleviate local mass-transfer resistances. Mass-transfer dynamics around hyaline hair tufts are thus more complex than hitherto assumed and may have important implications for algal physiology and plant-microbe interactions.
**Introduction**

Compared to terrestrial plants, aquatic macrophytes experience ~$10^4$ slower diffusion and a much lower solubility of gases in water than in air (Sand-Jensen & Krause-Jensen, 1997; Maberly & Madsen, 2002). The efficient exchange of nutrients and gases is further exacerbated by the diffusive boundary layer (DBL) surrounding all submerged surfaces (Jørgensen & Revsbech, 1985). The thickness and topography of DBLs around submerged impermeable objects is affected by flow velocity and surface topography (Jørgensen & Revsbech, 1985). Higher flow velocities decrease the DBL thickness by exerting a higher shear stress on the viscous sublayer near the surface. The effect of surface roughness on DBL thickness is variable, where angled planes facing the flow generally will have decreased boundary layers, while the DBL downstream of protruding structures will have increased boundary layers (Jørgensen & Des Marais, 1990). At the same time, the effect of surface roughness on mass transfer across a boundary layer is dichotomous, where a thicker DBL will decrease mass transfer, while surface roughness tends to increase the overall surface area, thus increasing mass transfer (Jørgensen & Revsbech, 1985). In microsensor-based studies of the DBL, it is important to note that the presence of the microsensor tip in itself can affect the local DBL thickness (Glud et al., 1994), where flow acceleration around the microsensor shaft will compress the local DBL thickness, leading to locally enhanced O$_2$ fluxes of the order of 10%. However, this effect is only significant when investigated on smooth surfaces, while a clear effect is apparently undetectable when measuring over tufts in for example a cyanobacterial mat (Lorenzen et al., 1995).

It has been estimated that the DBL accounts for up to 90% of the resistance to carbon fixation in freshwater plants (Black et al., 1981), and both structural- and biochemical regulations to alleviate such mass transfer resistance have evolved across lineages. Some aquatic macrophytes have e.g. developed i) thinner leaves and a reduced cuticle decreasing the diffusion path length to chloroplasts, ii) carbon concentrating mechanisms that increase internal CO$_2$ concentration, and iii) the ability to utilize HCO$_3^-$, which constitutes the largest fraction of dissolved inorganic carbon at ocean pH ((Pedersen & Colmer, 2014), (Pedersen et al., 2013) and references therein). In photosymbiotic corals, it has also been proposed that vortical ciliary flow can actively enhance mass transfer between the coral tissue and the surrounding water in stagnant or very low flow regimes with concomitant thick DBLs (Shapiro et al., 2014).

Hyaline hairs, that is, colourless, filamentous multicellular structures, are often present as whitish tufts on the thallus of brown macroalgae in the genus *Fucus*. The hairs originate in so-called cryptostomata, i.e. cavities on the apical and mid-regions of the thallus (Hurd et al., 1993). It is recognized that hyaline hairs
aid in the uptake of nutrients (Raven, 1981; Steen, 2003) e.g. during springtime, when photosynthetic potential is higher due to increased light levels, and the need for nutrients apparently triggers growth of hyaline hairs (Hurd et al., 1993).

How hyaline hairs affect solute exchange and nutrient acquisition in *Fucus* is still debated, and different functional roles have been suggested in the literature: i) the hairs might increase the algal surface area available for nutrient uptake, albeit this is probably not the major limitation on nutrient uptake (Raven, 1981); ii) the hyaline hairs might decrease the DBL due to turbulence created by the hairs as water flows across them, decreasing the mass transfer resistance imposed by the DBL (Raven, 1981); iii) the thin cell walls of the hairs relative to the thallus could have less resistance to the passage of ions (Oates & Cole, 1994; Hurd, 2000); iv) the hyaline hairs increase DBL thickness, thereby retaining the products of thallus surface-active enzymes such as extracellular phosphatases thereby ensuring more efficient nutrient uptake (Hurd, 2000).

There can be no doubt, however, that the DBL has great importance for macroalgal growth rates. The mass transfer resistance imposed by the DBL has been correlated with nutrient limitation in the giant kelp *Macrocystis pyrifera* (Wheeler, 1980), and a considerable spatial variation of the DBL over the thallus and cryptostomata of *Fucus vesiculosus* has been observed (Spilling et al., 2010). However, current knowledge of the DBL characteristics of aquatic plants is largely based on point measurements with O$_2$ microsensors (Hurd, 2000), while it is known from boundary layer studies in biofilms (Jørgensen & Des Marais, 1990), corals (Kühl et al., 1995; de Beer et al., 2000; Jimenez et al., 2011) and sediments (Gundersen & Jørgensen, 1990; Jørgensen & Des Marais, 1990; Røy et al., 2002; Røy et al., 2005) that the DBL exhibits a spatio-temporal heterogeneity which, is modulated by both flow velocity and surface topography. Similar studies of DBL topography are very limited in aquatic plant science (Brodersen et al., 2015), and the aim of this study was to explore how the DBL thickness and the local O$_2$ flux varied spatially over the thallus of *F. vesiculosus* with and without tufts of hyaline hairs. The exploration of the three-dimensional (3D) boundary layer topography was done with O$_2$ microsensors mounted in a fully automated motorized micromanipulator system that allowed measurements of O$_2$ concentration gradients in transects and grids over the thallus of *F. vesiculosus* (Fig. S1). Our results reveal a complex boundary layer landscape over the algal thallus, where mass transfer across the DBL apparently can be supplemented by advective processes due to the presence of hyaline hairs.
Materials and Methods

Sampling and experimental setup

Specimens of Fucus vesiculosus and seawater used in the experimental setup were sampled on the day of usage at <1 m depth at Kronborg, Helsingør, Denmark, from May to August. When studying the influence of a single tuft of hyaline hairs on the mass transfer boundary layer, excess hair tufts were carefully shaved off with a scalpel during observation under a dissection microscope to avoid thallus damage. Previous studies showed that mechanical removal of hyaline hairs does not induce defense mechanisms in Fucus (Jormalainen et al., 2003). When the influence of multiple tufts was analysed, the thallus was left intact. Prior to measurements, a piece of F. vesiculosus thallus with hyaline hairs was fixed on a slab of agar (~1.5% w/w in seawater) in a small flow chamber, creating a defined unidirectional flow of seawater across the thallus surface (Spilling et al., 2010). The flow chamber was connected via tubing to a submersible water pump in a continuously aerated and thermostated seawater reservoir tank underneath the flow chamber. Flow velocity was adjusted by restricting water flow to the flow chamber with a needle valve. By collecting water from the flow chamber outlet for one minute and dividing the sampled volume per time by the cross sectional area of the flow chamber we could estimate the mean free flow velocity. All measurements were carried out with mean free flow velocities in the flow chamber of either 1.65 cm s\(^{-1}\) or 4.88 cm s\(^{-1}\).

The sample was illuminated from above with light from a halogen lamp (Schott KL-2500LCD), equipped with a collimating lens, that produces a bell-shaped wavelength distribution in the PAR region (See the Supplementary Information in Lichtenberg et al. (2016) for spectrum). The spectral light composition was constant in all treatments and yielded a downwelling photon irradiance (400-700 nm) of ~350 µmol photons m\(^{-2}\) s\(^{-1}\), as measured with a quantum irradiance meter (LI-250, LiCor Inc., USA).

Microsensor measurements

Measurements of O\(_2\) concentration above the thallus of F. vesiculosus were carried out with Clark-type O\(_2\) microelectrodes (tip diameter 10 µm, OX10, Unisense A/S, Denmark; Revsbech (1989)) with a response time of <1-3 seconds and low stirring sensitivity (<2%). The microelectrode was connected to a pA meter (PA2000, Unisense A/S, Denmark), and sensor signals from the pA meter were acquired on a PC via a parallel port-connected A/D converter (ADC-101, Pico Technologies Ltd., England). The O\(_2\) microsensor was mounted in a custom-built micromanipulator setup enabling motorized positioning at defined x, y and z coordinates at ~1 µm resolution by use of 3 interconnected motorized positioners (VT-80, Micos GmbH, Germany) and controllers (MoCo DC, Micos GmbH, Germany). Data acquisition
and positioning were controlled by a custom-built software (Volfix) programmed in LabView (National Instruments, Japan). The $O_2$ microelectrode signal was linearly calibrated at experimental temperature ($\sim 17^\circ C$) and salinity ($S=16$) from measurements in air-saturated seawater and in seawater made anoxic by the addition of sodium dithionite. The $O_2$ concentration in air-saturated seawater, $C_0$, and the molecular diffusion coefficient of $O_2$, $D_0$, in seawater at experimental temperature and salinity were taken from tabulated values (Unisense A/S, Denmark) as $C_0 = 274 \mu \text{mol } O_2 \text{ L}^{-1}$ and $D_0 = 1.87 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$. No significant sensor drift was observed during measurements as judged from the signal stability in the constantly aerated thermostated water above the DBL between profile measurements.

**Mapping of boundary layers**

The boundary layer around tufts of hyaline hairs anchored in cryptostomata of *F. vesiculosus* was mapped by 2D transect and 3D grid measurements of $O_2$ concentration profiles towards the thallus surface. The Volfix software enabled us to specify a measuring grid/transect with any number of sampling points in the $x$, $y$, and $z$-directions. In this study, the $y$-direction corresponds to the direction of flow (where negative values indicate the distance behind a single tuft of hyaline hairs), the $x$-direction corresponds to the width of the flow chamber, and the $z$-direction corresponds to the height above the thallus surface (Fig. S1). The approximate height and radius of the hyaline hairs were estimated by manual manipulation of the microelectrode tip relative to the structures as observed under a stereomicroscope (SV6, Zeiss, Germany). Thallus samples were placed in the flow chamber with the length of the thallus oriented along the direction of flow, i.e., the $y$-direction.

**Two-dimensional transect measurements.** For 2D transect measurements, the $O_2$ microelectrode tip was positioned manually as close to the centre of the selected cryptostomata as possible using a dissection microscope for observation; this position was set to $y=0$ in the Volfix measuring software. The transect measurements started 2 mm upstream ($y=2$ mm) from the cryptostomata and 1 mm above the hyaline hairs (point A in Fig. S1D), and ended 4 mm downstream ($y=-4$ mm). Transects of $O_2$ concentration profiles were measured at a lateral resolution of 0.5 mm in the $y$-direction, with vertical $O_2$ concentration profiles measured at each transect point in steps of 0.1 mm in the $z$-direction. All profile measurements started at the same $z$-position and ended in the upper thallus layer, where a characteristic jump in the $O_2$ concentration, due to the physical impact of the $O_2$ microsensor and the solid thallus surface, enabled precise determination of the thallus surface.

**Three-dimensional grid measurements.** For measuring 3D grids of $O_2$ concentration profiles over thallus areas with only one tuft of hyaline hairs, 9 transects covering a $24 \text{ mm}^2$ sampling grid area were measured.
around a central tuft of hairs (Fig. S1E) with a lateral resolution of 0.5 mm (x- and y-direction) and a vertical resolution of 0.1 mm (z-direction). Measurements started ~1 mm above the hyaline hairs (Fig. S1E). For measurements of 3D grids of O₂ concentration profiles over larger thallus areas with multiple tufts of hyaline hairs, a 12 x 2 mm measuring grid was set up and measurements were performed at a lateral resolution of 0.5 mm (x and y directions) and a vertical resolution of 0.2 mm (z direction). Tufts of hyaline hairs were scattered across the thallus, and the starting point for the grid measurement was set randomly, but with the same starting point for measurements at a flow velocity of 1.65 cm s⁻¹ and 4.88 cm s⁻¹. Due to the length of measurements, different fresh thallus samples were used for individual 2D and 3D experiments.

**Diffusive boundary layer thickness and calculations**

There are different ways of determining the effective thickness of the diffusive boundary layer from O₂ microsensor measurements (Jørgensen & Des Marais, 1990). The DBL thickness is often found by extrapolating the linear O₂ gradient in the DBL to the bulk concentration of the free-flow region. The distance from the surface to the intersection of the extrapolated linear gradient and the bulk concentration is denoted the effective diffusive boundary layer thickness, Zₜ (Jørgensen & Revsbech, 1985). However, analysing large numbers of O₂ profiles in this manner is very time consuming, and a somewhat faster determination can be carried out by defining Zₜ as the distance between the surface and the vertical position above the surface where the O₂ concentration has changed by 10% relative to the O₂ concentration in the bulk water. Estimations of Zₜ via this method were found to differ by <10% from more precise determinations (Jørgensen & Des Marais, 1990). The diffusive flux of O₂ across the DBL, J (in units of nmol O₂ cm⁻² s⁻¹), was calculated from steady-state O₂ concentration profiles using Fick’s 1st law:

$$J = D₀(C∞ - C₀)/Zₜ$$  \hspace{1cm} (Eq. 1)

where C∞ is the O₂ concentration in the free-flow region (µmol O₂ L⁻¹ = nmol O₂ cm⁻³), C₀ is the O₂ concentration at the thallus surface (µmol O₂ L⁻¹), Zₜ is the effective DBL thickness (cm), and D₀ is the molecular diffusion coefficient of O₂ in seawater (cm² s⁻¹).

We note that the fluxes calculated with Fick’s 1st law are only valid in a flow regime with diffusive mass transport and negligible advection close to the thallus surface. A thicker DBL will be present under laminar flow conditions, but even under turbulent flow a thin diffusive sublayer will be present lining the thallus surface (Hurd, 2000). We also note that our DBL thickness determination method (see above)
can yield overestimates in flow regimes exhibiting a broad transition layer between the DBL and the fully mixed turbulent water phase. That is, the region where the measured O₂ concentration profiles show a curvature towards the constant O₂ concentration in the mixed water, and where there is a gradual transition from mass transfer dominated by molecular diffusion towards eddy diffusivity. Such conditions can potentially lead to an underestimation of local fluxes calculated with Eq. 1.

To compensate for the uneven surface of thalli, measurements below the thallus surface are not shown on final transects. The depth axis on transects is therefore denoted $z' = z - z₀$, where $z$ is the z-coordinate from the sample data and $z₀$ is the z-coordinate of the thallus surface. The thallus surface position was determined from the intermittent sudden drop in O₂ concentration when the microsensor pushed against the thallus surface cortex. Maps of O₂ concentration, $Zδ$, and $J$ were generated from measured transects and grids using data interpolation software (Kriging gridding method using default settings, i.e. Linear Variogram and Point Kriging, Surfer v.8, Golden Software Inc., USA).

**Statistics**

Two-way ANOVAs tested differences in the mean $Zδ$ over *F. vesiculosus* between flow rates and light conditions (light/dark). For significant main effects (flow rate and/or light condition) and interaction effects, Tukey’s multiple comparisons post hoc test was applied. Two-way ANOVAs tested the differences between mean O₂ flux values between flow rates and thallus condition (single or multiple tufts). For significant main effects (flow rate and/or thallus condition) and interaction effects, Tukey’s multiple comparisons post hoc test was applied. Statistical analysis was performed using Rstudio (Rstudio version 0.99.491, 2016) with the level of significance set to $p < 0.05$.

**Results**

*The diffusive boundary layer around single tufts of hyaline hairs*

Isopleths of O₂ concentration in the water column above the thalli showed a local increase in effective DBL thickness, $Zδ$, associated with the hyaline hair tuft (Fig. 1). The highest $Zδ$ values were located downstream from the hyaline hairs, either directly behind the tuft or even within the expanse of the hyaline hairs.
Figure 1. Fine-scale mapping of DBL around a single tuft of hyaline hairs on an illuminated *F. vesiculosus* thallus (350 µmol photons m$^{-2}$ s$^{-1}$). (A) and (B), 3D plots of *F. vesiculosus* thallus surface (grey area) and upper extension of DBL (coloured area) around a single tuft of hyaline hairs, at flow velocities of 1.65 (left panels) and 4.88 cm s$^{-1}$ (right panels). Colour bars depict the effective DBL thickness, $Z_δ$ (mm), and arrows indicate the direction of flow. (C) and (D), Transects in the x-direction (perpendicular to the flow), at position $y=-2$ mm from Fig. 1 A,B, respectively, normalized to thallus surface showing the local O$_2$ concentration. The zero position (0,0) indicates the position of the cryptostomata. Colour bars denote O$_2$ concentration (in µmol O$_2$ L$^{-1}$). (E) and (F), Transects of O$_2$ concentration (in µmol L$^{-1}$) measured across a single tuft of hyaline hairs in *F. vesiculosus* measured at flow velocities of 1.65 (E) and 4.88 cm s$^{-1}$ (F), in light (350 µmol photons m$^{-2}$ s$^{-1}$). The arrows indicate the flow direction. The zero position (0,0) indicates the position of the cryptostomata, and transects were adjusted to the thallus surface. Colour bars denote O$_2$ concentration (in µmol O$_2$ L$^{-1}$).
In light (350 µmol photons m\(^{-2}\) s\(^{-1}\)), and at a flow of 1.65 cm s\(^{-1}\), \(Z_\delta\) reached a maximum thickness of 1.2 mm downstream relative to the tuft at \(y = -0.5\) mm (Fig. S2, S3A), while under a flow of 4.88 cm s\(^{-1}\), the maximum \(Z_\delta\) was reduced to 0.6 mm at \(y = -2.5\) mm (Fig. S3A). In darkness, the maximal \(Z_\delta\) values were 0.9 mm and 0.4 mm at \(y = -2\) mm and \(y = 0\) mm under flows of 1.65 and 4.88 cm s\(^{-1}\), respectively. The mean \(Z_\delta\) did not change significantly (\(p < 0.05\)) between measurements in light and darkness (Fig. S3B) under low flow (\(Z_\delta\)(Light) = 0.72 mm, \(Z_\delta\)(Dark) = 0.58 mm; \(p_{adj} = 0.26\)) or high flow (\(Z_\delta\)(Light) = 0.36 mm, \(Z_\delta\)(Dark) = 0.18 mm; \(p_{adj} = 0.13\)). However, flow velocity had a significant effect on the mean \(Z_\delta\) that was significantly thinner under high flow (4.88 cm s\(^{-1}\)) than under low flow (1.65 cm s\(^{-1}\)) (\(p_{adj} < 0.001\) for both main effects).

The hyaline hairs affected \(Z_\delta\) downstream from the hair tuft (Fig. 1A,B) and caused a thickening of the boundary layer that also expanded perpendicular to the flow direction (Fig. 1C,D), reaching a maximum expansion at \(y = -2\) mm for both flows (\(Z_{\delta_{max}}\) = 1.8 mm for 1.65 cm s\(^{-1}\) and \(Z_{\delta_{max}}\) = 0.9 mm for 4.88 cm s\(^{-1}\)). Beyond the local peak in boundary layer thickness, the DBL closely followed the contours of the thallus surface topography. Two transects measured at higher resolution along the x-axis at \(y = -2\) mm showed that the increase in \(Z_\delta\) was roughly identical and extended ~1 mm on both sides of the hyaline hair tuft (Fig. 1C,D). At distances >1 mm away from the local maximum, the DBL approached a lower, more homogeneous thickness over thallus areas unaffected by the hair tuft.

**Figure 2.** Local transects of O\(_2\) concentration around single hair tufts from two different measurement series over an illuminated *F. vesiculosus* thallus (350 µmol photons m\(^{-2}\) s\(^{-1}\)). (A) shows a transect taken from Fig. 1A at \(x = -0.5\) mm under a flow velocity of 1.65 cm s\(^{-1}\), while (B) was measured similarly to Fig. 1E, also at a flow velocity of 1.65 cm s\(^{-1}\). The hair tufts were 2.5-3 mm in diameter and protruded 3-3.5 mm from the thallus. Both transects were adjusted to the thallus surface. The black arrow indicates the flow direction. Colour bars denote O\(_2\) concentration (in µmol O\(_2\) L\(^{-1}\)).

The hyaline hairs affected \(Z_\delta\) downstream from the hair tuft (Fig. 1A,B) and caused a thickening of the boundary layer that also expanded perpendicular to the flow direction (Fig. 1C,D), reaching a maximum expansion at \(y = -2\) mm for both flows (\(Z_{\delta_{max}}\) = 1.8 mm for 1.65 cm s\(^{-1}\) and \(Z_{\delta_{max}}\) = 0.9 mm for 4.88 cm s\(^{-1}\)). Beyond the local peak in boundary layer thickness, the DBL closely followed the contours of the thallus surface topography. Two transects measured at higher resolution along the x-axis at \(y = -2\) mm showed that the increase in \(Z_\delta\) was roughly identical and extended ~1 mm on both sides of the hyaline hair tuft (Fig. 1C,D). At distances >1 mm away from the local maximum, the DBL approached a lower, more homogeneous thickness over thallus areas unaffected by the hair tuft.
Unexpectedly, a transect of O₂ concentrations above the illuminated thallus at y=−2 mm in the x direction, i.e., perpendicular to the flow, showed a local area of increased O₂ concentration apparently separated from the DBL (Fig. 1C). A longitudinal transect at x=−0.5 mm along the y-direction, i.e., the flow direction, showed further indications of an apparent local “upwelling” of O₂ into the transition zone between the DBL and the fully mixed water column downstream from the hyaline hair tuft (Fig. 2). We found such “upwelling” zones most pronounced under low flow located around 2 mm downstream from the centre of the tuft and extending several millimetres into the water column with O₂ concentrations reaching up to >2 times air saturation in some cases (Fig. 2A).

The boundary layer around multiple tufts of hyaline hairs

To investigate the combined effects of multiple tufts on the boundary layer, 3D grid measurements of O₂ concentration were carried out over a thallus with several tufts of hyaline hairs spaced at approximately 2-5 mm distance. Such measurements showed that the smooth local thickening of the DBL around a single hyaline tuft relative to the DBL over the smooth thallus was altered in the presence of multiple tufts (Fig. 3). The boundary layer topography was more heterogeneous, with $Z_δ$ varying by more than 1 mm, reaching a maximum thickness of >2.5 mm under low flow and >1.5 mm under high flow. The DBL topography was thus strongly determined by the interaction between flow and the tufts of hyaline hairs under low flow, while we observed local minima in $Z_δ$ in-between individual tufts at higher flow velocity (Fig. 3B). Transects of O₂ concentrations at x=1 mm (extracted from the 3D grids in Fig. 3A,B) gave detailed information on how the O₂ concentration varied over the thallus with distance along the thallus in the flow direction (Fig. 3C,D). In light, the thallus surface O₂ concentration reached >900 µM in both flows, while the O₂ concentration in the transient zone of the DBL (z=0.7 mm) varied between 350 and 750 µM under low flow and between 300 and 550 µM under high flow. This demonstrated an overall compression of the boundary layer and a more effective O₂ exchange between thallus and water under higher flow. However, the thickening of the 300-350 µM O₂ contour areas e.g. at y=−11 mm and y=−6 mm (Fig. 3C), was due to gradually increasing O₂ concentrations from the bulk water towards the upper part of the DBL (data not shown). This creates an artefact in the precise determination of $Z_δ$ by the method proposed by Jørgensen and Des Marais (1990) that will overestimate the local DBL thickness e.g. compared with the local profile in y=−1 mm where a more steady O₂ increase was measured.
Diffusive $O_2$ fluxes over the Fucus thallus with single and multiple tufts

Although inconsistencies were found (e.g. in the area around $x = -1.5$ mm, $y = 1.5$ mm in Fig. 4A,B), increases in DBL thickness generally correlated with a decrease in $O_2$ flux, and the flow-dependent boundary layer topography strongly affected the $O_2$ flux from the illuminated $F. vesiculosus$ thallus. Comparing the $O_2$ fluxes in transects over the $F. vesiculosus$ thallus with single and multiple tufts of hyaline hairs (Fig. 5A,B) showed an increased $O_2$ flux just upstream from the position of the hyaline hair tufts independent of the flow velocity. The flux values generally correlated with the boundary thickness and the apparent $O_2$ flux gradually decreased downstream relative to the hair tuft. However, local variations were found in areas exhibiting less uniform increases in $O_2$ concentration towards the thallus surface.

**Figure 3.** Boundary layer mapping over several tufts of hyaline hairs on an illuminated $F. vesiculosus$ thallus (350 $\mu$mol photons m$^{-2}$ s$^{-1}$). (A) and (B), 3D plot of the thallus surface (grey area) and the upper extension of the DBL (coloured area) of multiple tufts of hyaline hairs under a flow velocity 1.65 (left panels) and 4.88 cm s$^{-1}$ (right panels). Colour bars depict the effective DBL thickness, $Z_\delta$ (mm). (C) and (D), Transects of $O_2$ concentration at position $x=1$ mm (along the $y$-axis direction) normalized to the thallus surface. Colour bars denote $O_2$ concentration (in $\mu$mol $O_2$ L$^{-1}$).
Figure 4. O$_2$ fluxes and DBL thicknesses (A) and (C) Isopleths of O$_2$ flux (in nmol O$_2$ cm$^{-2}$ s$^{-1}$) and (B) and (D), the effective DBL thickness, Z$_\delta$ (in mm), measured over an illuminated F. vesiculosus thallus (350 µmol photons m$^{-2}$ s$^{-1}$) around a single tuft of hyaline hairs at flow velocities of 1.65 cm s$^{-1}$ (A, B) and 4.88 cm s$^{-1}$ (C, D). The hyaline hairs were rooted in the cryptostomata located at the (0,0) coordinate, as indicated by the black cross. Black arrows indicate the flow direction.
The average O$_2$ flux calculated from transects over _F. vesiculosus_ thalli with single and multiple hyaline hair tufts (Fig. 5C,D) showed that flow was the major determinant of gas exchange between macroalgae and the surrounding seawater. The O$_2$ flux values were higher in high flow treatments than in low flow treatments (p adj <0.001) in measurements over both single and multiple hair tufts.

The O$_2$ fluxes measured around a single hair tuft under high flow were higher than the corresponding measurements over multiple tufts (Fig. 5C,D; p adj <0.001). However, the apparent flux values in the multiple tuft measurements were averaged over a two times larger distance (Fig. 5C; 12 mm), and thus include the combined effect of multiple tufts and boundary layer variation over these, while the values of the single tuft treatments (Fig. 5D; 6mm) only reflect boundary layer effects on the apparent O$_2$ flux immediately downstream from the hair tuft.

**Figure 5.** Comparison of O$_2$ flux values (in nmol O$_2$ cm$^{-2}$ s$^{-1}$) calculated from transects of O$_2$ concentration profiles measured over an illuminated (350 µmol photons m$^{-2}$ s$^{-1}$) intact _F. vesiculosus_ thallus with several tufts of hyaline hairs (A) and a thallus with only a single hair tuft (B) measured under flow velocities of 1.65 cm s$^{-1}$ and 4.88 cm s$^{-1}$. Note the difference in the x-scale. The black arrow indicates the flow direction. The individual position of the multiple hair tufts in panel (A) were not mapped and the zero position on the x-axis thus only reflects the starting point of the transect. In panel (B), the zero position indicates the centre of the cryptostomata. (C) and (D) The average O$_2$ flux (±SEM) across (C) the intact thallus and (D) the thallus with a single hair tuft protruding.
Discussion

In measurements around single hyaline hair tufts, the DBL followed the contour of the smooth thallus surface except around the tufts where a thickening occurred downstream and perpendicular to the flow direction with a concomitant decrease in the DBL thickness 1-2 mm away from the hair tufts, depending on the flow-regime. In measurements over multiple tufts, the smooth apparent thickening of the DBL observed around isolated single tufts was absent. This more dynamic boundary layer landscape was probably caused by the close vicinity of neighbouring hyaline hair tufts creating a more complex flow field. Interestingly, this suggests that the effect of multiple hair tufts apparently leads to an overall increased boundary layer thickness across the thallus. Intuitively, a thin DBL would create physical conditions that could better avoid high detrimental O$_2$ concentrations and inorganic carbon limitations in light and O$_2$ limitation in darkness. So why does Fucus expend metabolic energy on production of hyaline hairs?

In early work by Raven (1981), it was suggested that i) hyaline hairs aid in nutrient uptake by having a highly decreased diffusion resistance over the plasmalemma as compared to the thick algal thallus, and ii) hairs could protrude through the viscous sublayer and into the mainstream flow with better nutrient access. However, as pointed out by Hurd (2000), the thin and flexible hairs are considered unlikely to disrupt the viscous sublayer and create turbulence themselves. Here we show that, across a thallus with multiple hair tufts, the overall DBL thickness is increased, which has a functional significance similar to the observed DBL effects of epiphytes on submerged macrophytes (Sand-Jensen et al., 1985; Brodersen et al., 2015). A thickening of diffusive boundary layers creates a mass transfer limitation that in light can lead to high thallus surface O$_2$ concentrations (Brodersen et al., 2015; Lichtenberg & Kühl, 2015), potentially inducing photorespiration (Falkowski & Raven, 2007) and limiting the inorganic carbon supply (Larkum et al., 2003) to the thallus. However, such a mass transfer limitation would also maintain higher nutrient concentrations due to surface-associated enzyme activity that can aid in the uptake of e.g. phosphorous and other nutrients (Raven, 1992; Hurd, 2000).

A thicker DBL over thalli with tufts of hyaline hairs could also create a niche for epibiotic bacteria, and the presence of bacteria on algal thalli is well known (Cundell et al., 1977; Bolinches et al., 1988; Egan et al., 2013). In light of the recently developed ‘holobiont’ concept (Egan et al., 2013; Bordenstein & Theis, 2015) a physical structure facilitating an altered chemical microenvironment, such as demonstrated here for tufts of hyaline hairs, could provide a competitive advantage e.g. by providing algal-associated bacteria with e.g. metabolic compounds or by enhancing extracellular hydrolytic activities on the thallus surface due to impeded mass transfer and thus removal of dissolved exoenzymes or...
hydrolysis products. Studies of the role of bacteria in the algal life-cycle and metabolism have shown that a strong host specificity of epiphytic bacterial communities exists, possibly shaped by the algal metabolites as the primary selective force (Lachnit et al., 2009). Previous studies have e.g. demonstrated the presence of N₂-fixing cyanobacteria as part of the algal microbiome, and it has also been shown that native bacteria are required for normal morphological development in some algae (Provasoli & Pintner, 1980). However, the actual distribution and ecological niches of such macroalgae-associated microbes are not well studied. Spilling et al. (2010) found more pronounced O₂ dynamics, reaching anoxia during darkness, in the cryptostomata cavities of _F. vesiculosus_, wherein the hyaline hairs are anchored. Cryptostomata could thus represent potential niches for aerobic and anaerobic bacterial degradation of organic substrates or O₂-sensitive N₂ fixation that warrant further exploration.

In some transects measured on light-exposed _Fucus_ thalli, we observed areas of enhanced O₂ concentration detached from the boundary layer (Fig. 2). In a previous study, it was shown that nutrient uptake rates could increase 10-fold when the boundary layer was periodically stripped by passing waves (Stevens & Hurd, 1997). However, in our case the flow upstream from the tuft was laminar and no waves or DBL stripping occurred. The observed phenomenon of enhanced O₂ above the DBL could be explained by a combination of factors. As flow is obstructed by a physical object, a differential pressure field is created where a local drop in pressure is created around the hyaline hairs due to the locally smaller cross section of unobstructed flow. Such a pressure gradient could create a local advective upwelling around the area of low pressure, thus affecting the O₂ transport. This phenomenon is well described in e.g. sediment transport (Huettel et al., 1996) and plumes of O₂ release have also been observed before in coral-reef-associated algae _Chaetomorpha sp._ using planar optodes (Haas et al., 2013). In addition, so-called vortex shedding (von Kármán vortex sheets) could also be a factor influencing the observed O₂ release. Shedding of vortices can occur at certain Reynolds numbers at the transition between laminar and turbulent flow when the pressure increases in the direction of the flow, i.e., in the presence of a so-called adverse pressure gradient (Bearman, 1984). In our study, the flow upstream from the hyaline hairs was laminar but a transition to turbulent flow can occur, even at low Reynolds numbers, when a certain surface roughness is present and vortex shedding can initiate at Reynolds numbers of ~50 (Nepf, 1999). Using characteristic scales from this study (hyaline hair tuft diameter = 2 mm; free-stream velocity = 1.65 or 4.88 cm s⁻¹; fluid density = 1 kg L⁻¹ and a dynamic fluid viscosity of 1.08 × 10⁻³ Pa s (Kaye & Laby, 1995)), we calculated Reynolds numbers of ~30-90. Von Kármán vortices have previously been connected to flow patterns on the lee side of plant parts (Nikora,
2010) and based on the calculated Reynolds numbers the theoretical basis for the generation of vortex shedding (Nepf, 1999) due to tufts of hyaline hairs is present in our experimental setup. However, it is important to note that if a combination of diffusive and advective mass transfer exists downstream from the hair tufts then the calculated O\textsubscript{2} flux from Fick’s 1\textsuperscript{st} law in this area will be subject to large uncertainties and will underestimate the combined diffusive and advective exchange of O\textsubscript{2} between the thallus and the overlying water. These values should therefore be interpreted with caution and we note that, in case of such a mixed flow regime, an apparent increase in boundary layer thickness is not necessarily linked to reductions in flux. This underscores our findings that the O\textsubscript{2} dynamics in the presence of hyaline hairs on the Fucus thallus are more complex than hitherto assumed.

We speculate that a combination of pressure-gradient-mediated upwelling of O\textsubscript{2} and vortex shedding (Fig. 6) could explain the observed phenomena in Fig. 2, and the local mass transfer related to the presence of hyaline hair tufts on fucoid macroalgae may thus be more complex than previously thought. A more detailed investigation of such mixed diffusive and advective mass transfer phenomena was, however, beyond the scope of the present study and requires a more detailed characterization of the hydrodynamic regimes over thalli with and without tufts of hyaline hairs.

**Figure 6.** Conceptual drawing showing possible scenarios for the observed upwelling of O\textsubscript{2} downstream of the hyaline hairs. Flow velocity (straight black lines) decreases from the free stream velocity toward the thallus surface through the diffusive boundary layer (DBL). The hyaline hair tuft protruding from cryptostomata alters the local boundary layer thickness and creates a differential pressure field (shown in gradient blue and red colours) due to the smaller cross section of unobstructed flow. Flow acceleration over tufts result in local low pressure driving local advective upwelling. In addition, an adverse pressure gradient downstream from the hair tuft potentially results in vortex shedding.
In conclusion, our study of the chemical boundary layer landscape over the thallus of *F. vesiculosus* revealed a strong local boundary layer heterogeneity over and around tufts of hyaline hairs anchored in cryptostomata. Single tufts showed an apparent thickening of the DBL downstream and horizontally relative to the thinner DBL over the smooth thallus surface, while areas with multiple tufts exhibited an apparent overall thickening of the boundary layer that may affect gas and nutrient exchanges between the alga and seawater. Furthermore, we also observed complex solute exchange phenomena that could be driven by local pressure gradients and/or vortex shedding over the hyaline hair tufts. Altogether, this study demonstrates that interactions between flow and distinct macroalgal surface structures give rise to local heterogeneity in the chemical landscape and solute exchange. This may allow for microenvironmental niches on the thallus that can harbour epiphytic microbes with a diversity of aerobic and anaerobic metabolism. Further microscale studies of such niches in combination with e.g. microscopy and molecular detection of microbes in relation to hyaline hairs and cryptostomata thus seem an important next step to reveal further insights into the presence and role of the microbiome of fucoid algae.

**Acknowledgements**

This study was supported by grants from the Danish Council for Independent Research | Natural Sciences (MK), and a PhD stipend from the Department of Biology, University of Copenhagen (ML). We thank Roland Thar (Pyro-Science GmbH) for his help in establishing the 3D microsensor measurement setup and software and Erik Trampe for help with photography of Fig. S1C.
References


Supplementary information

Figure S1. Photographs of *Fucus vesiculosus* showing hyaline hairs and schematic plots of the measurement details of 2D transects and 3D grids. a) Stand of the brown alga *Fucus vesiculosus*, and b) close-up photograph showing whitish tufts of hyaline hairs protruding from the thallus. c) Cross-section through a thallus showing a single tuft of hyaline hairs anchored in a cryptostomata cavity. d) Schematic drawing of the spatial orientation of flow direction, thallus surface and tufts of hyaline hairs during microsensor measurements of transects, where point A indicates the starting point of transect measurements. e) Grids of O$_2$ concentration profiles used for mapping the diffusive boundary layer over the *F. vesiculosus* thallus, where point B indicates the starting position in grid measurements.
Figure S2. Transects of O₂ concentration (in µmol L⁻¹) measured across a single tuft of hyaline hairs in *Fucus vesiculosus* measured at flow velocities of 1.65 (a, c) and 4.88 cm s⁻¹ (b, d), in light (350 µmol photons m⁻² s⁻¹) (a, b) and darkness (c, d). The arrows indicate flow direction. The zero position (0,0) indicates the position of the cryptostomata, and transects were adjusted to the thallus surface. Colour bars denote O₂ concentration (in µmol O₂ L⁻¹).
Figure S3. Effective diffusive boundary layer thickness, \( Z_\delta \) (in mm) over a *Fucus vesiculosus* thallus measured as a function of the distance from the center of the cryptostomata with a tuft of hyaline hairs in light (350 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\)) and in darkness under flow velocities of 1.65 cm s\(^{-1}\) and 4.88 cm s\(^{-1}\) (a). The average \( Z_\delta \) (± SEM) over four transects measured both in light and in the dark at flow velocities of 1.65 cm s\(^{-1}\) and 4.88 cm s\(^{-1}\) (b).
Chapter 9

Fiber-optic probes for small-scale measurements of scalar irradiance

Published in *Photochemistry and Photobiology*

by

Lars Fledelius Rickelt, Mads Lichtenberg,
Erik Christian Løvbjerg Trampe and Michael Kühl
Fiber-optic probes for small-scale measurements of scalar irradiance

Lars Fledelius Rickelt¹, Mads Lichtenberg¹, Erik Christian Løvbjerg Trampe¹ and Michael Kühl¹²

¹Marine Biological Section, University of Copenhagen, Denmark
²Plant Functional Biology and Climate Change Cluster, University of Technology Sydney, Australia

Abstract

A new method for producing fiber-optic microprobes for scalar irradiance (=fluence rate) measurements is described. Such fine-scale measurements are important in many photobiological disciplines. With the new method, it is possible to cast spherical 30–600 µm wide light integrating sensor tips onto tapered or untapered optical fibers. The sensor tip is constructed by first casting a clear polymethyl methacrylate (PMMA) sphere (~80% of the size of the final probe tip diameter) onto the optical fiber via dip-coating. Subsequently, the clear sphere is covered with light diffusing layers of PMMA mixed with TiO₂ until the fiber probe exhibits a satisfactory isotropic response (typically 5–10%). We also present an experimental setup for measuring the isotropic response of fiber-optic scalar irradiance probes in air and water. The fiber probes can be mounted in a syringe equipped with a needle, facilitating retraction of the spherical fiber tip. This makes it, e.g. possible to cut a hole in cohesive tissue with the needle before inserting the probe. The light collecting properties of differently sized scalar irradiance probes (30, 40, 100, 300 and 470 µm) produced by this new method were compared to probes produced with previously published methods. The new scalar irradiance probes showed both higher throughput of light, especially for blue light, as well as a better isotropic light collection over a wide spectral range. The new method also allowed manufacturing of significantly smaller scalar irradiance microprobes (down to 30 µm tip diameter) than hitherto possible, and such sensors allow minimally invasive high-resolution scalar irradiance measurements in thin biofilms, leaves and animal tissues.
Introduction

Light is essential for life on Earth and is an important environmental parameter in biology, but also in medicine, where light is used for diagnosis and treatment, e.g. in photodynamic therapy (PDT). It is a challenge to measure light in dense media such as sediments, biofilms and tissues, where intense scattering and absorption results in strong light attenuation and steep light gradients, and where phenomena such as photon trapping and path length enhancement come from multiple scattering and internal reflections at optical boundaries with variations in refractive indices (Star & Marijnissen, 1989; Kühl et al., 1994a; Flemming & Wingender, 2001). It is thus essential in both biomedical dosimetry and photosynthetic studies in plant physiology and microbiology to determine the total light the cells receive (Kühl & Jørgensen, 1992; Kühl et al., 1994a; Björn, 2008). This involves measuring the integral quantum flux from all directions about a point; this parameter ($E_0$) is often denoted as the photon scalar irradiance ($\mu$mol photons m$^{-2}$ s$^{-1}$) in environmental research or the radiant energy fluence rate (in W m$^{-2}$) in biomedical research.

The light field in a given medium can be described by detailed measurements of field radiance ($L$) with flat-cut optical fibers that have well-defined light acceptance characteristics (Kühl et al., 1994b; Kühl, 2005). The field radiance, $L(\theta, \phi)$ from a given direction specified by the zenith and azimuth angles ($\theta$, $\phi$) in a spherical coordinate system is defined as:

$$L(\theta, \phi) = \frac{d^2\Phi}{dA d\omega}$$

where $\Phi$ is the radiant flux from that direction per unit solid angle, $d\omega$, per unit area perpendicular to the direction of light propagation, $dA$.

The scalar irradiance ($E_0$) or fluence rate at a given point can be expressed as the field radiance integrated over the whole sphere of $4\pi$ solid angle (Kühl & Jørgensen, 1992; Kühl et al., 1994a; Björn, 2008):

$$E_0 = \int_{4\pi} L(\theta, \phi) d\omega$$

Scalar irradiance probes are usually built as spherical light collectors exhibiting an isotropic angular response to incident light, i.e. light from all directions is captured by the probe tip and channeled to the detector with equal probability. Scalar irradiance probes can be manufactured by fixing a light diffusing sphere with isotropic light collection properties at the end of an optical fiber. The size of the diffusing sphere, its angular isotropic response, the transmittance at different wavelengths, and the mechanical
stability are important parameters for the choice of probe for a particular application. Ideal scalar irradiance probes should have a very good isotropic response, i.e. a standard deviation <10% of the mean detector response for different incident light angles as well as a small blind angle, where the optical fiber is in contact with the spherical tip. All wavelengths should be transmitted identically. Ultraviolet (UV) radiation is usually not transmitted well due to strong absorption in the probe material, and special optical fibers with high OH-content for transmission in the UV region are needed. To resolve the steep light gradients in scattering media, a small spherical tip is crucial for measurements in sediments, biofilms and tissues. A small tip diameter also minimizes local impact at the insertion point, especially in cohesive media where it can be necessary to precut a hole with a needle. However, untapered fibers with larger spheres are less fragile than tapered fibers with small spheres.

Several different methods for preparing fiber-optic scalar irradiance probes with spherical tip diameters <1000 µm have been described for application in biomedical and environmental sciences. Marijnissen and Star developed scalar irradiance probes for applications in tissue optics and PDT (Marijnissen & Star, 1987; van Staveren et al., 1995). The sensors were constructed from a light diffusing plastic, Arnite (polyethylene terephthalate), sphere with ~800 µm diameter that was machined on a lathe, and glued to a 200 µm wide flat-cut optical fiber. The probes exhibited a good isotropic response of 10% and good mechanical strength; however, their size is quite large for most biological applications. They can be used in clinical medicine, and are commercially available (MedLight SA, Switzerland; PDT Systems, Buellton, CA, USA).

Henderson developed an alternative manufacturing method based on a light-cured polymer probe using a white dental fissure sealant (Helioseal; Ivoclar Vivadent) to form a <800 µm wide sphere on the tip of an untapered optical fiber (Henderson, 1990). These sensors exhibited an angular light collection isotropy of 5% at 488 nm, and 7% at 632 nm. A similar method was used to cure a resin mixed with TiO₂ forming a 50 µm wide light-collecting sphere on a tapered fiber, albeit with a less ideal isotropic response (Holt et al., 2014).

Using a fundamentally different approach, Lilge et al. developed two different types of scalar irradiance probes based on the use of a dye-doped measuring tip fixed at a distance from the light-collecting optical fiber by a transparent material (Lilge et al., 1993). Type I was made from polymethyl methacrylate (PMMA) and different fluorescent dyes with measuring tip diameters of 265–615 µm and a light collection isotropy of 10% in water. In type II, the fiber cladding was removed by HF etching, and inserted in a capillary tube with a “fluorescent dye-doped UV curing glue” at the end. The result was a cylindrical isotropic probe with diameter of 170 µm, and a sensing length of 200–400 µm showing an isotropy of
20% in air. The responsivity of these designs was, however, two orders of magnitude lower than for probes made according to Marijnissen and Star (Marijnissen & Star, 1987; Lilge et al., 1993; van Staveren et al., 1995). Their angular response was detected by rotating the light source around the probes. For measurements in water, the probes were held in a round container filled with water. The material of the container was not explained, and no comparison of probes measured in both air and water were given.

Dodds fixed a drop of Titanium White acrylic paint at the end of a 125 µm step-index optical fiber that dried to a scattering sphere of ~250 µm in diameter (Dodds, 1992). Such a probe was used to measure scalar irradiance in sediments and microbial mats. The angular response was less ideal and to make up for that, four measurements were done turning the sensor 90° along its axis and integrating the results, when a light profile was done.

Instead of paint, Lassen et al. used polymethyl, and polybutyl methacrylate (PMMA and PBMA) dissolved in xylene (Plexisol, PM 560 and PM 709) and mixed with TiO$_2$ powder to cast a 70–100 µm wide scattering sphere on the end of a tapered fiber (Lassen et al., 1992). These microprobes show a good isotropic angular response of 10% at 450, 650 and 850 nm both in air and water and they have been widely used for visible and near-infrared light measurements in aquatic photosynthesis studies (Kühl, 2005).

To alleviate the bad transmission in the UV region of the methacrylate-based scalar irradiance microprobes, Garcia-Pichel produced an UV transmitting ~100 µm wide vitro-ceramic spherical tip by back melting of a long optical fiber taper coated with MgO crystals (Garcia-Pichel, 1995). Such sensors enable UV scalar irradiance measurements down to 250 nm with an isotropic angular response of 15%.

The mentioned scalar irradiance probes have been applied in a variety of biomedical and ecological applications, where the small probe size has allowed new insights to the light microenvironment, and optical properties of biofilms, sediments and tissues (Star et al., 1988; Kühl & Jørgensen, 1992; Ploug et al., 1993; Kühl et al., 1994b; Bebout & Garcia-Pichel, 1995; Kühl et al., 1996). Some recent examples include the investigation on the effect of light on in vitro cultivated blastocysts in mouse embryos (Li et al., 2014), detailed measurements of vertical, and lateral light gradients within and across coral tissues in different scleractinian species (Wangpraseurt et al., 2012), characterization of the biophotonic properties of iridocytes in photosymbiotic giant clams (Holt et al., 2014), and measurements of radiative energy budgets in different photosynthetic microbial mats (Al-Najjar et al., 2012).

A comparison of different types of fiber-optic scalar irradiance probes in terms of their measuring characteristics and material properties has to our knowledge not been reported in the literature. In this
study, we compare four different types of scalar irradiance probes, and present a further development of the type invented by Lassen et al. (1992), along with details on a setup for measuring the isotropic performance of scalar irradiance probes in air and water. The new manufacturing method enables construction of ultrasmall scalar irradiance microprobes with 30 - 150 µm wide spherical light collectors cast onto tapered fibers, or 220–600 µm wide spherical collectors cast onto untapered fibers. These sensors show ~5 times less light attenuation, and good isotropic responses at different wavelengths. The optical fiber can be fixed within a syringe equipped with a needle, facilitating a retractable probe. This makes the microprobe easier to handle, which is crucial in various applications, such as for measuring in cohesive microbial mats, leaves and various tissues. The needle with the retracted probe can cut a hole in the object before the probe is inserted.

Materials and methods

Fixation in syringe, cutting and fabrication of tapered fiber tips.

A 5 m long single strand fused-silica multimode optical fiber patch cord with standard SMA-connectors was used for manufacturing all scalar irradiance probes in this study. The optical fiber was a step-index fiber with 105/125 µm core/cladding diameter ratio, and a numerical aperture in air of NA = 0.22 (FG105LCA, Thorlabs, USA). The patch cord was cut in two, and the protective PVC coating and Kevlar fibers were removed over a length of ~15 cm from one end. The underlying Tefzel polymer jacket enclosing the fiber was removed over ~7 cm with a mechanical fiber stripping tool (Micro-Strip; Micro Electronics Inc., USA). For better handling, the fiber was fixed to the piston in a 1 mL syringe painted with an opaque black paint (Conductive Carbon Paint; SPI Supplies, West Chester, PA, USA); this also made the fiber retractable (see Fig. 1, where components are indicated with letters):

A 5 mm hole was drilled in the piston head (A). The black rubber gasket (B) was removed from the piston (C), and about a third of the gasket holder side was cut off with a Stanley knife. The gasket was put back, and a hypodermic needle with removed Luer connector (D) (Sterican 21G, 0.80 x 80 mm; B. Braun Melsungen AG, Germany) was pushed through the gasket. Then the fiber (E) was first put through the hole of the piston head (A), and then through the needle tube (D). The sharp end of the tube was pushed into the fiber protection, and the fiber protection was fixed to the piston (C) with black tape (Vinyl Electric Tape, Scotch Super 33+; 3M Electrical Products Division, USA). The piston including the fixed fiber was put back into the black-painted syringe (F) with the fiber end (E) protruding from the opening. A hypodermic needle (G) (23G, 0.6 x 25 mm, Fine-Ject; Henke-Sass Wolf GmbH, Germany) was attached to the syringe with the bare fiber pushed through the needle. The syringe was then
mounted vertically in a micromanipulator (MM33; Märtzhäuser, Wetzlar, Germany) with a small weight (3.75 g) attached to the bare fiber end. A taper was made by heating the fiber with a small oxygen/propane flame from a miniature brazing and welding set (Roxy-Kit; Rothenberger, Frankfurt a. M., Germany). Thereafter, the syringe was mounted horizontally in a micromanipulator with the fiber tip placed under a dissection microscope. The fiber outside the needle was painted with opaque black paint diluted 1:1 with isopropanol, and the taper was cut back manually with a sharpened forceps to the desired diameter (10–15 µm) of the tapered tip. Untapered fibers were cut with an optical fiber cleaving tool (Thomas & Betts, Rantanz, New Jersey) to obtain a straight and flat-cut tip before it was put through a hypodermic needle (23G, 0.6 x 25 mm, Fine-Ject). The outermost 2–3 cm of the fiber was coated with an opaque layer of the black paint diluted 1:1 with isopropanol. Finally, the plastic Luer connector of the needle was painted with undiluted black paint.

**Casting of light-collecting spheres onto fiber tips - PMMA/TiO<sub>2</sub> - based probes.**

For the construction of the light-collecting spheres, two stock solutions of 25% w/w of polymethyl methacrylate (PMMA) (Goodfellow Cambridge Ltd., UK; refractive index n = 1.49), were made in chlorobenzene: Solution A consisted of 5 g PMMA in 15 g chlorobenzene, and solution B consisted of 3 g PMMA 1 g TiO<sub>2</sub>, 9 g chlorobenzene (25% w/w TiO<sub>2</sub> in the dry PMMA). From solutions A and B, three other solutions were made with a final content of TiO<sub>2</sub> in dry PMMA of 12.5% (solution C), 6.25% (solution D) and 3.15% (solution E), respectively. Chlorobenzene was chosen as the solvent, because it dissolves PMMA and evaporates with an adequate speed. It can be exchanged with xylene without problems.
The syringe with the fiber protruding out of the needle was mounted in a micromanipulator, and the tip was observed under a dissection microscope. Any black paint was carefully removed from the flat-cut fiber end, and a clear sphere was cast by dip coating the fiber tip in solution A aiming after placing the center of this sphere at the tip of the fiber. For this, a drop of the solution was placed on a small spatula, and was moved to the fiber until the drop on the spatula touched it. After retraction from the polymer droplet, the surface tension of the adhered material formed a small sphere on the fiber tip. After drying for 3–5 min, the process continued until the desired sphere size was obtained. The final sphere size usually was ~20–30% wider than the diameter of this clear sphere. A schematic drawing of the final result for a 470 µm probe with an inner 360 µm clear sphere is shown in Fig. 2. The casting process was then continued with mixtures containing different concentrations of TiO₂. The isotropic response was quickly checked after each adherence of a new layer (see 'Measurement of isotropic response') and the process was stopped when a sufficient isotropic response was obtained. If no satisfactory result could be obtained, the sphere was removed completely with CHCl₃, and the process repeated.

For spheres on both tapered and untapered fibers, the best result was obtained using mixture C for the first layers, and D or E for finer adjustments. Using this approach, scalar irradiance probes with five different sizes were used in this study: three supported by tapered fibers (30, 40 and 100 µm) and two supported by untapered fibers (300 and 470 µm). We also manufactured a large scalar irradiance probe (500 µm), with an untapered fiber and using only solution C for the sphere, following the casting method of (Lassen et al., 1992). The critical angle for light reflected out of PMMA was calculated to 42.9° in air and 64.8° in water.
Helioseal curing-based scalar irradiance probes.

We manufactured scalar irradiance probes according to Henderson (1990) and van Staveren et al. (1995) in the following way: After removal of the black paint at the fiber tip, the distal end of the fiber was connected to a UV light source (UV glue lamp, Dymax, Germany), and the fiber tip was placed into a small fissure sealant (Helioseal, amount of an UV light curing white dental Ivoclar Vivadent, Liechtenstein; refractive index \( n_{25} = 1.51 \)). The curing light was applied for 10 s; thereafter the fiber was pulled out of the sealant solution creating a 500 µm wide sphere of Helioseal at the tip. The surface of the sphere was not completely cured, but was finished by turning the sphere directly in front of the UV source for some minutes.

Helioseal is cured with 400-500 nm light. The cured sphere was then washed in ethanol, and the fiber immediately behind the spherical tip was painted with diluted black paint. It was not possible to produce isotropic spheres on tapered fibers with this method. The critical angle for light reflected out of Helioseal was calculated to 41.4° in air and 61.6° in water.

Scalar irradiance probes based on gluing a diffusing sphere to fibers.

A scalar irradiance probe with a diffusing sphere glued to a step-index optical silica glass fiber was compared to the other types of probes. We used a commercially available version of a probe developed by Marijnissen and Star (1987) (Medlight isotropic probe model IP850 with a gold radiomarker, tip diameter 850 µm, isotropy of ±10% in air 40 – 360°, wavelength range 480–800 nm; silica, low OH-, 400 µm core, NA = 0.37; SMA-connector, Medlight S.A., Switzerland). After the sphere was machined on a lathe out of Arnite™, a blind hole was drilled toward the sphere center and the fiber was glued into the hole with a transparent UV adhesive (van Staveren et al., 1995). The diameter of the fiber was measured between the sphere and the gold radiomarker to 450 µm. The refractive index of Arnite™ was 1.51 (Marijnissen & Star, 2002). The critical angle for light reflected out of Arnite™ was calculated to 41.4° in air, and 61.6° in water.

The probe was mounted within a hypodermic needle (17G, 1.5 x 50 mm) mounted on a 1 mL black-painted syringe for easier handling during measurements. It was also necessary to paint the fiber between the needle and the sphere including the part between the radiomarker and the sphere for correct measurements. Due to the good mechanical stability of the sphere, stains of paint on the probe could be removed by carefully scraping with a small dissection knife.
Probe characterization - detectors and light sources.

A custom-built light meter, with a relatively flat spectral quantum responsivity for 400–700 nm light, developed for microscale measurements of photosynthetically active radiation (PAR, 400–700 nm) (Kühl et al., 1997), was used for characterizing the fiber-optic scalar irradiance probes. The light meter signal was recorded on a strip-chart recorder (BD25; Kipp & Zonen, Netherlands).

A fiber-optic spectrometer (USB2000 operated with the Spectra Suite software; Ocean Optics, Dunedin, USA) was used to record the spectral response of the probes and bare fibers. All spectra were recorded as an average of 10 scans, using a boxcar smoothing width of 4, and with the spectrometers nonlinearity and stray light correction enabled. The integration time was set as high as possible, and the corresponding dark noise was automatically subtracted for each recorded spectrum.

A light meter (Universal Light Meter, ULM-500; Walz, Germany) equipped with a calibrated photon irradiance sensor (LI-190; Li-Cor, USA) was used for measurements of the absolute photon irradiance of incident PAR (400–700 nm) from the collimated light source in units of µmol photons m⁻² s⁻¹.

Collimating optics was used for all sensor characterization measurements (Ovio Collimated Source; Ovio Optics, France) together with different light-emitting diodes (LED). The LED’s were connected to a trigger box controlled by the software Look@RGB (both available from www.fish-n-chips.de), which enabled PC-controlled adjustment of the LED intensity (Larsen et al., 2011). Three different LEDs were used in connection with the collimation optics: Two different white LEDs, one for low light intensities (Oslo 1 LED; ILH-OW01-STWH-SC211-WIR200; RS-Components, UK), and one for high light intensities (Oslo 4 LEDs; ILH0004-ULWH-SC211-WIR200; RS-Components, UK), and a 405 nm LED (Oslo 4 LEDs; ILH-OW04-UVBL-SC211; RS-Components, UK). The opening diameter of the Ovio optics was 32 mm, and at the backside of the box (at a distance of 54.5 cm) the beam diameter was 55 mm in air. The beam divergence for the collimated light was calculated to be <2.5° in air. The value was similar in water after a glass plate was placed in front of the collimation lens.

Measurement of isotropic response. For characterizing the angular light-collecting properties of fiber-optic scalar irradiance probes in air and water, a device was constructed from 1 cm thick black PVC (parts are identified by letters in Fig. 3): It consisted of a 60 x 60 x 10 cm box with a 10 cm disk (A) glued to the bottom in the middle of the box. A needle was put vertical through the center of the disk. Another 23.5 cm disk (B) with a 10 cm hole in the center exactly fitting the first disk was placed so it could be revolved freely. A holder (C) for the sensors was fixed on the outer disk with a metal rod so it could be rotated around its own axis. A plastic screw was used to lock the holder after centering of
the probe sphere. A laminated print of a graduated circle was glued to the central disk (A). A pointer (D) was placed in front of the sensor holder for easy reading of the measurement angle.

The collimation optics was mounted water-tight on one side of the box in a holder (E) that was sealed with an O-ring (Simmerring). A glass plate was placed in front of the collimator lens to prevent water from leaking, dew on the backside of the lens, and to keep the light collimation similar with air and water in the measuring chamber.

\[ \text{Figure 3. Setup for measuring the light-collecting isotropy of scalar irradiance probes. Comprised of a flat black box with a fixed disk (A) placed in the center with an angular scale and a needle, a rotational disk (B) on which a scalar irradiance probe is mounted in a holder (C) with the probe tip placed over the center of the fixed disk, a read out pointer (D), and the collimated light source mounted water-tight in a holder (E). During probe readout, the box is covered with a black lid, and the box can also be filled with water.} \]

The collimated light source and probe holders were constructed in a way, making the spherical probe tip position adjustable, as to be put in the centre of the light beam precisely over the needle in the centre of the disk. The chamber could be closed with a light-tight lid to prevent ambient light from disturbing the measurements.

For measurements, the scalar irradiance probe was attached to the holder on the revolving table with the sphere exactly over the guiding needle placed at the centre of the disk (Fig. 3). The distal end of the
fiber-optic scalar irradiance probe was connected to a light meter. The directional response of the probe was determined by rotating the revolving table from +160° to -160° in angular steps of 10° in a beam of collimated light. At 0° the probe fiber and the light source were aligned. To check for possible variations in the measuring setup such as light source fluctuations, fiber bending effects or air bubbles in the water, measurements at -90°, 0°, 90° and 160° were measured several times for each probe. Four series of measurements were recorded with the PAR-meter, and two series with the scalar irradiance probes connected to the spectrometer. All probes were first measured in air, where after the chamber was filled with water and measurements continued. After a measurement series, the probe was turned 90° around its longitudinal axis in the sensor holder, and the angular light collection was measured again. The water was then drained from the chamber and the probe properties was measured once more in air.

**Calibration of scalar irradiance probes for photon irradiance measurements.**

A 7 x 7 x 7 cm black PVC box with a lid and two aligned holes in opposite walls was used to calibrate the response of scalar irradiance sensors in absolute units of µmol photons m⁻² s⁻¹. The collimated light source and a photon irradiance sensor (LI190, LiCOR, USA; Universal Light Meter, ULM-500, Walz, Germany) were placed in opposite holes. The photon irradiance (µmol photons m⁻² s⁻¹) was measured at eight different current intensities (100–800 mA) for each of the white, blue and red LED’s as adjusted by the LED power supply. Subsequently, the irradiance sensor was exchanged with a fiber-optic scalar irradiance probe with its measuring tip placed at exactly the same spot and distance in the light field relative to the collimator, and the sensor response was measured in mV with the PAR-meter. Measurements with the scalar irradiance probe could then be converted using the linear correlation between mV readings and µmol photons m⁻² s⁻¹.

**Light attenuation of scalar irradiance sensors.**

We compared the light attenuation in each of the four different types of scalar irradiance probes, based on different sphere types fixed on the same untapered fiber. The sensors were mounted in the light calibration box (see section above), and light spectra were recorded with the spectrometer system. The percentage of light passing from the light scattering sphere at the tip into the probe fiber was calculated by dividing the probe spectra with spectra recorded with bare fibers placed at similar distance and position in the light field (Fig. 4). A 400 µm core step-index fiber (Laser Components, Germany) was
used corresponding to the Medlight scalar irradiance probe. All probes were supported by untapered fibers, and the results were corrected for different cross-sectional areas due to different sphere diameters. Besides measuring probe spectra with a white LED light source, additional spectra were recorded with a 405 nm LED to obtain sufficient signal in the 380–450 nm region.

**Photography of sensors.**

The different types of scalar irradiance probes were photographed with a commercially available digital SLR camera (Canon EOS 7D MkII; Canon Europe Ltd., Middlesex, UK), connected via a 0.5x photoadapter (IMAG-AX; Heinz Walz GmbH, Effeltrich, Germany) to an epifluorescence microscope (Axiostar Plus FL; Carl Zeiss GmbH, Germany), fitted with either a 10x or 20x plan-APOCHROMATE objective (Carl Zeiss GmbH, Germany). Illumination of the probes was achieved by means of the light source of the microscope and a fiber-optic halogen lamp (KL-1500; Schott AG, Mainz, Germany) fitted with a double-light guide for side illumination.

**Application of new scalar irradiance microsensors.**

Spectral scalar irradiance was measured using a microprofiling setup with the sensor mounted in a motorized micromanipulator (MU-1; PyroScience GmbH, Germany) and controlled by PC-software (Profix; PyroScience GmbH, Germany); this allowed advances in 100 µm vertical steps through the sample. Spectra of the scalar irradiance were measured with the probe fiber connected to a fiber-optic spectrometer (USB2000+; Ocean Optics, FL, USA) that was interfaced to a PC running dedicated spectral acquisition software (SpectraSuite; Ocean Optics, FL, USA). Incident light was provided either with a fiber optic tungsten halogen lamp with a collimating lens (Schott KL2500LCD; Schott AG, Germany) or from a LED ring (Walz GmbH, Germany). Spectra of scalar irradiance can be integrated in the spectral region of interest, e.g. the PAR region (400–700 nm), and normalized to the incident downwelling irradiance measured over a black non-reflective light-well, to construct scalar irradiance profiles through tissues and characterize the amount of photosynthetically active radiation (PAR) in a certain tissue depth. For a detailed description of the collection and analysis of acquired spectra, see (Lichtenberg & Kühl, 2015).
Results

Light attenuation in the different scalar irradiance probes
A comparison of light attenuation in the four different probe materials showed that wavelengths <410 nm were strongly absorbed in probe tips made of PMMA or Helioseal, whereas the machined sphere probe made according to Marijnissen and Star (1987) exhibited a much better performance in the UV region. At wavelengths >415 nm, probes manufactured according to Henderson (1990) and Lassen et al. (1992) exhibited similar light attenuation, whereas probes made according to Marijnissen and Star (1987) and probes made with the new procedure described in this study showed about five times less light attenuation in the probe material (Fig. 4).

Angular light collecting properties of scalar irradiance probes
We manufactured different types of scalar irradiance sensors with spherical tip diameters of 30–850 µm (Fig. 5). The isotropic response of the different scalar irradiance probes revealed relatively large differences in isotropy between different probes when measured in air and water and for different wavelength ranges (Table 1; Figs. 6–9).
Sensors manufactured according to Marijnissen and Star (1987) and Lassen et al. (1992) showed similar isotropy for blue, green and red light, whereas the Helioseal-based sensor manufactured according to Henderson (1990) showed a large color dependence, where the isotropy for green light was 3–5 times smaller than for blue and red light (Fig. 8C). Except for some minor peaks in water, for some sensors.

Figure 4. A comparison of spectral light attenuation in scalar irradiance probes made of four different types of light-collecting sphere materials, all supported by the same type of untapered fibers. (A) Measurements using a 405 nm LED as light source. (B) Measurements using a white LED light source. The light intensity is expressed relative to the measuring signal obtained with bare untapered fibers in the light path.
in the region -120° to -160°, and 120° to 160°, the sensors manufactured with the new method presented here generally exhibited a good isotropic light collection in both air and water that was similar or better than the other types of scalar irradiance probes (Figs. 6 and 8) over a wide range of tip diameters (Figs. 7 and 9). The angular light response of most probes was symmetrical about 0°. The probe manufactured according to Marijnissen and Star (1987) showed a characteristic parabolic shape, whereas the other types of scalar irradiance probes exhibited a more periodical variation in angular response.

Figure 5. Photographs of different fiber-optic scalar irradiance probes. Sensors made with the new manufacturing procedure are shown in panels (A, B) 40 µm sphere diameter at two different magnifications, where B was illuminated through the fiber, C) 100 µm, and D) 470 µm. Panel E shows a probe made according to Lassen et al. (1992) (500 µm). Panel F shows a probe (500 µm) made according to Henderson (1990). Panels G and H show a commercial probe (850 µm) made according to Marijnissen & Star (1987) with and without a black overcoat of the fiber, respectively.
Application of scalar irradiance microprobes

The new manufacturing procedure presented in this study enabled production of small scalar irradiance microprobes with tip diameters <50 µm, which can be used for measuring scalar irradiance attenuation profiles in thin specimens. We successfully tested such probes in the tough thallus of the brown macroalga Fucus serratus and in the cohesive tissue of the reef-building coral Montastrea curta (Fig. 10). In both specimens, it was possible to measure detailed light attenuation profiles of photon scalar irradiance of photosynthetically active radiation (PAR, 400–700 nm). The light microprofiles showed local enhancement of scalar irradiance near the surface of the tissue due to photon trapping, created by multiple scattering and enhancement of the photon pathlength (Kühl, 2005) followed by an exponential attenuation of light in deeper tissue layers.

Discussion

We present the first comparison of the light-collecting properties of different types of fiber-optic scalar irradiance probes along with a new manufacturing method that enables fabrication of scalar irradiance microprobes with a good isotropic response and significantly smaller tip diameters than previously realized. Furthermore, the new scalar irradiance probes exhibited a higher throughput of light in the visible to near-infrared spectral range, thus alleviating constraints on detector sensitivity. The detailed characterization of probe performance, using a new set-up for measuring the angular light collection of scalar irradiance probes, revealed distinct difference between the different probe types, as discussed below.

Characterizing the angular light collection of scalar irradiance probes

To our knowledge, a thorough comparison of different types of scalar irradiance sensors has not previously been published. With the construction of a new device enabling angular response measurement in air and water (Fig. 3), it was possible to compare the performance of different scalar irradiance probes. If the probes and sensors are manufactured correctly, there is not a significant difference in the behaviour of the sensors, whether they are measured in air or water, but such comparative measurements can effectively reveal any flaws in the production that can direct optimization of probe construction.
Table 1. Light-collecting properties of different types of scalar irradiance probes. Probe isotropy was measured in the setup shown in Figure 3 in air as well as in water with the sensor turned 90° around the longitudinal fiber axes between measurements. The isotropy was quantified as the standard deviation of probe signal normalized to the highest angular signal reading. The probes were manufactured with four different methods: Probe no. 1–5 (different diameters) were made with the new fabrication method based on casting first an inner sphere of PMMA and then a diffusing shell of PMMA doped with TiO₂ onto the fiber tip (the first number is the size of the clear sphere and the last number is the final probe diameter); Probe no. 6, was obtained with a 850 µm wide machined diffusing plastic sphere fixed to an optical fiber according to Marijnissen and Star (1987); Probe no. 7 was made by casting 500 µm wide sphere of PMMA doped with TiO₂ onto the fiber tip according to the method of Lassen et al. (1992); Probe no. 8 was made by curing a 500 µm wide sphere of Helioseal on the fiber tip according to Henderson (1990).

<table>
<thead>
<tr>
<th>Probe No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (µm)</td>
<td>n.d./30</td>
<td>35/40</td>
<td>80/100</td>
<td>n.d./30</td>
<td>360/470</td>
<td>850</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Fiber tip geometry</td>
<td>Tapered</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air 90°</td>
<td>9.3</td>
<td>4.6</td>
<td>7.2</td>
<td>5.5</td>
<td>6.8</td>
<td>17.2</td>
<td>8.9</td>
<td>7.3</td>
</tr>
<tr>
<td>Water 90°</td>
<td>7.5</td>
<td>15.0</td>
<td>7.3</td>
<td>8.3</td>
<td>7.3</td>
<td>18.5</td>
<td>14.7</td>
<td>7.8</td>
</tr>
<tr>
<td>Air blue (450nm)</td>
<td>13.2</td>
<td>9.2</td>
<td>7.8</td>
<td>6.9</td>
<td>8.0</td>
<td>18.7</td>
<td>13.1</td>
<td>27.4</td>
</tr>
<tr>
<td>Air green (550nm)</td>
<td>7.5</td>
<td>5.6</td>
<td>6.6</td>
<td>6.8</td>
<td>7.0</td>
<td>19.5</td>
<td>13.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Air red (650nm)</td>
<td>7.6</td>
<td>7.2</td>
<td>8.7</td>
<td>7.7</td>
<td>9.3</td>
<td>20.2</td>
<td>13.8</td>
<td>23.5</td>
</tr>
<tr>
<td>Water blue (450nm)</td>
<td>18.9</td>
<td>21.1</td>
<td>11.0</td>
<td>9.4</td>
<td>9.4</td>
<td>21.4</td>
<td>8.9</td>
<td>24.5</td>
</tr>
<tr>
<td>Water green (550nm)</td>
<td>12.1</td>
<td>15.7</td>
<td>6.5</td>
<td>6.8</td>
<td>7.5</td>
<td>22.6</td>
<td>8.8</td>
<td>11.4</td>
</tr>
<tr>
<td>Water red (650nm)</td>
<td>12.3</td>
<td>15.0</td>
<td>4.6</td>
<td>5.1</td>
<td>7.8</td>
<td>23.3</td>
<td>9.9</td>
<td>34.0</td>
</tr>
</tbody>
</table>

As an example, it was necessary to paint the fiber all the way to the sphere on the probe manufactured according to Marijnissen and Star (1987), before a reasonable isotropic angular response was obtained; while the standard deviations in the isotropy plot were not improved by the painting, the shape of the plot became much different. Before painting, the plot in air was relatively flat from -90° to 90° with a decreasing angular response at the ends. In water, peaks appeared around -130° and 130°. After painting, the plots were similar in air and water (Fig. 6D; data before painting not shown). This underscores the importance of avoiding light entry or leakage via the fiber behind the spherical probe tip or via the optical fiber strand.
When the first recording of the “Marijnissen and Star” sensor was done, no protective painting was applied, not even on the syringe. This resulted in a large peak at -140° to -150° in both air and water, but only in one position of the syringe. This was not seen when the syringe was turned 90° in the holder. After painting of the syringe, this peak disappeared, but the overall plot still looked strange although the overall variability in angular response was ok. We concluded that the minor peaks in the angle response plots (Figs. 6 and 7) are artefacts due to flaws in the black painting of the fibers very close to the sphere where it is difficult to see (Fig. 5) and from which some light can leak out.

Figure 6. The isotropic light-collecting properties of four different types of scalar irradiance probes. The relative response was measured with the probes connected to a PMT light meter with a flat quantum response over 400–700 nm and illuminated with a collimated white LED light source. Each probe was mounted in the angular calibration setup shown in Figure 3 and the probe response was measured as a function of incident light angle in 10° steps. Measurements were done in air and water. After each measurement, the sensor was turned 90° around its own axis and another set was recorded to check for spherical homogeneity in light collection.
The peaks arise because more light is apparently leaked out upon direct illumination of the front of the spherical tip and less when the light comes from the back. This may also explain the observed difference in angular response found in air and water, where in the latter case more light can be coupled out of the fiber more easily due to a lower difference in refractive index between fiber and water as compared to fiber and air. For a fiber with a 105/125 µm core/cladding ratio, 30% of the fiber cross section is cladding and the cladding refractive index is only a little smaller than the core refractive index.

This indicates that a little <30% of the light from the sphere will enter the cladding. In air most of this light will rapidly be caught by the core, but in water the acceptance angle is ~20° wider and more of the light will escape out if the protective painting is insufficient. For tapered fibers, light coupling across the sides will be more pronounced than for untapered fibers. The general problem with light coupling

![Figure 7. The isotropic light-collecting properties of four different sizes of scalar irradiance probes manufactured with the new method. The relative response was measured with the probes connected to a PMT light meter with a flat quantum response over 400-700 nm and with a collimated white LED light source. Probes were mounted in the setup shown in Figure 3 and the angular response was measured as a function of incident light angle in 10° steps. Measurements were done in air and water. After each measurement, the probe was turned 90° around its own axis and another set was recorded to check for spherical homogeneity in light collection.](image-url)
out of the fibers was confirmed by redoing the 100 µm probe (Star & Marijnissen, 1989) (Fig. 7C), and the 500 µm Lassen probe (Kühl et al., 1994b) (Fig. 6B), where preliminary results showed large differences in isotropic response in air and water (data not shown). When the new probes were produced, great attention was put into ensuring a flawless painting of the fibers. Lassen et al. (1992) showed an isotropic plot of the same sensor in both air and water, but did not describe how it was carried out. Lilge et al. (1993) measured some sensors in water and some in air, but did not present any comparison of the same sensor in both air and water. The set-up was described in some detail but it seems problematic as the probe was held in a cylindrical water bath and the collimated light was moved around it. It cannot be assumed that the light will still be collimated inside the water bath described due to focusing effects. The probes measured in air, was excited with a 351 nm laser, however, light attenuation in water is to strong (Lilge et al., 1993). van Staveren et al. (1995) used a similar set-up as Lilge et al. (1993) but placed a lens in front of the water bath to counteract the above mentioned focusing effects. They found differences in isotropy for probes inserted in different media and gave some correction factors for absolute calibration depending on the refractive index of the probe material and the surrounding medium. The probe fibers did not have any protection against light collection or leakage (Star et al., 1988; Star & Marijnissen, 1989; van Staveren et al., 1995). They also compared isotropic properties for probes acting either as a light detector or as a light source, and measured large differences due to dissimilar light paths. A probe with an isotropic light emission (<15%) could be anisotropic for light detection (>35%), and vice versa (van Staveren et al., 1995). The isotropic response of a 50 µm scalar irradiance microprobe mentioned in the introduction produced by UV curing a resin mixed with TiO$_2$ on a tapered fiber was measured by emitting light out of the probe, and the result was plotted as logarithmic values (Holt et al., 2014). It is problematic to assess the isotropic response this way due to the different geometry for the light path as described by van Staveren et al. (1995).

**Optical throughput and spectral differences in isotropic response**

The difference in the angular response to blue, green, and red light of the various scalar irradiance probe types can largely be explained by absorption differences in the sphere materials. This affects the light attenuation in the light-collecting sphere due to scattering enhancing differences in the spectral absorption characteristics of the probe sphere material. That is, the path length enhancement for photons in the scattering sphere material increases the probability of photon absorption at wavelengths overlapping with the absorption maxima of the sphere material, while this effect is much smaller for photons in spectral ranges outside characteristic absorption maxima.
In this way, both material properties, the homogeneity of the sphere in terms of material thickness, and the way the sphere is fixed onto the fiber will affect the probability of photons collected and channeled to the detector via the optical fiber.

The refractive index of the sphere material can also affect the light-collecting properties of scalar irradiance probes. The difference in refractive indices of air and water has no major significance for the light penetrating the probes but for the radiation reflected out of the probe there is a difference in the critical angle. For Helioseal and Arnite™, the difference is ~20°, and for PMMA it is a little larger, ~22°. Photons collected in the sphere material are thus more efficiently retained in the sphere when measuring in air as compared to measurements in water. On the other side, backscatter of incident light in the sphere surface will be higher in air than in water. Such immersion effects are well known, also from

Figure 8. The spectral dependence of the isotropic light-collecting properties of four different types of scalar irradiance probes. The relative response was measured with the probes connected to a spectroradiometer and illuminated with a collimated white LED light source. Probes were mounted in the setup shown in Figure 3 and the probe response was measured as a function of incident light angle in 10° steps. Measurements were done in air except for A.

In this way, both material properties, the homogeneity of the sphere in terms of material thickness, and the way the sphere is fixed onto the fiber will affect the probability of photons collected and channeled to the detector via the optical fiber.

The refractive index of the sphere material can also affect the light-collecting properties of scalar irradiance probes. The difference in refractive indices of air and water has no major significance for the light penetrating the probes but for the radiation reflected out of the probe there is a difference in the critical angle. For Helioseal and Arnite™, the difference is ~20°, and for PMMA it is a little larger, ~22°. Photons collected in the sphere material are thus more efficiently retained in the sphere when measuring in air as compared to measurements in water. On the other side, backscatter of incident light in the sphere surface will be higher in air than in water. Such immersion effects are well known, also from
other types of scalar irradiance probes, and for probes made according to Marijnissen and Star (1987), such effects can lead to minor uncertainties in the range of <5% when measuring in turbid media (Marijnissen & Star, 1996; Marijnissen & Star, 2002). Probes manufactured according to Marijnissen and Star generally exhibit a small uniform absorption of visible light (van Staveren et al., 1995), hence the observed difference in the three colors was very small (Fig. 8D). PMMA-based sensors (Lassen et al., 1992) showed stronger absorption of blue light as compared to green and red light. The spectral variation in the isotropic response of such sensors could be related to the length of the path the light must travel inside the probe.

Such effect can be illustrated by looking at the construction of the new probes (Fig. 2), where the fiber tip is not placed in the center of the probe, but in the center of the clear sphere so the path through the TiO$_2$-doped PMMA is longer for light coming from the front than from the rear. This can give a somewhat stronger attenuation of the frontal light. In probes made according to Lassen et al. (1992), the fiber tip is placed more central in the PMMA sphere, and such differences in path lengths are less pronounced. But as mentioned above, the solid PMMA + TiO2 sphere of these probes causes a much higher overall attenuation of incident light as compared to probes made with the novel method presented here. Scalar irradiance probes made according to Henderson (1990), displayed a much stronger absorbance of blue light because the Helisoseal matrix is produced to absorb in this wavelength range as it is used for curing of the matrix (Henderson, 1990; van Staveren et al., 1995).

**Fabrication of scalar irradiance microprobes**

The variance in isotropic light collection of the different scalar irradiance probes is strongly affected by the actual position of the fiber tip inside the spherical light collector casted on the fiber. This is also relevant for the Marijnissen-Star-type probes (van Staveren et al., 1995), where the parabolic shape of their angular light-collecting function is caused by the placement of the sphere on the fiber, as well as a small ratio between the sphere diameter (850 µm), and the fiber diameter (450 µm); in the original publications the ratio was recommended to be 4–5 (van Staveren et al., 1995). The insertion point of the fiber itself creates a blind angle for light collection by the sphere and will in most cases set a limit for how efficient light is collected at incident angles >120°. However, during the fabrication of scalar irradiance probes made according to the new method, one can partially compensate for such effects. The construction based on a clear inner sphere coated with a scattering shell thus makes it possible to produce small probes down to 30 µm on tapered fibers with a tip diameter of ∼15 µm, and on
untapered fibers down to 240 µm with good isotropic response (Table 1). It has not previously been possible to create probes made according to Lassen et al. (1992) on untapered fibers. This is mainly due to the concentration of PMMA in xylene (40–45% w/w) of the used polymer solutions, which leads to a high surface tension in the material and larger spheres will not dry with a smooth surface (Lassen et al., 1992). For larger spheres, the idea behind the Henderson method is elegant, but the optical properties of the probe material are not ideal for broad spectral light measurements as their angular light collection varies strongly with wavelength (Henderson, 1990).

With the new method presented here, it is both faster to produce scalar irradiance probes with good isotropic performance and a higher optical throughput as compared to probes based on casting a sphere of only PMMA + TiO2 (Lassen et al., 1992)(14). The new method makes it easier to vary the tip

Figure 9. The spectral dependence of the isotropic light-collecting properties of four different sizes of scalar irradiance probes manufactured with the new method. The relative response was measured with the probes connected to a spectroradiometer and illuminated with a collimated white LED light source. Probes were mounted in the setup shown in Figure 3 and the angular response was measured as a function of incident light angle in 10° steps. Measurements were done in air.
dimensions of the probe, and it is easier to predict the size of the final sphere during the fabrication process.

**Mechanical stability of scalar irradiance probes**

It is difficult to give an exact evaluation of the mechanical stability of the probes without destroying them, but the material, and diameter of the employed optical fiber, and how well the sphere material sticks to the fiber are essential parameters. The fiber material is silica-glass for all probes compared in this study. Naturally, tapered fibers are thinner and more fragile than untapered fibers. van Staveren *et al.* (1995) found that the mechanical strength of sensors made according to Marijnissen and Star (1987), and Henderson (1990) was similar. Sensors made with PMMA spheres cast on the fiber tip are less sturdy, especially when measuring in very cohesive media, such as dense tissue, where there can be a substantial drag on the sphere when retracting the probe, with a potential loss of the spherical tip. Etching of the fiber tip (Rickelt *et al.*, 2015) prior to casting the sphere results in better adhesion, and thus more robust scalar irradiance probes. Furthermore, the smaller probes made with the new method also appeared sturdier when used for measurements in plant and animal tissues (Fig. 10).

**Application of new scalar irradiance probes**

Collection of scalar irradiance profiles in thin specimens, e.g. in the tough thallus of the brown macroalga *Fucus serratus*, and in the cohesive tissue of the reef-building coral *Montastrea curta* (Fig. 10) is only possible using very small sensors. This is due to (1) the thickness of the tissue under investigation, which is often <1 mm; and (2) the toughness of the outer tissue layers (epidermis in corals and outer cortex in algae), which makes it difficult for larger objects to penetrate. In addition, larger spheres are often

---

**Figure 10.** Two examples of scalar irradiance microsensor measurements in biological tissues. Vertical profiles of photon scalar irradiance (400–700 nm) in (A) the living tissue of the scleractinian coral *Montastrea curta* and (B) the thallus of the brown macroalga *Fucus serratus*. Values are expressed in % of the incident downwelling photon irradiance as measured with the fiber probe positioned over a black non-reflective light well.
actively pulled off the fiber tip by coral mesenterial filaments acting as a defense mechanism to fiber
insertion, but apparently such filament attachment are unable to pull off the small spheres (<50 µm)
(Daniel Wangpraseurt and Mads Lichtenberg, personal communication).

**Conclusion**

The new fabrication method yields scalar irradiance probes with excellent isotropic light-collecting
properties, being comparable or better than previously developed scalar irradiance probes, and spherical
tip diameters ranging from as small as 30 µm on tapered fibers up to ~600 µm on untapered fibers.
The presented experimental setup for measuring the isotropic light collection of the scalar irradiance
probes in air and any relevant liquid is easy to build and operate. If probes are carefully produced, e.g.
by an experienced technician, it is not necessary to test them both in air and water. However, testing
can effectively reveal flaws in the probe performance due to imperfect coating/isolation of fiber tips or
damage of the spherical tip. The possibility of making well-functioning scalar irradiance microprobes with
tip diameters <50 µm now enables light measurements at higher spatial resolution in thin objects, such
as terrestrial leaves and thin-tissued corals, and aquatic macrophytes. This new probe thus gives rise to
many new fields of application in photobiology.

**Acknowledgements**

This study was supported by grants from the Danish Council for Independent Research | Natural
Sciences (MK) and an Eliteforsk talent grant from the Danish Council for Independent Research (ECLT).
The authors thank Egil Nielsen for help with the mechanical construction of the isotropic calibration
box and Bjørn Jacobsen for assisting with photography.
References


Chapter 10

Multicolor light sheet microscopy-based imaging of absorption- and photosynthesis distribution in plant tissue

Intended for submission to Plant Physiology

by

Mads Lichtenberg, Erik C. L. Trampe, Thomas C. Vogelmann and Michael Kühl
Multicolor light sheet microscopy-based imaging of absorption- and photosynthesis distribution in plant tissue

Mads Lichtenberg, Erik C. L. Trampe, Thomas C. Vogelmann and Michael Kühl

1Marine Biological Section, University of Copenhagen, Denmark
2Department of Plant Biology, University of Vermont, USA
3Climate Change Cluster (C3), University of Technology Sydney, Australia

Abstract

In vivo chlorophyll fluorescence measurements of quantum yields of PSII photochemistry in optically dense systems are complicated by the presence of steep light gradients due to strong scattering in concert with absorption. Consequently, the externally measured effective PSII quantum yield may be composed of signals derived from cells differentially exposed to actinic light, where cells located deeper inside tissues will receive lower actinic irradiance than cells closer to the surface, thus displaying distinct photophysiological status. Here, we demonstrate how quantum yields of PSII photochemistry change under natural tissue light gradients compared to conventionally measured quantum yields. This was done by applying actinic irradiance perpendicular to one side of a thallus cross section of the aquatic macrophyte Fucus vesiculosus with a laser light sheet of defined spectral composition while imaging variable chlorophyll fluorescence from the cross section with a pulse-amplitude-modulated (PAM) imaging system mounted on a microscope. We show that quantum yields are highly affected by light gradients and that traditional surface-based variable chlorophyll fluorescence measurements result in substantial under- and/or over-estimations, depending on incident actinic irradiance levels. We present a method for using chlorophyll fluorescence profiles in combination with integrating sphere measurements of reflection and transmission to calculate depth-resolved photon absorption profiles, which can be used to correct apparent PSII electron transport rates to photons absorbed by PSII. Absorption profiles of the investigated aquatic macrophyte were different in shape from what is typically observed in terrestrial leaves, and based on this finding we discuss strategies for optimizing photon absorption by the ability of structurally organizing phytoelements according to in situ light environments.


**Introduction**

Estimating photosynthetic parameters using variable chlorophyll fluorescence techniques has become increasingly popular due to its ease of use and non-invasive nature. The basic fluorescence signals of 'open' or 'closed' reaction centers (F and Fm, respectively) change according to actinic irradiance and are powerful monitors of the status and activity of the photosynthetic apparatus (Baker, 2008). Most measurements of variable chlorophyll fluorescence in complex plant tissues, and in other surface-associated cell assemblages like biofilms and sediments, rely on external measurements with fiber-optic or imaging fluorimeters under the assumptions that i) different cells are subjected to the same amount of measuring light and actinic irradiance, ii) that saturating pulses are indeed saturating all cells, and iii) that the fluorescence detected is emitted equally from all sampled cells (Serodio, 2004). These assumptions are usually valid in optically dilute samples (Klughammer and Schreiber, 2015), whereas steep light gradients in densely pigmented tissues or algal biofilms will distort the measurements of maximal and effective PSII quantum yields. Cells located deeper inside tissues will receive less actinic irradiance than cells close to the surface. Thus, externally integrated measurements of variable chlorophyll fluorescence contain a complex mixture of signals originating from different layers in the structure exposed to different levels of measuring and actinic light, and the actual operational depth of such measurements remains unknown. This inherent limitation of such measurements can e.g. lead to light dependent overestimations of effective PSII quantum yields of up to 40% e.g. in microphytobenthic assemblages (Serodio, 2004).

Previous efforts to describe the internal gradients of photosynthetic efficiencies have used microfiber based pulse amplitude modulation (PAM) techniques (Schreiber et al., 1996) revealing distinct differences between such internal and external variable chlorophyll fluorescence measurements (Oguchi et al., 2011). Another challenge is to quantify the internal light gradients to qualify the actinic light exposure in different tissue layers. This can be done by applying fiber optic scalar irradiance microprobes (Rickelt et al., 2016), and such measurements enabled estimates of internal rates of PSII electron transport corrected for the specific tissue light gradients in corals and plants (Lichtenberg and Kühl, 2015; Lichtenberg et al., 2016). However, to obtain absolute electron transport rates (ETR) through PSII, it is necessary to know the absorption factor, which describes the PSII absorption cross section and the balance between PSI and PSII photochemistry, and these parameters cannot be calculated from measurements of light availability. In addition, due to the small tip size of fiber optic microprobes (usually <50 µm) used to detect the fluorescence, these measurements are also prone to reflect the natural heterogeneity of such systems (Lichtenberg and Kühl, 2015; Lichtenberg et al., 2016). A method was
recently proposed for calculating absolute electron turnover rates of PSII but was limited to surface measurements or optically thin systems, due to the measurement dimensions of the used methodology (Szabó et al., 2014). It is thus of great importance to further explore how steep gradients of light influence photosynthetic efficiencies in complex photosynthetic tissues and surface associated phototrophic communities.

Internal gradients of light absorption have been quantified from fluorescence profiles in terrestrial leaves (Takahashi et al., 1994; Vogelmann and Han, 2000) and this technique has been combined with fine scale measurements of CO₂ fixation to investigate the relationship between chlorophyll concentration, light absorption and photosynthesis at high spatial resolution (Vogelmann and Evans, 2002; Evans and Vogelmann, 2003). These studies generally found a good correlation between the light absorption of different spectral ranges, and the associated CO₂ fixation profiles. However, the CO₂ fixation rates relied on freeze clamping ¹⁴CO₂ pre-incubated leaf samples with concomitant paradermal sectioning, and measurements by scintillation counting, which is a laborious process that is e.g. prone to tissue compression during sectioning (Vogelmann and Evans, 2002).

The lower community photosynthesis often observed in aquatic systems as compared to terrestrial systems (Sand-Jensen and Krause-Jensen, 1997) can be largely explained by the inability of aquatic macrophytes to obtain an optimal 3D structural organization of the phytoelements in relation to the incident irradiance (Binzer and Sand-Jensen, 2002, 2002), unlike their terrestrial counterparts that e.g. can regulate leaf inclination to increase community light utilization (McMillen and McClendon, 1979; Myers et al., 1997). In addition, specialized cell/tissue structures in terrestrial plants can increase photon absorption, e.g. in sun adapted leaves that possess well-developed palisade cells that can act as light funnels directing light into the photosynthetically active mesophyll layer (Vogelmann and Martin, 1993), while some shade adapted understory plants have convex epidermal cells that can focus light and thus increase the limited available incident photons to the position of the chloroplasts (Vogelmann et al., 1996; Brodersen and Vogelmann, 2007). In contrast, most macroalgae are not recognized to have specialized tissue structures to facilitate penetration of light, although there has been reports of light guides in some green algae (Ramus, 1978).

Macroalgal members of the Fucales have morphological differentiated tissues such as the basal thallus, the growing sterile frond, and fertile receptacles. Previous studies have shown a longitudinal gradient in photosynthetic potential of tissue types, with a ~2 times higher photosynthetic carbon assimilation rate in the sterile apical growing thallus as compared to the basal and fertile thallus parts (Küppers and
Kremer, 1978). On the tissue scale, cells are differentiated into meristoderm, cortex, and medullary layers (Garbary and Kim, 2005). While all cell types contain plastids (Moss, 1983) the outer meristoderm and cortex cells contain more chloroplasts and thylakoids than the medullary filaments. It has been suggested that the medullary filaments could play a role in longitudinal translocation of materials (Moss, 1983; Raven, 2003), and further that they may play a structural role in providing elasticity in terms of a 'cushion-like' effect protecting against wave action (Moss, 1983). The medulla layer is surrounded on both sides by anatomically similar layers of cortex, meristoderm, and epidermis cells (henceforth referred to as cortex) in contrast to e.g. bifacial terrestrial plant leaves that display morphologically and physiologically differentiated abaxial- and adaxial domains. In *Fucus*, steep gradients of light have been measured using fiber-optic microprobes, although this approach is rather challenging in such cohesive tissues (Spilling et al., 2010; Lichtenberg and Kühl, 2015).

In this study, we used a novel multicolor laser light sheet microscopy setup to image the distribution of light absorption and photosynthetic activity over transverse sections of an aquatic macrophyte to resolve how photosynthetic efficiencies are affected by steep light gradients in different spectral regions. We describe for the first time how PSII quantum yields are affected by natural light gradients in optically dense tissues using a novel method that can resolve such gradients routinely compared to other laborious approaches such as mapping with fiber-optic probes (Kühl and Jørgensen, 1994; Lichtenberg et al., 2016).
Figure 1. Experimental setup for light sheet microscopy in combination with variable chlorophyll fluorescence imaging. A) The sample holder consisted of a cuvette cut down to 11 mm height (internal dimensions of 10 × 10 × 10 mm). The sample was mounted in agar in the bottom of the sample holder, which was filled with seawater and then closed with a coverslip. B) Spectral composition of the laser-light settings used for measurements of chlorophyll fluorescence profiles and the actinic light in measurement of variable chlorophyll fluorescence. The laser was adjusted to have the same absolute photon irradiance independent of spectral composition. C) Schematic drawing of the experimental setup for measuring chlorophyll fluorescence profiles. (a) The thallus sample positioned in the cuvette, (b) microscope objective, (c) filter cube with longpass filter, (d) CCD camera. Illumination of the sample was done with a PC-controlled super continuum laser connected to a spectral line filter unit and a laser sheet generator. D) Schematic drawing of the experimental setup for measuring variable chlorophyll fluorescence microscopy. (a) sample fixed in the cuvette, (b) microscope objective, (c) emission filter, (d) dichroic beam splitter cube, (e) dichroic filter, (f) mirror (to ocular), (g) CCD camera. Weak modulated measuring light was provided by a software-controlled RGB LED. Actinic light was provided perpendicular to one side of the thallus surface by a PC-controlled super continuum laser connected to a spectral line filter unit and a laser sheet generator.
Results

Cross-thallus chlorophyll fluorescence profiles

When illuminated homogeneously across the thallus cross section, both cortex layers displayed an equally high amount of chlorophyll that was 2.5-5 fold higher than in the central medulla (Fig. 2A and 3B), assuming that relative chlorophyll content can be estimated from fluorescence using epi-illumination (Vogelmann and Evans, 2002).

Figure 2. 3D surface plots showing distribution of Chl a fluorescence (normalized to max fluorescence) of A) a Fucus vesiculosus thallus cross section illuminated evenly over the cut side with 430 nm light from a Xe-lamp. The plot is composed of multiple images taken through a 10x objective, and stitched together using Adobe Photoshop. B, C, D, E) Thallus cross sections illuminated perpendicular to one side of the thallus surface with a super continuum laser sheet of different spectral compositions of B) blue (425-475 nm), C) green (525-575 nm), D) red (615-665 nm) and E) white (400-700 nm) light.

The fluorescence profiles under light sheet illumination perpendicular to one side of the cross section showed, that blue light (425-475 nm) was attenuated strongest in an exponential manner with depth and decreased to <21% of the maximum fluorescence (F_{max}) ~250 µm inside the thallus (Fig. 3D). Fluorescence profiles over the thallus cross section using green (525-575 nm), and red (615-665 nm) light showed similar attenuation but decreased to a minimum fluorescence >2 times higher than was found for blue light at a similar depth in the thallus. Blue, green and red light induced fluorescence profiles all displayed F_{max} values close to the thallus surface, while a different fluorescence profile was observed under broadband white light illumination (Fig. 3D). Here, a peak was located at the same position as the F_{max} of the blue, green and red profiles followed by a decrease and then an increase to another F_{max} peaking ~100 µm inside the thallus. Common for all profiles was that the fluorescence...
showed a peak close to the illuminated cortex followed by a decrease towards the center of the medulla before increasing again towards the shaded cortex. The relative largest increase towards the shaded thallus side was in the order of blue < red < green < white. The width of the peaks was of similar size and extended 150-200 µm from the surfaces towards the center of the thallus (Fig. 3).

**Figure 3. Cross sections of Chl a fluorescence.** A) Epifluorescence microscopy image (false colors) of a *F. vesiculosus* thallus cross section illuminated evenly with blue light (430 nm) from a Xe-lamp, and B) the associated fluorescence profile (normalized to max fluorescence). C) Example of a fluorescence image (false colors) of a thallus fragment irradiated perpendicularly to one side of the thallus surface (arrow) with a laser sheet of red light (615-665 nm) from a super continuum laser, and D) chlorophyll fluorescence profiles of cross sections of apical thallus fragments of *F. vesiculosus* irradiated perpendicularly to the thallus surface with different spectral bands of blue (425-475nm), green (525-575 nm), red (615-665) and white (400-700nm) light. Data was normalized to max fluorescence and actual data points are spaced 0.8 µm apart. (*n*=5. Error bars are not shown for clarity but mean relative S.D. was ±7.7%).

**Absorption profiles**

The integrating sphere measurements of thallus reflection, transmission and absorption displayed typical characteristics for densely pigmented opaque plant tissue (Fig. 4A). Reflection was relatively uniform at ~3% of the incident irradiance, although slightly higher in the green/yellow part of the spectrum (around
570 nm). The absorption spectra showed excitation peaks from major photopigments present in brown macroalgae, e.g., Chl a (absorption peaks at 430 and 672 nm), Chl c (absorption peaks at 460; 590; 635 nm (Shibata and Haxo, 1969; Kühl et al., 1995)), fucoxanthin (absorption peak at 425, 450, 475 nm; extends to 580 nm in vivo (Govindjee and Braun, 1974)) and carotenoids (430-470 nm). The mean absorption averaged over PAR (400-700 nm) using broadband white light was 92% of the incident irradiance. Transmission was highest (10-13%) in the green/yellow part (around 570 nm) of the spectrum and close to zero in the blue and red regions, while the mean transmission was 5% of the incident irradiance (Fig. 4A).

Figure 4. Reflection, transmission and absorption. A) Spectral measurements of reflection, transmission and absorption from a *F. vesiculosus* thallus fragment using an integrating sphere (see Fig. S1). Data was recorded using either incident blue (425-475 nm; blue lines), green (525-575 nm; green lines), red (615-665 nm; red lines) or white (400-700 nm; black lines) laser light. Dashed lines indicate ± 1 S.D.; n=3 B) Calculated absorption profiles of cross sections of apical thallus fragments of *F. vesiculosus* irradiated perpendicular to one side of the thallus surface with different spectral bands of blue (425-475 nm), green (525-575 nm), red (615-665 nm) and white (400-700 nm) light. Absorption was calculated from the measured chlorophyll fluorescence profiles (Fig. 3D) and normalized to the bulk absorption measured with an integrating sphere (A). The dashed lines indicate the borders of the cortex and medulla tissue layers. Data points are spaced 0.8 µm apart (n=5).

By normalizing the chlorophyll fluorescence profiles (Fig. 3D) to the total absorption measured for blue, green, red, and white light with an integrating sphere (Fig. 4A) we could calculate the depth specific photon absorption inside the thallus (see also Fig. S3). The different thallus regions (cortex/medulla) were estimated to be on average 150 µm in thickness (Fig. 3). When illuminating the thallus with the laser sheet, the apparent absorption of photons was always highest in the upper and lower cortex as compared to the medulla, where the fractional absorption was lowest (Fig. 4B; Table 1). We modelled the light availability in the *F. vesiculosus* thallus by using measured attenuation coefficients of cortex and
medulla layers from a closely related brown alga *F. serratus* (Lichtenberg and Kühl, 2015) assuming monoexponential attenuation of light in the thallus (Fig. 5; see text in Materials and Methods).

**Figure 5. Internal light availability.** Left panel shows plots of attenuation profiles of blue light (420-520 nm) calculated using attenuation coefficients from the cortex (blue triangles) and medulla (red circles) layers (Lichtenberg and Kühl, 2015), and a profile of the attenuation due to absorption of blue light (425-475 nm) estimated from the observed fluorescence profile (black squares). Right panel shows the natural logarithm transformed absorption profile with linear fits in the cortex and medulla layers ($R^2$>0.95 for all fits).

These modelled curves of light attenuation were compared with curves of attenuation due to absorption found in this study (Fig. 5) to test if the found absorption profiles were in the same order as the attenuation profiles, as would be expected for densely pigmented systems. We found an average light attenuation coefficient of 5.64 mm$^{-1}$ ($R^2$=0.97) over the entire thallus, with higher attenuation coefficients in the cortex layers (upper cortex = 6.0 mm$^{-1}$ ($R^2$=0.99); lower cortex = 9.8 mm$^{-1}$ ($R^2$=0.96)) than in the medulla layer (4.3 mm$^{-1}$ ($R^2$=0.99)) (Fig. 5). These values were in the same order as the light attenuation coefficients of cortex and medulla layers in *F. serratus* (6.8 mm$^{-1}$ and 3.4 mm$^{-1}$ for cortex and medulla, respectively (Lichtenberg and Kühl, 2015)), suggesting that the distribution of photon absorption can be found by combination of chlorophyll fluorescence profiles and measurements of total absorption.

**PSII quantum yields and photosynthetic electron transport**

In the dark-adapted state, all thallus layers displayed a maximal PSII quantum yield of >0.6 indicating no major stress factor on photosynthetic performance due to cutting or sample handing (Fig. S4). When applying actinic irradiance homogeneously over the cross sections via the build in LEDs of the
microscope PAM system, the effective PSII quantum yield decreased in all thallus layers but more so in the medulla as compared to the cortex layers. The highest decrease was found under high incident irradiance, where the effective PSII quantum yield in the medulla decreased to <0.3 (Fig. 7A). This pattern changed under actinic laser sheet illumination of the cross section from one side. While the PSII quantum yield distribution were apparently unaffected by the changed actinic light geometry in the dark adapted state and under very low irradiance, the PSII quantum yield decreased rapidly over the illuminated cortex and reached levels of <0.1 under the highest irradiance. Due to the strong light attenuation across the thallus, the PSII quantum yields in the medulla and the shaded cortex layers decreased less than when illuminated homogeneously via the Imaging PAM actinic light source, and the effective PSII quantum yield in the shaded cortex remained at levels similar to dark adapted states (>0.6) even at the highest irradiance (Fig. 7B).

Figure 6. Apparent electron transport rates through PSII corrected for absorbed photons (Fig. 4). Measurements were performed with 20 s acclimation (RLC) to increasing actinic irradiance of A) blue (425-475 nm), B) green (525-575 nm), C) red (615-665 nm) and D) white (400-700 nm) light provided by a laser sheet illuminating a thallus fragment perpendicular to one side of the thallus surface. Data point represents means ± S.E. (n=5 except panel A where n=4).
Figure 7. Isopleths and images of effective PSII quantum yield in apical thallus fragments of *F. vesiculosus* illuminated evenly on a cross section or perpendicular to one side of the thallus surface. Images were acquired under red light using either direct light from the build-in LEDs of the microscope PAM system (590-650 nm), or light perpendicular to the thallus surface provided by a super continuum laser (615-665 nm) connected to a tunable single line filter and delivered via a laser sheet generator. The isopleths (A, B) show the influence of actinic irradiance on the effective PSII quantum yield (in µmol electrons m$^{-2}$ s$^{-1}$ (µmol photons m$^{-2}$ s$^{-1}$)$^{-1}$) as function of depth in the tissue when illuminated either directly on the cross section (A) or perpendicular to the surface of the thallus (B). Illumination in B was given from left towards right of the panel. Line profiles (line width = 15 pixel) were taken on thallus parts with similar thickness (~250 µm) with cortex layers also displaying similar thicknesses (~50-75 µm). Panels C-H show images of effective PSII quantum yield in darkness, moderate (567 ± 18 µmol photons m$^{-2}$ s$^{-1}$) and, saturating irradiance (1087 ± 30 µmol photons m$^{-2}$ s$^{-1}$) under direct even illumination of the cross section (C-E) and with laser light sheet illumination perpendicular to the thallus surface (arrows) (F-H). Illumination in F-H was given from the bottom towards the top of the panels. Scalebar = 0.2 mm.
Apparent electron transport rates (ETR) through PSII were calculated for the illuminated (upper) cortex, the medulla, and the shaded (lower) cortex, and the rates were corrected for the amount of absorbed photons in the respective tissue layers. In all cases, the ETR rates in the upper- and lower cortex layers were very similar when corrected for absorbed light. The medulla ETR rates were similar to the cortex activity on the sub-saturated part of the ETR vs. irradiance curve but saturated at higher irradiance (Fig. 6). The slope of the ETR vs. absorbed light curve under blue light was lower than for red and white light but reached higher ETR rates (Fig. 6; Table 1). The curves appeared similar under green and red light although green light yielded a lower slope on the sub-saturated part of the ETR vs. absorption curve. The ETR curves measured under broadband white light appeared qualitatively as a combination of the curves measured under blue, green and red light, where saturation occurred at higher irradiance, similar to the green and red curves, but reaching higher ETR values probably caused by the blue light component. However, the slopes on the subsaturated part of the ETR vs. irradiance curves were significantly different between white light and the average of the blue, green and red curves in all thallus layers (Table 1). The green and red ETR vs. absorbed light curves were similar in appearance in correspondence with their associated absorption profiles that were also similar (Fig. 4B and Fig. 6B, C). Surprisingly, the ETR curves under blue light did not reach saturation and ETR rates in the cortex and medulla layers were very similar except at the highest irradiance, where a decrease in the medulla layer was observed (Fig. 6A). Even at the highest irradiance, PSII quantum yields in the cortex layers remained high, and only a small increase in the NPQ was observed (Fig. S4), which could point to better light handling properties of blue light compared green and red light.
Discussion

A novel combination of multi-color light sheet microscopy with variable chlorophyll fluorescence imaging enabled the mapping of light absorption and photosynthetic efficiencies in densely pigmented tissues. By combining well tested methods of integrating sphere measurements and the chlorophyll fluorescence profile technique (Takahashi et al., 1994; Vogelmann and Han, 2000) we propose a method for calculating profiles of photon absorption across plant tissue sections, which can be combined with variable chlorophyll fluorescence imaging of photosynthetic efficiency across tissue light gradients. Conversion of PSII quantum yields measured by variable chlorophyll fluorescence to absolute rates of photosynthetic electron transport activity requires precise measurements of i) mean effective PAR, ii) the PSII absorption cross-section, and iii) knowledge about the partitioning between PSI and PSII photochemistry (Klughammer and Schreiber, 2015). While such information can be obtained in dilute suspensions of chloroplasts and microalgae (Klughammer and Schreiber, 2015), to measure these parameters in dense algal solutions, plant tissue and algal biofilms is not trivial (Szabó et al., 2014; Klughammer and Schreiber, 2015). In optically dense systems, light gradients are affected by both multiple scattering and absorption and it is important to take diffuse light into account when quantifying actinic light levels, i.e., by measuring the incident photon flux from all directions with scalar irradiance microprobes (Kühl, 2005). While such sensors have tip diameters down to 30 µm (Rickelt et al., 2016), it is difficult to perform scalar irradiance measurements in thin, cohesive plant tissues, where measurements can be biased by tissue compression due to the physical impact of the microprobe (Spilling et al., 2010; Lichtenberg and Kühl, 2015). The mean effective PAR can also be calculated from complex measurements of the angular radiance distribution with field radiance microprobes (Vogelmann and Bijörn, 1984; Vogelmann et al., 1989; Kühl and Jørgensen, 1994). Alternatively, information on the cell-size distribution, and the inherent optical properties, i.e., the scattering phase function, and the scattering and absorption coefficients allow calculations of PAR gradients, but these parameters are difficult to determine in optically dense media (Privoznik et al., 1978; Berberoglu et al., 2009; Klughammer and Schreiber, 2015), albeit recent experimental and theoretical advances in biomedical optics have allowed detailed characterization of tissue optics using combinations of optical reflection spectroscopy, optical coherence tomography and Monte Carlo simulations (Wang et al., 1995; Wangpraseurt et al., 2016; Wangpraseurt et al., 2016).

However, an easy experimental solution to the abovementioned complications relies on measuring the chlorophyll fluorescence profile, which represents the net outcome of photon absorption along the actinic light gradient in the tissue (Takahashi et al., 1994; Vogelmann and Han, 2000). By correlating
fluorescence profiles to total absorption we could measure the direct result of absorption and the values obtained are thus only affected by the quantum yield of fluorescence and energy transfer between antenna pigment molecules and PSII and PSI. Thus, this method allows estimates of the distribution of photon absorption, although it does not allow to separate possible changes in the absorption cross section or balance between PSI and PSII absorption in different tissue layers.

**Table 1: ETR vs. photon absorption curve parameters and fractional photon absorption** (in % of total absorption) calculated for the upper cortex, medulla and lower cortex under blue (425-475 nm), green (525-575 nm), red (615-665 nm) and white (400-700 nm) irradiance applied perpendicular to one side of the thallus surface of *F. vesiculosus*. Slopes on the subsaturated part of the curve, maximum ETR values and the light acclimation index Ek were estimated from curve fitting of the ETR vs. photon absorption curves with an exponential function (Webb et al., 1974) using a non-linear Levenberg-Marquardt fitting algorithm. The RGB values were calculated as average curves of blue, green and red. Pairwise superscript letters indicate statistically significant differences (One-way ANOVA; p<0.01; n=5 for white; n=4 for RGB).

<table>
<thead>
<tr>
<th></th>
<th>Upper Cortex</th>
<th>Medulla</th>
<th>Lower Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Slope</strong></td>
<td>ETR&lt;sub&gt;max&lt;/sub&gt;</td>
<td>E&lt;sub&gt;k&lt;/sub&gt;</td>
<td>Abs (%)</td>
</tr>
<tr>
<td><strong>Red</strong></td>
<td>0.60</td>
<td>197.09</td>
<td>327.23</td>
</tr>
<tr>
<td><strong>Green</strong></td>
<td>0.47</td>
<td>225.31</td>
<td>482.46</td>
</tr>
<tr>
<td><strong>Blue</strong></td>
<td>0.54</td>
<td>878.52</td>
<td>1620.05</td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>378.40</td>
<td>629.79</td>
</tr>
<tr>
<td><strong>RGB</strong></td>
<td>0.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

We found total absorption values from integrating sphere measurements that were similar to terrestrial leaves (Gorton et al., 2010), although the absorption of green/yellow light was higher in *F. vesiculosus* due to the presence of accessory brown algal pigments such as fucoxanthin that displays a high efficiency of energy transfer to Chl a (70-80% *in vivo* (Govindjee and Braun, 1974)). Due to the low reflection and transmission in the thallus, the absorption will be in the same order as the light attenuation. Comparing, the calculated attenuation of light due to absorption with the calculated light attenuation using scalar irradiance attenuation coefficients from a previous study (Lichtenberg and Kühl (2015); see Materials and Methods) we found a whole-thallus absorption coefficient that was lower than cortex attenuation coefficients and higher than medulla attenuation coefficients (Fig. 5). We predicted identical absorption coefficients in the cortex layers since these layers are not anatomically different and in addition displayed similar levels of chlorophyll fluorescence under uniform epi-illumination. Surprisingly, the absorption coefficient of the shaded cortex was larger than the one found in the illuminated cortex (Fig. 5). However, our calculations were based on profiles of blue light, which was almost completely absorbed, making the calculations of absorption in the shaded cortex more prone to errors due to the
lower signal/noise ratio. Additionally, we used a long-pass filter (>670 nm) to detect fluorescence, and the resulting profiles therefore comprised both red- and far-red chlorophyll fluorescence. Due to self-absorption of red fluorescence by chlorophyll, red fluorescence profiles are likely to better represent absorption profiles than profiles of far-red fluorescence (Vogelmann and Han, 2000) and thus, absorption of red fluorescence in the shaded cortex could cause the apparently higher absorption coefficient. Future studies should therefore divide the detected fluorescence signals into red- and far-red fluorescence to avoid this possible overestimation. We also note that our laser sheet had a Gaussian beam profile which makes positioning close to the edge of the cut thallus surface difficult and may create a potential spill-over of photons onto the cut side. This limitation could be solved by shaping the beam e.g. by the generalized-phase-contrast method (Banas et al., 2014) to transform the Gaussian beam profile to a sharp rectangular shape and such work is now underway.

The shape of the white fluorescence profile across the thallus was slightly different in appearance than the profiles for blue, green and red light. Previously, it was shown that profiles of carbon assimilation and chlorophyll fluorescence profiles followed each other closely depending on the spectral quality (Sun et al., 1996; Vogelmann and Han, 2000) and it has been proposed that profiles of carbon fixation under white light can be described as the mean when using blue, green and red light (Sun et al., 1998; Vogelmann and Han, 2000). Here we show that for plant tissue harboring a range of accessory pigments such as e.g. fucoxanthin, the absorptive properties are more complex resulting in a different response to white light than what can be expected from the combination of measurements made under monochromatic light. Furthermore, it was shown that green light drives photosynthesis more efficiently deeper in terrestrial plant tissue than blue and red light due to a larger penetration depth of green light in leaf tissues (Sun et al., 1998; Terashima et al., 2009), but the presence of fucoxanthin and carotenes in Fucus caused the green light (525-575 nm) to be absorbed equally effective as red light. However, not all wavelengths were absorbed equally effective, and e.g. the spectral region around 570-605 nm displayed reduced absorption as compared to the rest of the PAR region. Since only the white light treatment covered this part of the spectrum, it is possible that illumination with broadband white light caused the differently shaped absorption profile. This could be confirmed by measuring additional chlorophyll fluorescence profiles using yellow light (e.g. 570-605 nm) to validate if this would result in fluorescence profiles with $F_{\text{max}}$ located deeper in the thallus, similar to fluorescence profiles in terrestrial leaves illuminated with green light (Vogelmann and Han, 2000).
Photosynthesis

We demonstrate that PSII quantum yields and derived apparent ETR across the thallus cross sections strongly differed between homogeneous actinic light illumination of the cross section and unidirectional actinic light illumination on one side of the thallus with a laser light sheet. Under low incident irradiance, the yields were very similar in all layers and between measurements. However, as incident light directly on the cut side increased, the yields decreased across the tissue, with the highest decreases found in the medulla (Fig. 7A, C-E). This was not the case under laser light sheet illumination perpendicular to one side of the thallus surface, where we found strongly reduced yields in the illuminated cortex, while yields in the shaded cortex were unaffected (Fig. 7B, F-H), even at the highest incident irradiance (1108 µmol photons m\(^{-2}\) s\(^{-1}\)). Thus, when applying actinic light directly on a cross section we show that it is possible to both underestimate and overestimate PSII quantum yields as compared to yields found under natural light gradients.

The differences in PSII quantum yield measured under illumination directly on the cross section or perpendicular to the side of the thallus surface indicate the difference between the photosynthetic potential under equal illumination and the realized photosynthesis under tissue light gradients. We show here that even under high incident irradiance, photosynthetic electron transport in the lower cortex was not saturated, and even illumination of tissue cross sections will therefore underestimate the PSII quantum yield as compared to shaded parts during high unidirectional illumination. Apparent ETR rates in the cortex layers were very similar when corrected for absorption, while the medulla layer displayed saturation and lower ETR rates at increasing irradiance indicating a lower photosynthetic capacity, probably due to lower pigment content (Fig. 3B; Fig. 6). The slopes of the initial part of the ETR curves, which is a measure of the light utilization efficiency at subsaturating photon flux, were similar in all thallus layers albeit consistently slightly lower in the medulla (Table 1). In a recent study, absolute ETR rates of thin-tissued corals were calculated (Szabó et al., 2014) and the rates found in this study were very similar to the rates found in corals, albeit slightly smaller. Szabo et al. (2014) also found initial slopes of the sub-saturated part of the ETR vs. irradiance curves that were slightly higher, probably reflecting differences in the photochemical adaptation that have been shown to be tightly linked to the optical properties of coral tissues (Wangpraseurt et al., 2016; Wangpraseurt et al., 2016). On a daily basis, the medulla layer of *Fucus* will on average experience the lowest photon irradiance compared to the cortex layers and could therefore be thought of as shade adapted compared to the cortex layers. Shade adapted phytoelements will normally display higher light use efficiencies but lower photosynthetic capacity due to increased pigment content and biochemical regulations in the photosynthetic machinery and/or
ultrastructural changes in the chloroplasts (Lichtenthaler et al., 1981; Lichtenthaler and Babani, 2004; Lichtenthaler et al., 2007; Sarijeva et al., 2007). However, as the initial slopes of the ETR vs. absorbed light curves in the medulla were both lower and displayed saturation at lower irradiances, medulla layers cannot be described as photosynthetically shade adapted in conventional terms. Conversely, it appears that the structural organisation of the thallus layers could be adapted to maximize photon absorption in the outer cortex layers, while having a relatively translucent central medulla with low absorptive properties. This is in contrast to terrestrial leaves, where even illumination of tissue layers is achieved by increased internal scattering due to intercellular airspaces with the concomitant absorption profiles following an exponential attenuation with depth (Vogelmann and Han, 2000). This fundamental difference is in good agreement when considering their respective position in terrestrial and aquatic habitats, as terrestrial leaves can organize their position according to the angle of solar radiation, whereas aquatic macrophytes are limited in their structural organization by strong drag and shearing forces imposed by waves and currents, randomly exposing both sides of the thallus to direct light. By maximizing absorption in the outer layers and having a translucent central layer, *F. vesiculosus* can thus maximize light harvesting by allowing photons not absorbed in the illuminated thallus to propagate to tissue layers with unused photosynthetic potential thereby ensuring a more efficient resource distribution.

The ETR vs. absorbed light curves measured in different tissue layers under laser light sheet illumination were similar to what was previously found in *Fucus* (Lichtenberg and Kühl, 2015) although these microfiber PAM-based measurements were lower and associated with high standard deviations due to the small measurement volume of the fiber-optic microprobe which makes such measurements prone to microscale tissue heterogeneity effects. With our new approach, it is now possible to integrate photosynthetic responses from specific tissue layers much more precisely, and in addition to correct them for the amount of photons absorbed by that given tissue layer. Here we used a 10x microscope objective, but in principle such measurements could be performed at even higher magnification e.g. to investigate single cell gradients of light and photosynthetic efficiencies e.g. in large algal cells. The combination of multi-color light sheet microscopy with variable chlorophyll fluorescence imaging on plant tissue cross-sections provides an alternative to more destructive methods such as constructing profiles of CO₂ fixation from paradermal sectioning (Evans and Vogelmann, 2003) or nanoscale secondary-ion-mass-spectroscopy (Kilburn et al., 2010; Wangpraseurt et al., 2016) and allows sequential measurements on the same sample e.g. under different levels of actinic irradiance or comparisons of diffuse vs. collimated light fields (Brodersen et al., 2008). Here we demonstrated the application on
aquatic macrophyte tissue but the technique is readily applicable to many other types of plant tissues including terrestrial leaves.

**Conclusion**

The combination of multi-color light sheet microscopy and variable chlorophyll fluorescence imaging is a powerful technique that enables fine scale characterization of light absorption and photosynthesis across plant tissue sections under experimental light gradients. Hitherto, variable chlorophyll fluorescence imaging of plant tissue has relied on even illumination of samples with actinic light, which is not representative of the natural internal light fields in illuminated plants. Furthermore, the quantification of photon absorption from light sheet induced cross-tissue fluorescence profiles can be used in concert with variable chlorophyll fluorescence imaging enabling calculations of ETR rates that otherwise require knowledge of the absorption cross-section, and the mean effective PAR.

The spectral flexibility of the white super continuum laser source allows the use of this method in literally any photosynthetic system with different photo-pigmentation. In this manner e.g. the role of specific accessory pigments in light propagation and photosynthesis can be investigated.

**Materials and Methods**

**Sample collection and preparation**

Stands of the macroalgae *Fucus vesiculosus* were collected in the littoral zone at various locations around Helsingør, Denmark during late summer and were maintained in 10 liter buckets continuously flushed with 0.2 µm filtered aerated seawater (temperature=16°C; salinity=32) for up to one week prior to experiments. Samples were kept under a 12:12 h light:dark cycle under a photon irradiance of ~50 µmol photons m⁻² s⁻¹ (PAR, 400-700 nm) as provided by a fluorescent tube (Philips Master TL-D90, 18W; Philips, Amsterdam, the Netherlands).

Prior to measurements, an apical thallus fragment was cut ~1 cm from the thallus tip with a razor blade, and the cut side was rinsed in seawater with a transfer pipette to wash away pigments leaking from cut chloroplasts. The sample holder (Fig. 1A) consisted of a standard plastic cuvette, cut down to a height of 11 mm to allow insertion under the microscope (internal size = 10 x 10 x 10 mm). To fix the sample in the cuvette, ~300 µL of 20 g L⁻¹ agar (Sigma-Aldrich) was transferred to the cuvette and allowed to cool to 20°C after which a slit was cut parallel to the cuvette window to allow insertion of the thallus fragment. The thallus was inserted flush with the edge of the cuvette, filled with seawater (16°C; salinity=32) and closed with a coverslip.
Profiles of absorbed light as estimated from chlorophyll fluorescence profiles across tissue sections were imaged with a customized microscope setup (Fig. 1C). The sample cuvette (see above) was mounted on an inverted microscope (IX81; Olympus, Japan) with a 10x objective (UPlanSApo 10x/0.40; Olympus, Japan). The sample was illuminated perpendicular to one side of the thallus surface by a supercontinuum laser (SuperK Extreme, EX-B, NKT Photonics, Denmark). The laser was connected to a tunable single line filter module (SuperK Varia, NKT Photonics, Denmark) via a single mode fiber with a collimated output. The tunable single line filter could be tuned from 400-840 nm to produce bandwidths from 1-400nm. Light from the single line filter module was delivered, via an alignment tool (SuperK Connect, NKT Photonics, Denmark), to an endlessly-single-mode-large-mode-area photonic crystal fiber (FD7, NKT Photonics, Denmark) with a collimated output. The collimated output was connected to a cylindrical laser sheet generator (NKT Photonics, Denmark) with a 14° “light-sheet half angle” yielding a 5 cm longitudinal line at 10 cm distance. The generated laser sheet had a Gaussian beam profile of ~1 mm on the latitudinal axis. The output laser optics was mounted on a manual micromanipulator (MM33; Märzhäuser, Wetzlar, Germany) that allowed easy positioning of the laser sheet in the focal plane of the microscope.

The sample was positioned with the cut side facing the microscope objective and the laser sheet was adjusted to hit as close to the edge of the cut as possible without illuminating the cut side itself. After positioning, the sample was allowed to dark adapt for 15 min.

Images of chlorophyll fluorescence from the cross-section were taken with a sensitive charge-coupled device (CCD) camera (iXon, Andor, UK) using a fixed exposure time of 70 ms. Chlorophyll fluorescence was detected by placing a ultra-steep longpass edge filter (BLP01-664R, Semrock, USA) in the light path between objective and the camera with a transmission close to 100% at wavelengths >670nm and close to 0% at wavelengths <670nm. Illumination in different spectral bands was achieved by control of the laser and the spectral filtering module with the manufacturers software (NKTP Control, NKT Photonics, Denmark). Four different spectral illumination bands were composed (Fig. 1B) and adjusted to the same photon irradiance (1190.3 ± 1.9 µmol photons m⁻² s⁻¹) as measured with a micro quantum sensor (MC-MQS, Walz GmbH, Germany) connected to a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany).

Illumination of the sample during individual image acquisitions was limited to <2 s per image, and the spectral sequence was randomized between replicates. Images were analyzed in ImageJ (v. 1.50B), where
grey values were extracted either by the line profile tool or by extracting all grey values. The images had a spatial resolution of 0.8x0.8 µm pixel⁻¹. Maximum chlorophyll fluorescence was normalized to 1 in all images to allow comparison between images. Plots were made in OriginPro (v. 9.3, Origin Lab, MA, USA). The thickness of the thallus varied, both between samples and depending on location in the cross section (Fig. 2A). Therefore, profiles of fluorescence were taken at thallus thicknesses of ~450 µm to allow comparison of thallus light gradients over the same tissue thickness.

**Integrating sphere measurements of reflectance and transmittance**

The thallus reflectance and transmittance was measured using an integrating sphere (diameter = 10 cm; port diameters = 2.5 cm, Labsphere Instruments Inc., USA). The sphere had 3 port openings; two located opposite each other and one orthogonal to the two other openings (Fig. S1). The incident light from the supercontinuum laser was tuned to different spectral ranges each with the same photon irradiance (see text above and Fig. 1B). Light was measured with a calibrated spectral irradiance meter (MSC15, Gigahertz Optik GmbH, Germany) connected to the orthogonally located port on the integrating sphere. For transmittance measurements, a thallus fragment was mounted in front of the entrance port between the light source and the integrating sphere and the port opposite to the entrance was covered with a white reflecting plate. For reflectance measurements, a thallus fragment was placed in the port opening opposite to the incident laser beam at an angle of 5-10° to capture both the specular and diffuse reflection. Total absorption (A) by the thallus was estimated as:

\[
A = \int_{\lambda_i}^{\lambda_f} \frac{(I - R - T)}{I} d\lambda
\]

where I is the incident photon irradiance, R is the reflection and T is the transmission, all integrated over the spectral region of interest [\(\lambda_i - \lambda_f\)].

**Modelling of light attenuation**

Light attenuation profiles were modelled using attenuation coefficients, \(\alpha\), of blue irradiance (420-520 nm) in the cortex and medulla layers measured in a previous study (Lichtenberg and Kühl, 2015). The model assumed monoexponential attenuation of incident irradiance, \(I_0\), over tissue depth intervals \(\Delta z\) and the light availability in different tissue depths was then calculated as:
These data were compared to the estimated attenuation, due to absorption quantified as induced fluorescence and corrected for total absorption (Fig. S3) across the thallus under blue irradiance (425-475 nm) in this study. The attenuation due to absorption was calculated by subtracting the cumulative absorption (Fig. 4B), integrated in 50 µm increments, from the incident irradiance.

**Variable chlorophyll fluorescence imaging**

Pulse-amplitude-modulated variable chlorophyll fluorescence imaging (Imaging-PAM) with the saturation pulse method (Schreiber et al., 1995; Schreiber, 2004; Kühl and Polerecky, 2008) was used to assess the photosynthetic performance over cross-sections of the thallus mounted in cuvettes as described above. Measurements were performed with a microscope Imaging-PAM system (Fig. 1D) fitted with a Red-Green-Blue (RGB) LED excitation lamp (IMAG-RGB; Heinz Walz GmbH, Effeltrich Germany) as described in detail elsewhere (Trampe et al., 2011). The microscope was fitted with a high numerical aperture objective (10x/0.8, Plan-Apochromate, Carl Zeiss GmbH, Germany). Fast measurements of the effective PSII quantum yield under increasing photon irradiance (each irradiance step was applied for 20 s) were used to measure so-called rapid light curves (RLC) (White and Critchley, 1999) of relative PSII electron transport vs. irradiance curves. Two different approaches were applied: i) Using increasing actinic irradiances of red light (590-650nm) as provided by the system-default internal RGB-LED lamp for equal excitation of the exposed cross-section of the thallus from above, in combination with the customizable automated light curve function of the software provided by the system software (Imaging Win, Heinz Walz GmbH, Germany); ii) Using the supercontinuum laser setup as described above as an external actinic light source, illuminating the thallus with a light sheet perpendicular to the thallus surface. The actinic light sheet was controlled manually in stepwise increments in sync with the custom defined automated light curve function of the ImaginWin software facilitating a semi-automated acquisition of RLC’s, while the system-default internal actinic RGB-LED light source was disconnected. RLC’s with the laser light sheet were obtained with increasing actinic irradiance of blue (425-475 nm), green (525-575 nm), red (615-665 nm) or white (400-700 nm) light, respectively. Non-actinic modulated blue measuring light was provided by the build-in LEDs of the ImagingPAM system during both approaches. The two setups were calibrated using a light-meter connected to a cosine corrected mini quantum PAR sensor, (ULM-500, MQS-B, Walz GmbH, Germany). All samples were allowed to dark adapt for 15 min before
the measurements started. When in the dark-adapted state, all reactions centers of PSII were open, enabling imaging of the minimal fluorescence yield (F₀). Upon exposure to a high-intensity saturation pulse, all PSII reaction centers closed permitting imaging of the maximal fluorescence yield (Fₘ) over the thallus cross section. From these images, the maximum PSII quantum yield could be calculated as in Schreiber (2004):

\[
\frac{F_V}{F_m} = \frac{(F_m - F_0)}{F_m}
\]

From imaging of the fluorescence yield, F, while the sample was illuminated with a predefined level of actinic light (PAR, in µmol photons m⁻² s⁻¹), and the maximum fluorescence yield during a saturation pulse, Fₘ′, images of the effective PSII quantum yield could be calculated as:

\[
\Phi_{PSII} = \frac{(F_m' - F)}{F_m'}
\]

From these values, the relative photosynthetic electron transport rate (rETR), is usually derived using the equation:

\[
rETR = \Phi_{PSII} \times PAR \times AF
\]

where PAR is the incident photon irradiance, and AF (the absorption factor) is a constant set to 0.42 assuming that 84% of the incident light is absorbed (Demmig and Björkman, 1987) and an even charge separation between photosystem II and I (Schreiber et al., 2012). In our study, we estimated internal ETR rates (in units of µmol electrons m⁻² s⁻¹) in different regions of the thallus (cortex/medulla) by multiplying average \( \Phi_{PSII} \) values (in units of µmol electrons m⁻² s⁻¹ (µmol photons m⁻² s⁻¹)⁻¹) for a given area with the total amount of absorbed photons (in units of µmol photons m⁻² s⁻¹) in that area. The wavelength dependent absorption was calculated by normalizing the chlorophyll fluorescence profiles to the total absorption (Fig. S3). This essentially gives an estimate of the wavelength dependent photon absorption at any given depth in the thallus, assuming that one unit of fluorescence was the direct result of one unit of absorption. In reality however, this is affected by factors such as the quantum yield of fluorescence and energy transfer between antenna pigment molecules and PSII/PSI. The true photon absorption will therefore vary slightly, and the calculated photon absorptions are thus only estimates.
The light saturation coefficient, i.e., the photon irradiance at onset of light saturation of photosynthesis, $E_k$ was calculated as $E_k = \frac{ETR_{max}}{\alpha}$, where $ETR_{max}$ is the maximum activity, and $\alpha$ is the initial slope of the ETR vs. photon absorption curve; both parameters were obtained by curve fitting of ETR measurements using an exponential function (Webb et al., 1974) by means of a non-linear Levenberg-Marquardt fitting algorithm (OriginPro 2015, OriginLab Corporation, Northampton, MA, USA).

Statistics

One-way ANOVAs were applied to test differences in the slopes of the ETR curves between different light treatments. Data was first tested for normality (Shapiro-Wilk) and for equal variance. Statistical analysis was performed in SigmaPlot (SigmaPlot v. 12.5) and significance level was set to $p<0.01$.

Acknowledgements

This study was supported by a Sapere-Aude Advanced grant from the Danish Council for Independent Research | Natural Sciences (MK). We thank NKT Photonics, Denmark, for generous loan of the Supercontinuum Laser and for excellent technical support.
References


Lichtenberg M, Larkum AWD, Kühl M (2016) Photosynthetic acclimation of Symbiodinium in hospite depends on vertical position in the tissue of the scleractinian coral Montastrea curta. Frontiers in Microbiology 7: 230


273


Supplementary information

**Integrating sphere measurements**

Light was provided by a supercontinuum laser (EX-B SuperK Extreme, NKT Photonics, Denmark) tuned by a single line filter unit (Super K Varia, NKT Photonics, Denmark) to defined spectra of blue (425-475 nm), green (525-575 nm), red (615-665 nm) or white (400-700 nm) light. Transmission (Fig. S1, left) was measured with an apical thallus fragment of *Fucus vesiculosus* placed in the port opening in front of the integrating sphere, while the port opening in the back was covered with a white reflecting cover plate. Reflection (Fig. S1, right) was measured by placing an apical thallus fragment in the port opening on the back of the integrating sphere at a 5-10° angle to capture both the specular and diffuse reflection. Transmitted and reflected light was detected by a spectral light meter (MSC15; Gigahertz Optik Gmbh, Germany) connected to the port located orthogonally to the other port-openings. Incident light was measured, without the sample, by covering the port opening in the back with a white reflecting cover plate.

![Schematic drawing of integrating sphere measurements of transmission (left) and reflection (right).](image)

**Figure S1.** Schematic drawing of integrating sphere measurements of transmission (left) and reflection (right).

**Fluorescence profiles of DCMU treated thallus**

We measured chlorophyll fluorescence profiles across thallus cross sections incubated in 10 µM DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethyl-urea; Sigma-Aldrich) for 1 h. DCMU is a specific PSII electron transport inhibitor and should therefore produce relatively stable fluorescence profiles with irradiation time (Vogelmann & Han, 2000). The fluorescence profiles of the DCMU treated thallus followed the shape of the untreated thallus but were slightly higher (Fig. S2). Assuming tissue layers of 150 µm
thickness (Fig. 3), the effect was largest in the medulla where the relative fluorescence of the DCMU treated thallus was 25.5 ± 3.6 % higher than in the untreated thallus while, the fluorescence in the upper cortex was 9.3 ± 3.7 % higher and 13.8 ± 2.5 % higher in the lower cortex. The fluorescence profiles of the untreated thallus could thus have been somewhat affected by photosynthetic electron transport and therefore not solely a product of the actinic light and chlorophyll concentration (Vogelmann & Han, 2000). This could be a result of the experimental procedure, where the variable chlorophyll fluorescence measurements were carried out before the measurements of fluorescence profiles, and even though a 30 min dark period was given between the two measurement series this might have affected the profiles.

Absorption profile calculations
Absorption profiles were calculated from measured fluorescence profiles and integrating sphere measurements of whole thallus absorption (Fig. S3). First, grey values of fluorescence were normalized to the maximal fluorescence, Fmax, to get the distribution of relative fluorescence over the thallus cross section. By measuring the whole-thallus absorption, we could then normalize the fluorescence distribution to the absolute absorption such that the integrated area under the curve (Fig. S3D) was equal to the total thallus absorption.
Effective quantum yield and non-photochemical quenching

Apical thallus fragments of *Fucus vesiculosus* were illuminated perpendicular to one side of the thallus surface with different colors of increasing actinic irradiance (see main text) and variable chlorophyll fluorescence parameters were monitored (Fig. S4). The curves were corrected for the depth specific photon absorption, calculated as above (Fig. S3). Yields generally decreased with increasing irradiance with concomitant increases in the NPQ. In all cases, the yield of the medulla was lower and the NPQ was higher than in the cortex layers when corrected for absorption. This points to a lower
photosynthetic potential of the medulla probably due to the much lower pigment content (Fig. 3 main text).

**Figure S4.** Quantum yield of PSII (Y(II)) and NPQ curves of the upper cortex, medulla and lower cortex of *Fucus vesiculosus* irradiated perpendicular to one side of the thallus surface with blue (425-475 nm) green (525-575 nm), red (615-665 nm) or white (400-700 nm) light as provided by a supercontinuum laser connected to a tunable single line filter unit. See main-text for measurement details.

**References**

Chapter 11

Nanoparticle-based measurements of pH and O₂ dynamics in the rhizosphere of Zostera marina L.: Effects of temperature elevation and light-dark transitions

Published in Plant, Cell and Environment

by

Kasper Elgetti Brodersen, Klaus Koren, Mads Lichtenberg and Michael Kühl
Nanoparticle-based measurements of pH and O$_2$ dynamics in the rhizosphere of *Zostera marina* L.: Effects of temperature elevation and light-dark transitions

Kasper Elgetti Brodersen$^{1,2}$, Klaus Koren$^{2,3}$, Mads Lichtenberg$^2$ and Michael Kühl$^{1,2}$

$^1$Plant Functional Biology and Climate Change Cluster, University of Technology, Sydney, Australia
$^2$Marine Biological Section, University of Copenhagen, Denmark
$^3$These authors contributed equally to this work

Abstract

Seagrasses can modulate the geochemical conditions in their immediate rhizosphere through the release of chemical compounds from their below-ground tissue. This is a vital chemical defense mechanism, whereby the plants detoxify the surrounding sediment.

Using novel nanoparticle-based optical O$_2$ and pH sensors incorporated in reduced and transparent artificial sediment, we investigated the spatio-temporal dynamics of pH and O$_2$ within the entire rhizosphere of *Zostera marina* L. during experimental manipulations of light and temperature. We combined such measurements with O$_2$ microsensor measurements of the photosynthetic productivity and respiration of seagrass leaves.

We found pronounced pH and O$_2$ microheterogeneity within the immediate rhizosphere of *Z. marina*, with higher below-ground tissue oxidation capability and rhizoplane pH levels during both light exposure of the leaf canopy and elevated temperature, where the temperature-mediated stimuli of biogeochemical processes seemed to predominate. Low rhizosphere pH microenvironments appeared to correlate with plant-derived oxic microzones stimulating local sulphide oxidation and thus driving local proton generation, although the rhizoplane pH levels generally where much higher than the bulk sediment pH.

Our data show that *Z. marina* can actively alter its rhizosphere pH microenvironment alleviating the local H$_2$S toxicity and enhancing nutrient availability in the adjacent sediment via geochemical speciation shift.
Introduction

To accommodate growth in often highly reduced, sulphidic sediment environments, seagrasses possess aerenchymal tissue composed of a system of interconnected gas channels facilitating rapid transport of $O_2$ from the seagrass leaves to the below-ground tissue (Larkum et al. 1989; McComb et al. 1999). Aerenchymal $O_2$ supply supports aerobic metabolism at the root apical meristems, and also facilitates radial $O_2$ loss (ROL) to the immediate rhizosphere from the basal meristems with leaf sheath, rhizome and the root apical meristems (Pedersen et al. 1998 and 1999; Jensen et al. 2005; Frederiksen & Glud, 2006; Brodersen et al. 2014, 2015a; Koren et al. 2015). The below-ground ROL drives local chemical oxidation of the surrounding sediment in plant-derived oxic microniches, wherein new actively growing roots can form and reach maturity with protective barriers to ROL and sulphide intrusion (Barnabas, 1996; Enstone et al. 2003; Brodersen et al. 2014, 2015a). Most of these barriers to ROL are induced by anoxic, sulphidic conditions (Armstrong & Armstrong, 2001 and 2005) and inhibit gas-exchange over most of the root surface area ensuring an efficient internal gas transport to the apical parts of growing roots (Colmer, 2003).

Seagrasses can thus actively alter their rhizosphere microenvironment through the release of $O_2$ from their below-ground tissue, thereby enhancing the redox potential of the immediate rhizosphere and stimulating re-oxidation of sediment-produced reduced phytotoxins, such as $H_2S$ (Lamers et al. 2013; Brodersen et al. 2014, 2015a). The oxidation capacity of the below-ground tissue is determined by numerous $O_2$ sources and sinks (Borum et al. 2006), where the most important regulating parameters include the $O_2$ conditions in the water column during night-time as the plants are completely dependent on passive diffusion of $O_2$ into their leaves when photosynthesis ceases ($O_2$ source) (Greve et al. 2003; Pedersen et al. 2004; Borum et al. 2005; Frederiksen & Glud, 2006; Brodersen et al. 2015a), the light availability and quality during day-time strongly regulating rates of shoot photosynthesis ($O_2$ source) (Brodersen et al. 2015a,b), ambient water temperature affecting plant and sediment respiratory needs and reaction kinetics (mainly regulating the $O_2$ sinks, but also affects rates of leaf photosynthesis) (Raun & Borum, 2013), as well as the thickness of the seagrass leaf diffusive boundary layer impeding gas and nutrient exchange with the surrounding water-column and thereby the water flow (thus negatively affecting the $O_2$ source) (Binzer et al. 2005; Brodersen et al. 2015b).

Recently, Brodersen et al. (2015a) showed that the seagrass *Zostera muelleri* subsp. *capricorni* can modulate the pH microenvironment in its immediate rhizosphere, further alleviating the risk of $H_2S$ intrusion through local sediment pH enhancements. This chemical defense mechanism, whereby pH
enhancement changes the sulphide speciation in the rhizoplane towards non-permeable HS- ions, is still poorly understood and there is therefore a need to elucidate the sediment pH microheterogeneity on a whole rhizosphere-scale.

Possible mechanisms behind such pH changes in the immediate rhizosphere are plant-derived allelochemicals. Rhizome/root exudation of organic carbon to the rhizosphere, as a result of internal carbon translocation, leads to enhanced bacterial productivity and growth in the seagrass rhizosphere (Moriarty et al. 1986). Rates of sulphate reduction have been coupled to plant photosynthesis and below-ground biomass (Pollard & Moriarty, 1991; Blaabjerg et al. 1998; Blaabjerg & Finster, 1998; Hansen et al. 2000; Nielsen et al. 2001) and young seagrass roots have also been found to stimulate the growth of epsilon- and gamma-proteobacteria that can utilize O2 and nitrate as electron acceptors to re-oxidize sulphide (Jensen et al. 2007). Interestingly, the younger plant structures often leak O2 from around the root-cap, where the presence of sulphide oxidizers overlaps with the plant-derived oxygenated microniches (Jensen et al. 2005; Frederiksen & Glud, 2006; Brodersen et al. 2014).

The root-shoot junctions (including the basal leaf meristem) and the root apical meristems (Moriarty et al. 1986) have been suggested as sites of exudation, with rhizome/root organic carbon exudation amounting up to 18% of the total carbon fixed by the seagrass host (Hansen et al. 2000). The highest sulphate reduction rates in the seagrass rhizosphere have correspondingly been observed at the seagrasses rhizomes and roots, where, for example, Pollard and Moriarty (1991) found 6 times higher sulphate reduction rates in seagrass-vegetated sediment as compared to non-vegetated areas. Sulphate reducing bacteria associated with the below-ground tissue of seagrasses show high O2 tolerance (Blaabjerg & Finster, 1998), and several studies have shown that increasing temperature and light exposure of the seagrass leaf canopy has a pronounced positive impact on the rhizosphere sulphate reduction rate (Isaksen & Jørgensen, 1994; Isaksen & Finster, 1996; Blaabjerg et al. 1998). Sulphate reduction can have a positive impact on the availability of phosphate in marine sediment owing to its reducing properties (Pollard & Moriarty, 1991), adding to the growing evidence of a specific relationship between the seagrass host and sulphate reducing bacteria based on a reciprocal exchange of nutrients (Moriarty et al. 1986; Blaabjerg et al. 1998; Hansen et al. 2000; Nielsen et al. 2001).

The consumption or production of protons as a result of microbial metabolisms and/or plant-derived allelochemicals plays an important role in the determination of sediment pH (Srinivasan & Mahadevan, 2010; Brodersen et al. 2015a). Such sediment pH alterations can influence the chemical speciation and availability of vital nutrients (e.g. ammonium and phosphate) at the plant/sediment interfaces (Pollard & Moriarty, 1991; Pagès et al. 2011, 2012; Brodersen et al. 2015a). Yet the understanding of rhizosphere
pH dynamics in seagrasses is underexplored and data on pH microheterogeneity at plant/sediment interfaces are lacking. In present study, we used novel O$_2$ and pH sensitive optical nanosensors incorporated in artificial, transparent sediment to investigate the pH and O$_2$ microdynamics in the rhizosphere of *Zostera marina* L. during light/dark transitions and temperature elevations. Our results provide new insights into the pH microheterogeneity and O$_2$ distribution in the *Zostera marina* L. rhizosphere during changing environmental conditions. We discuss how such pH and O$_2$ microgradients may alter the geochemical speciation of vital chemical species at plant/sediment- and oxic/anoxic interfaces.

**Materials and Methods**

**Seagrass sampling**

*Zostera marina* L. specimens were collected in shallow waters (less than 2 m depth) near Rungsted Harbour, Denmark and were transported in seawater from the sampling site to the laboratory within 1h of sampling. The collected seagrass specimens were transplanted into sieved sediment from the sampling site to exclude burrowing animals from the holding tank. Specimens were held in a 30 L aquarium continuously flushed with aerated seawater (5 L h$^{-1}$; salinity of 34‰; temperature of ~12°C) under a 14:10 h light/dark cycle. Illumination with a photon irradiance (400-700 nm) of ~200 µmol photons m$^{-2}$ s$^{-1}$ was provided by a combination of fluorescent and halogen lamps. Prior to experiments, selected plants were gently washed free of any adhering sediment particles and rhizome ends were carefully sealed with petroleum jelly to avoid gas leakage from damaged older rhizome parts, before placement in the custom-made, narrow split flow chamber (described below; Fig. 1). Relative small *Z. marina* specimens were used owing to the chamber dimension restrictions.

**Experimental setup and artificial, transparent sediment**

The applied experimental chamber consisted of a custom-made narrow, transparent acrylic split flow chamber attached to the side of a 30 L aquarium (inner dimensions 1×13×12 cm; Fig. 1). The split flow chamber was divided into an upper and lower compartment by means of an acrylic wall with numerous holes (inner diameter of ~1 mm) and was equipped with a removable front window for ease of access when casting the sediment and positioning the seagrass. A seagrass specimen was positioned in the upper compartment with the above-ground tissue in the free-flowing seawater phase and the below-ground tissue embedded in reduced, artificial sediment (Fig. 1).
The artificial, transparent sediment with embedded nanosensors was designed to mimic chemical settings in natural marine sediment (Brodersen et al. 2014), while enabling direct visual assessment of the below-ground tissue during measurements (Fig. 1; further described in Koren et al. 2015). The transparent artificial sediment consisted of a deoxygenated ~0.5% (w/v) agar-seawater gel, buffered with HEPES (final concentration of 10 mM; pH ~7), amended with O$_2$ or pH sensitive nanoparticles (~3 and 7 % v/v, respectively) and Na$_2$S*9H$_2$O to a final H$_2$S concentration of 500 µM (at pH 7). The agar powder was pre-washed in continuously stirred cold seawater to improve clarity. The lower compartment of the split flow chamber contained a highly sulphidic (final H$_2$S concentration of 2500 µM) deoxygenated ~0.5% (w/v) agar-seawater solution buffered with HEPES (10 mM), ensuring a continuous supply of H$_2$S to the above artificial sediment with nanosensors during experiments, thereby

**Figure 1:** A) Schematic diagram of the experimental setup, showing the custom-made aquarium equipped with the narrow split flow-chamber and the ratiometric bio-imaging camera system. B) Image of the below-ground plant tissue structure during O$_2$ measurements. C) Image visualizing the below-ground plant tissue structure during pH measurements. Note that the difference in brightness seen on the structural images (B, C) is due to the specific long pass filters used for luminescence imaging.
maintaining a constantly high $O_2$ demand in the sediment (Brodersen et al. 2014, 2015a). After positioning of the plant and casting the sediments, the chamber was sealed and placed in front of the imaging system (described below).

Illumination of the leaf canopy was provided by a fibre-optic tungsten halogen lamp (KL-2500; Schott GmbH, Mainz, Germany) equipped with a collimating lens. The incident photon irradiance (PAR, 400-700 nm) at the level of the seagrass leaf canopy was measured with a calibrated irradiance sensor (Walz GmbH, Effeltrich, Germany) connected to a quantum irradiance meter (LI-250; LiCor, Lincoln, NE, USA).

A constant flow of seawater (salinity of 34‰) was maintained in the water-column of the upper flow chamber compartment via a connected pump submerged in an aerated and temperature-controlled seawater tank. The below-ground pH and $O_2$ microenvironment within the *Zostera marina* L. rhizosphere was investigated during light/dark transitions (incident photon irradiance of 500 µmol photons m$^{-2}$ s$^{-1}$) and at two different experimental temperatures (~16 and 24°C). Plants were acclimatized to the experimental conditions for a minimum of 4h prior to start of measurements to ensure steady-state biogeochemical conditions in the rhizosphere (as confirmed from repetitive image recordings). Temperature changes were induced by slowly increasing the temperature of the seawater reservoir for ~3h until the desired temperature was reached and the plants were then allowed to acclimatize to the experimental temperature and irradiance for another 4h before image recordings commenced.

**Optical nanoparticle-based sensors**

The optical nanoparticle-based pH sensors were prepared based on a modified literature method (Xie et al. 2013; Wang et al. 2012). Briefly, 1 mg of perylene (Sigma-Aldrich), 1 mg of lipophilic indicator 1-hydroxypyrene-3,6,8-tris(2-ethylhexyl)sulfonamide (lipo-HPTS) (generously provided by Dr. Sergey Borisov TU Graz; Borisov et al. 2009) and 100 mg of the triblock copolymer Pluronic® F-127 (Sigma-Aldrich) were dissolved in 15 mL of tetrahydrofuran (THF). The mixture was poured into 100 mL of continuously stirred distilled water, the THF was evaporated under an air stream, and the particle suspension was concentrated to a final concentration of 5 mg mL$^{-1}$ at 60 °C. The obtained pH sensor nanoparticles had an average size of <100 nm as shown in the literature (Xie et al. 2013). The pH sensor nanoparticles were added to the pre-heated and previously deoxygenated artificial sediment in the last stage of the casting procedure, i.e., during cooling at ~38 °C to obtain a final concentration of ~7 % (v/v) in the agar matrix.
A detailed description of the optical nanoparticle-based pH sensors, including optical properties and calibration procedures is provided in the supporting information (Fig. S1-4 and S6). Artificial sediment with optical O2 sensor nanoparticles was prepared according to Koren et al. (2015). Briefly, 3 mg of platinum(II) meso-(2,3,4,5,6-pentafluoro)phenyl porphyrin (PtTFPP; indicator dye), 3 mg of Macrolex fluorescence yellow 10GN (MY; reference dye) and 200 mg of the styrene maleic anhydride copolymer (PSMA with 8% MA) XIRAN were dissolved in 20 g of Tetrahydrofuran (THF). This mixture was then poured into 200 mL of continuously stirred distilled water. THF was evaporated under an air stream, and the particle suspension was concentrated to a final concentration of 5 mg mL\(^{-1}\) at 60°C. The optical O2 sensor nanoparticles were added to the pre-heated and previously deoxygenated artificial sediment in the last stage of the casting procedure at an agar temperature of ~38°C to obtain a final concentration of ~3% (v/v) in the agar matrix. Calibration curves of the optical O2 sensor nanoparticles at the two different experimental temperatures are provided in the supporting information (Fig. S5).

**Imaging setup and data acquisition**

A RGB camera setup (Larsen et al. 2011) was used for ratiometric pH and O2 imaging (Fig. 1). The imaging system consisted of a SLR camera (EOS 1000D, Canon, Japan) mounted on a tripod and equipped with a macro objective lens (Macro 100 f2.8 D, Tokina, Japan) and a long pass filter (pH imaging, 455 nm; O2 imaging, 530 nm; Uqgoptics.com). Excitation of the luminescent sensor nanoparticles was achieved by means of a multichip LED (LedEngin Inc, RS Components Ltd, Corby, UK) combined with a bandpass filter (pH imaging, 405 nm; O2 imaging, 455 nm). The applied LEDs were powered by a USB-controlled LED driver unit designed for luminescence imaging applications (imaging.fish-n-chips.de). Data acquisition and control of the SLR exposure and LED light were achieved with a PC running custom software “look@RGB” (imaging.fish-n-chips.de).

**Image calibration and analysis**

The obtained SLR images were first split into red, green and blue channels and were then analysed via the Java-based image processing software ImageJ (rsbweb.nih.gov/ij/). In order to achieve images of pH and O2 dependent ratios, raw images were divided using the ImageJ plugin Ratio Plus (rsb.info.nih.gov/ij/plugins/ratio-plus.html). For O2 imaging, this implied dividing the red channel (emission of the O2 sensitive dye) with the green channel (emission of the reference dye). For pH imaging, the red channel (indicator dye) was divided with the blue channel (reference dye). The obtained ratio images
were fitted with previously obtained calibration curves (Fig. S4 and S5) using the Curve Fitting function in ImageJ, by means of linking the ratio images to the respective O₂ concentrations or pH units (see further details in Larsen et al. 2011; Koren et al. 2015).

Net photosynthesis and plant respiration rates

A seagrass leaf was positioned in a custom-made sample holder consisting of two 2 mm plexiglass plates to ensure a steady sample during microsensor measurements. Profiles were made through a hole in the plates (Ø = 3 mm) towards the seagrass leaf surface. The sample holder was positioned in a flow chamber (25×8×5 cm), which was connected to an aquarium pump ensuring a steady flow of ~3 cm s⁻¹ of aerated seawater (salinity = 34) from a 25 L aquarium, wherein the temperature was kept constant at either ~16 or 24°C by a thermostat (F25-HD, Julabo GmbH, Germany). Light was provided with a fiber-optic tungsten halogen lamp (KL-2500 LCD, Schott GmbH, Germany) positioned at a 45° angle above the sample. The experimental photon irradiance (PAR) was 500 µmol photons m⁻² s⁻¹, measured at the position of the sample, i.e., the leaf canopy, with a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany) connected to a submersible spherical micro-quantum-sensor (US-SQS/L, Walz GmbH, Germany).

Vertical profiles of O₂ concentration were measured in 50 µm increments from 0.5 mm above the leaf towards the tissue surface, using a Clark-type O₂ microsensor with a tip diameter of <25 µm (OX-25, Unisense, Denmark; Revsbech, 1989), with a fast response time (t₉₀<0.5 s) and a low stirring sensitivity (1-2%). The microsensor was mounted on a motorized micromanipulator (MU-1, PyroScience GmbH, Germany) and connected to a pA-meter (OXY-meter, Unisense, Denmark) that was interfaced to a PC via an A/D converter (DCR-16, PyroScience GmbH, Germany). Microsensor positioning and data acquisition were controlled by dedicated software (Profix, PyroScience GmbH, Germany).

Net photosynthesis and dark respiration rates were calculated from Fick’s 1st law of diffusion:

\[ J_{O_2} = -D_0 \frac{\partial C}{\partial z} \]

where \( D_0 \) is the salinity and temperature dependent diffusion coefficient of O₂ in seawater (www.unisense.com) and \( \partial C/\partial z \) is the linear concentration gradient of O₂ in the diffusive boundary layer.
Results

Rates of photosynthesis and respiration

The net photosynthesis and respiration rates of *Zostera marina* L. at the two experimental temperatures were determined via O2 concentration microprofiles measured towards the leaf tissue surface (Fig. 2). Measurements revealed a 2.2 times higher net photosynthesis rate at 24°C as compared to 16°C, amounting to an increase in O2 efflux from 0.117 to 0.252 nmol O2 cm⁻² s⁻¹; and a 1.4 times higher respiration rate at 24°C as compared to 16°C, which amounted to an increase in O2 influx from -0.116 to -0.159 nmol O2 cm⁻² s⁻¹. The measured temperature-induced enhancement in the rate of net photosynthesis and respiration corresponded to Q₁₀ temperature coefficients of 2.6 and 1.5, respectively.

O2 distribution and microdynamics

The two-dimensional O2 distribution in the *Z. marina* L. rhizosphere at 16 and 24°C during light-dark transitions is shown in Fig. 3. The O2 images showed an O2 release, i.e., radial oxygen loss, especially from the root-shoot junctions (nodiums) and the rhizome, leading to several oxic microniches in the immediate rhizosphere of *Z. marina* L. The seagrass was able to maintain oxic conditions around the rhizome even without photosynthetic activity (Fig. 3). The O2 concentration images revealed a distinct increase in the belowground tissue oxidation capacity at 24°C as compared to 16°C; this temperature effect predominated over light stimulation of the plants photosystems (Fig. 3). The extent of oxygenated regions and the below-ground tissue surface O2 concentration did only increase slightly during light exposure of the leaf canopy (incident irradiance of 500 μmol photons m⁻² s⁻¹; Fig. 3). Some of the prophyllums (single leaves originating from the rhizome at the nodiums), as well as the leaf sheath at the base of the shoot also released O2 to the rhizosphere.
The maximal width of the oxic microniches around the rhizomes was ~5.0 mm at nodium 7 during light exposure at a temperature of 24°C, corresponding to an oxic microshield thickness of ~0.75 mm surrounding the respective root-shoot junction (data obtained by subtracting the diameter of the rhizome), which is similar to previous findings in natural sediment (e.g. Pedersen et al. 1998; Jensen et al. 2005). The O₂ concentrations determined within selected regions of interest (ROIs) in the Z. marina rhizoplane confirmed these observations (Fig. 4; Table 1). Based on O₂ concentration measurements in ROI 1-7, we calculated a mean of a 1.1-fold increase in the oxidation capability of the belowground tissue as a result of the dark/light transitions as compared to a 1.3-fold increase in response to the 8°C temperature elevation. The highest rhizome surface O₂ levels were found at the root-shoot junctions (nodium 4, 5 and 7) corresponding to O₂ concentrations reaching up to 122 µmol L⁻¹ (ROI 3, 4 and 5 in Fig. 4; Table 1). The O₂ imaging thus documented pronounced spatial microheterogeneity and high spatio-temporal microdynamics of the belowground oxic microzones around the rhizome of Z. marina that was modulated by changes in light and temperature.
Figure 4: Selected regions of interest (ROI) within the immediate rhizosphere of *Zostera marina* L. used to determine the O₂ distribution during light/dark transitions (incident irradiance (PAR; 400-700 nm) of 500 µmol photons m⁻² s⁻¹) at the experimental temperatures (~16 and 24 °C). Boxes and numbers indicate the measured ROI. Mean O₂ concentration values representing the entire ROI are presented in Table 1.

Table 1: O₂ concentrations at selected regions of interest (ROI) within the immediate rhizosphere of *Zostera marina* L. Boxes and numbers indicate the measured ROI. O₂ concentrations are given in both % air saturation and µmol L⁻¹ at ~16 and 24 °C during light-dark transitions.

<table>
<thead>
<tr>
<th>ROI</th>
<th>~16°C</th>
<th>~24°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% air sat.</td>
<td>µmol L⁻¹</td>
</tr>
<tr>
<td>ROI 1</td>
<td>5.8</td>
<td>(14.6)</td>
</tr>
<tr>
<td>ROI 2</td>
<td>8.9</td>
<td>(22.3)</td>
</tr>
<tr>
<td>ROI 3</td>
<td>37.2</td>
<td>(93.2)</td>
</tr>
<tr>
<td>ROI 4</td>
<td>32.3</td>
<td>(81.0)</td>
</tr>
<tr>
<td>ROI 5</td>
<td>34.6</td>
<td>(86.7)</td>
</tr>
<tr>
<td>ROI 6</td>
<td>12.2</td>
<td>(30.6)</td>
</tr>
<tr>
<td>ROI 7</td>
<td>24.0</td>
<td>(60.2)</td>
</tr>
</tbody>
</table>

*ROI 1 represents measurements at the non-illuminated part of the shoot; ROI 2 = at the root-shoot junction (nodium 2); ROI 3 = at the base of the prophyllum close to the root-shoot junction (nodium 4); ROI 4 = at the root-shoot junction (nodium 5); ROI 5 = at the root-shoot junction (nodium 7); ROI 6 = internode 7 with prophyllum; ROI 7 = at the rhizome-end.
**pH heterogeneity and dynamics**

We found a high degree of pH heterogeneity within the seagrass rhizosphere, with distinct microzones of very low pH (down to ~pH 4), as well as rhizome/rhizoplane pH levels well above the pH of the surrounding bulk sediment (up to pH > 8; Fig. 5).

Comparison of O₂ and pH images revealed that areas of low pH overlapped with oxic microniches in the seagrass rhizosphere, whereas the high pH levels predominantly were measured on the surface of the mature part of the roots, the prophyllums and at the end of the rhizome, although patchy distributions of relatively high pH levels (as compared to bulk sediment pH levels) were observed on the surface of the entire belowground tissue. Selected regions of interest (ROIs) within the immediate rhizosphere of *Z. marina* exhibited higher pH levels (ΔpH of 0.02 - 0.31) in the rhizoplane during light exposure of the leaf canopy as compared to dark conditions at both experimental temperatures (ROI 1-7; Fig. 6; Table 2). The light-driven pH microdynamics was surpassed by the effect of the 8°C temperature elevation showing much higher pH levels (ΔpH of 0.46 - 0.88) in the rhizoplane of *Zostera*.
Zostera marina L. at 24°C as compared to 16°C during both light exposure and darkness (ROI 1-3 and 5-7; Fig. 6; Table 2).

A distinct hotspot of low pH was measured in the region of nodium 7, internode 7 and nodium 8 with an up to 5.2 mm wide zone of pH <5. The lowest rhizosphere pH levels were measured within this distinct zone with pH levels reaching the lower detection limit (pH 4) of the pH indicator (Fig. 5 and 6). The region of the belowground tissue with the highest pH levels was also found adjacent to nodium 7, corresponding to ROI 7 in Fig. 6 (Table 2).

**pH microheterogeneity at interfaces**

Extraction of cross-tissue pH values along line profiles in the pH images revealed pronounced pH microheterogeneity at interfaces (Fig. 7). The pH increased relative to the ambient sediment across internode 3 with the surrounding prophyllum, reaching pH levels of up to 8.3 on the rhizome surface.
Table 2: pH values in selected regions of interest (ROI) within the immediate rhizosphere of Zostera marina L. Values are given as a mean of the entire ROI ± S.E; and as the relative difference in pH between the experimentally changed environmental conditions (ΔpH). n = 5-18. The average pH of the bulk, artificial sediment at similar vertical depth as the below-ground biomass was ~ 5.7±0.0 (includes all treatments).

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ROI 1</td>
<td>5.8±0.0</td>
<td>6.4±0.0</td>
<td>5.8±0.0</td>
<td>6.4±0.0</td>
<td>0.0</td>
<td>0.1</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>ROI 2</td>
<td>5.6±0.0</td>
<td>6.3±0.0</td>
<td>5.7±0.1</td>
<td>6.5±0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>ROI 3</td>
<td>5.6±0.0</td>
<td>6.3±0.0</td>
<td>5.7±0.1</td>
<td>6.4±0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 4</td>
<td>6.7±0.0</td>
<td>6.6±0.0</td>
<td>5.7±0.1</td>
<td>6.8±0.0</td>
<td>0.0</td>
<td>0.2</td>
<td>-0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>ROI 5</td>
<td>3.9±0.0</td>
<td>4.8±0.0</td>
<td>4.2±0.1</td>
<td>4.9±0.0</td>
<td>0.3</td>
<td>0.1</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 6</td>
<td>5.9±0.0</td>
<td>7.1±0.0</td>
<td>6.0±0.1</td>
<td>6.6±0.0</td>
<td>0.1</td>
<td>0.3</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>ROI 7</td>
<td>6.6±0.1</td>
<td>7.1±0.0</td>
<td>6.9±0.2</td>
<td>7.4±0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*ROI 1 represents measurements at the basal leaf meristem (nodium 1); ROI 2 = the root-shoot junction (nodium 4); ROI 3 = at the base of the prophyllum close to the root-shoot junction (nodium 4); ROI 4 = root-bundle at nodium 6; ROI 5 = internode 7 with prophyllum; ROI 6 = at the rhizome-end; ROI 7 = root-shoot junction (nodium 7).

and correlating with rapidly increasing pH levels at the rhizome/sediment interface (Fig. 7b; CTS 1). Interestingly, the cross tissue pH profile across internode 4 with prophyllum close to nodium 4 showed increasing pH levels at the approximate position of the oxic/anoxic interface with pH levels reaching up to 8.0 during light exposure of the leaf canopy (Fig. 7c; CTS2). This was contrary to the rhizome/sediment interface where decreasing pH levels down to 4.1 were observed on the rhizome surface (measured during light exposure at 16 °C), thus indicative of proton consuming and producing biogeochemical processes altering the geochemical microenvironment at this specific belowground oxic microniche (Fig. 7c; CTS2).

A line microprofile across a root from root-bundle 6 showed similar microheterogeneity as found at internode 3, with increasing pH levels at the root/sediment interface, and root surface pH levels of up to 7.6 (Fig. 7d; CTS 3). Cross tissue microprofile 4 across internode 7 with prophyllum showed a pronounced decrease in pH at the approximate position of the oxic/anoxic interface with pH levels within the low pH hotspot approaching the lower detection limit of the pH indicator (Fig. 7e; CTS 4).

Across nodium 9 at the end of the rhizome with a degraded prophyllum, pH increased at the approximate position of the rhizome up to pH 8.7 (Fig. 7f; CTS5). These observations were supported by vertical pH microprofiles measured from the seawater/sediment interface down to the bottom of the pH sensitive sediment (Fig. 8). A rapid decrease in pH was observed within the
Figure 7: Cross tissue line sections (CTS) determining the pH microdynamics at the plant/rhizosphere interface and on the plant tissue surface. The steady-state cross tissue line sections were determined at the two experimental temperatures (i.e. ~16 and 24 °C) during light-dark transitions (under an incident photon irradiance (PAR; 400-700nm) of 500 µmol photons m⁻² s⁻¹).

(a) Structural image of the seagrass *Z. marina* L. embedded in the artificial, transparent sediment with pH sensitive nanoparticles (pH colour coded image), illustrating the positions of the respective cross tissue line sections (CTS1-5).

(b) Line microprofile across internode 3 with attached prophyllum (CTS1).

(c) Line microprofile across internode 4 with prophyllum close to nodium 4 (CTS2).

(d) Line microprofile across root from root-bundle 6 (CTS3).

(e) Line microprofile across...
uppermost 5 mm as typically observed in natural marine sediments (Stahl et al. 2006; Zhu et al. 2006), with pH levels decreasing from about ~7 at the water/sediment interface down to pH ~6 at 5 mm depth where after it stabilised.

A vertical pH microprofile extracted from pH images (VM1; Fig. 8b) showed the pH microdynamics and microheterogeneity at the interfaces between the sediment and the first prophyllum, as well as between the sediment and the basal meristem with leaf sheath. An increase in pH was measured at the position of the basal meristem with leaf sheath, i.e., the meristematic region of the rhizome, and along roots of the first root bundle (Fig. 8b). This was in contrast to pH conditions at the prophyllum/sediment interface, where we observed a rapid increase in pH towards the leaf tissue surface followed by a rapid decrease across the prophyllum, possibly due to oxic conditions and/or biological re-oxidation of H₂S (Fig. 8b; VM1). Another vertical pH microprofile (Fig. 8c; VM2) showed a rapid pH decrease at the interface between the sediment and the base of the fifth prophyllum/internode 7. At nodium 8 (root-shoot junction), a rapid increase in pH was seen at the approximate position of the oxic/anoxic interface with pH levels up to 8.4, followed by a strong decrease in pH across the rhizome tissue with pH levels decreasing to ~4.6 (Fig. 8d; VM3). A root from root-bundle 8 may have interfered with the interpretation of the pH microdynamics at nodium 8 (see Fig. 8d; VM3; ~26 mm depth). Nevertheless, our results clearly showed that plant-derived alterations of the belowground chemical microenvironment caused pH changes in the rhizoplane with a high degree of spatial microheterogeneity.
Figure 8: Vertical pH microprofiles (VM) illustrating the pH heterogeneity and microdynamics in the rhizosphere of Z. marina L. The vertical pH microprofiles were determined at steady-state conditions during light-dark transitions (photon irradiance (PAR) of 500 µmol photons m⁻² s⁻¹) at ~16 and 24 °C. (a) Structural image of the Z. marina L. plant illustrating the spatial positions of the vertical pH microprofiles (colour coded image). (b) Vertical pH microprofile from the water/sediment interface across the first prophyllum and the basal meristem with leaf sheath to the bottom of the artificial sediment (VM1). (c) Vertical pH microprofile from the water/sediment interface across the base of the fifth prophyllum and the rhizome (internode 7) to the bottom of the artificial sediment (VM2). (d) Vertical pH microprofile from the water/sediment interface across the root-shoot junction at nodium 8 to the bottom of the artificial sediment (VM3). Y-axis = 0 indicate the artificial sediment surface. The approximate position of the below-ground tissue is indicated on the graphs by means of colour coded boxes (i.e. P = Prophyllum (blue), BM = Basal meristem with leaf sheath (green), R = Roots (brown); IN7P = Internode 7 at the base of the prophyllum (green); N = Nodium 8 (green)). n = 3. Note that the white areas on leaves/prophyllums (marked with black arrows on the figure) should be interpreted with caution, as some of these high pH microniches (pH of ≥9) seemed to be caused by epiphyte-derived red background luminescence (Fig. S6).
Discussion

Our results showed a high spatio-temporal pH and O\textsubscript{2} microheterogeneity in the rhizosphere of Z. marina, where the chemical conditions in the immediate rhizosphere were highly affected by the plant host (Fig. 3 and 5). Radial O\textsubscript{2} loss (ROL) from the below-ground tissue of Z. marina resulted in oxic microniches around the root-shoot junctions and the rhizome (Fig. 3 and 4). Such oxic microniches have recently been shown to facilitate chemical re-oxidation of sediment-produced H\textsubscript{2}S, and ROL is therefore an important chemical defence mechanism whereby the plants can actively detoxify phytotoxins in the surrounding sediment (Brodersen et al. 2014, 2015a).

**Oxidation capacity of the below-ground tissue**

The higher oxidation capacity of the below-ground tissue observed at 24°C as compared to 16°C (Fig. 4; Table 1) was due to a relatively higher rate of shoot photosynthesis (Fig. 2). The light-independent reactions, i.e., the enzyme-controlled reactions in the photosystems, are highly temperature dependent and the rate of photosynthesis, therefore, increases in direct proportion to temperature until it reaches a temperature optimum for the given plant, where after it rapidly decreases e.g. due to enzyme denaturation (Staehr & Borum, 2011). The optimum temperature for oxygenic photosynthesis in summer acclimated Z. marina plants is ~24°C (Staehr & Borum, 2011). The higher ROL from the rhizome in darkness at 24°C as compared to 16°C (Fig. 3 and 4) may be explained by a significantly higher O\textsubscript{2} diffusion coefficient in the temperature elevated water. As a water column temperature elevation of 8°C results in a ~25% increase in the rate of O\textsubscript{2} diffusion across the diffusive boundary layer (DBL) and into the above-ground tissue from the surrounding aerated water column (Ramsing & Gundersen, 2015), thus allowing enhanced internal O\textsubscript{2} supply through the aerenchyma (low-resistance internal gas channels) to the belowground tissue during darkness. This enhancement of the internal O\textsubscript{2} concentration gradient may be supported by a simultaneous temperature-induced increase in ROL owing to (i) the relatively increased lateral molecular O\textsubscript{2} diffusion rate across the epidermal layer of the belowground tissue at higher temperatures (although this might be counter-balanced by the higher tissue respiration), and (ii) the high leaf surface-to-volume ratio of the small Z. marina specimens used in this study leading to a relatively high efflux of O\textsubscript{2} from the leaves into the water column in light and a relatively high influx of O\textsubscript{2} from the water column into leaves in darkness. Most prophyllums seemed to release O\textsubscript{2} into the rhizosphere (Fig. 3), and where prophyllum 1-5 potentially could be fueled by O\textsubscript{2} from the water-column, the fully buried prophyllum 6 at nodium 9 must be supplied with O\textsubscript{2} from the rhizome. Only a minor O\textsubscript{2} leakage was detected from the roots of
the 2nd root-bundle close to the basal meristem during light exposure and a temperature of 24°C (Fig. 3). Structural tissue barriers to ROL (e.g. suberin; Barnabas, 1996) minimize cross tissue gas permeability of mature roots (e.g. Colmer, 2003; Jensen et al. 2005; Frederiksen & Glud, 2006; Brodersen et al. 2015a). Frederiksen and Glud (2006) found that the root oxygenated zones diminished with root age and suggested that O₂ leakage from Z. marina roots eventually ceased. Our results further support such anatomical root adaptation of Z. marina to a life in a hostile reduced sediment environment. Barriers to ROL protect the plants against exposure to sediment-derived reduced phytotoxins such as H₂S and increase the amount of internal O₂ transported to the apical root meristems ensuring aerobic metabolism in distal parts of the plants.

**pH microheterogeneity in the rhizosphere**

The novel pH sensitive nanosensors incorporated in the transparent sediment matrix enabled the first detailed mapping of the spatio-temporal pH microheterogeneity in the whole rhizosphere of Z. marina (Fig. 5). A similar pattern was recently observed in the rhizosphere of Zostera muelleri spp. capricorni by means of point measurements using electrochemical microsensors (Brodersen et al. 2015a). Regions in the immediate rhizosphere of Z. marina with very low pH levels (pH <5) seemed to correlate with the plant-derived oxic microniches. Such acidification could be due to proton formation as a byproduct of the spontaneous chemical reactions between plant-released O₂ and sediment H₂S within the oxic microzone (Fig. 5 and 6). We also measured slightly lower pH values in the immediate rhizosphere during darkness as compared to in light (Fig. 5 and 6), owing to plant and sediment respiration processes in addition to the aforementioned plant-derived spontaneous chemical re-oxidation of H₂S.

At the end of the rhizome around nodium 9, the pH imaging revealed high pH levels in the adjacent sediment (Fig. 7f). We speculate that such local pH enhancement may be due to high levels of accessible organic carbon in this specific region of the rhizoplane, as a result of tissue degradation and rhizome exudates, leading to proton consumption through microbial metabolisms such as sulphate reduction (Isaksen & Finster, 1996; Blaabjerg et al. 1998; Hansen et al. 2000; Nielsen et al. 2001). These plant-microbial mediated local changes in the rhizosphere pH microenvironment are potentially very important for seagrasses as enhanced pH levels in the immediate rhizosphere lead to a shift in the sulphide speciation away from H₂S and towards non-permeable and thus non-phytotoxic HS⁻ ions. Besides formation of oxic microniches due to ROL (see above), rhizosphere pH changes represent another chemical defense mechanism, whereby the plants further detoxify the surrounding sediment to accommodate their own growth in the often reduced, anoxic environments (Brodersen et al. 2015a).


**Biogeochemical processes**

The enhanced photosynthetic activity of *Z. marina* L. at its photosynthetic temperature optimum (~24°C) (Fig. 2), positively affects the production of photosynthates and thereby lead to diurnal increases in the secretion of root/rhizome exudates and ROL (Moriarty et al. 1986; Blaabjerg et al. 1998; Nielsen et al. 2001) that may stimulate the microbial activity (such as sulphate reduction and sulphide oxidation, respectively) on the root/rhizome surface and in the immediate rhizosphere. The overall higher pH levels measured in the immediate rhizosphere at 24°C as compared to 16°C (Fig. 5 and 6), may thus be a result of a temperature-induced enhancement in the plants photosynthetic activity leading to increased rhizome/root exudation of organic carbon to the rhizosphere (Moriarty et al. 1986; Blaabjerg et al. 1998). Such exudation could either directly increase the pH levels in the immediate rhizosphere and on the below-ground tissue surface through secreted allelochemicals like amines (although this would be an expensive chemical defence mechanism for the plants) and other alkaline substances, and/or indirectly via stimulation of microbial processes such as sulphate reduction (as indicated at the plant-derived oxic/anoxic interfaces (Fig. 7c and 8d), in combination with the generally temperature-mediated increase of the sulphate reduction rates owing to reaction kinetics (Isaksen & Finster, 1996; Blaabjerg et al. 1998). Sulphate reduction rates associated with rinsed *Zostera muelleri* spp. *capricorni* roots/rhizomes have been found to be up to 11 times higher than in the bulk sediment (Hansen et al. 2000), and both rhizome and roots have been shown to be important habitats for sulphate-reducing and N₂-fixing bacteria (Blaabjerg & Finster, 1998; Nielsen et al. 2001). Sulphate-reducing bacteria associated with rhizomes/roots possess a high N₂-fixing activity that can cover up to 65% of the nitrogen needed by the seagrass plants (Hansen et al. 2000; Nielsen et al. 2001). Notably, high sulphate reduction rates in the seagrass rhizosphere, furthermore, leads to a sulphide-induced release of sediment-bound phosphorus, as the reduction of Fe(III)(oxyhydroxides) to Fe(II) results in phosphate release to the pore water, which then becomes available for plant growth (Pollard & Moriarty, 1991; Pagès et al. 2011; Pagès et al. 2012). A mutual beneficial relationship between the *Zostera marina* L. plant host and sulphate reducing bacteria in the rhizoplane seems therefore likely during non-stressed environmental conditions, where the sulphate reducing bacteria provides nutrients in the form of nitrogen and phosphate to the plant host as a response to plant-mediated rhizome/root exudates. However, we note that this hypothesis remains speculative and needs further experimental support. Our study did not aim to investigate the role of sulphate reducing bacteria in the *Z. marina* rhizosphere, and as we have used a sterile artificial sediment any sulphate reducing bacteria in the
immediate rhizosphere must have originated from the non-sterile plant tissue. Future studies could e.g. involve artificial sediment based on extracted pore water or even cultures of sulphate reducing bacteria in combination with quantification of bacteria around the root biomass, e.g. using FISH with group-specific probes.

In other microniches associated with the formation of oxic microzones (Fig. 7c and 8d) biological and/or spontaneous chemical sulphide re-oxidation processes reduced the rhizoplane pH levels (Fig. 5). Such hotspots of low pH may well be due to a relatively higher abundance of sulphide oxidizing bacteria at that specific region, as microbes associated with the below-ground tissue of seagrass show a patchy distribution (Nielsen et al. 2001).

**Optical nanoparticle-based sensors incorporated into transparent artificial sediment**

The combined use of O$_2$ and pH sensitive nanoparticles with transparent artificial sediments enabled combined chemical and structural imaging on the whole rhizosphere level. This novel application of optical nanoparticle-based sensors represents an important supplement to existing methods, such as planar optodes and microsensors, when elucidating the rhizosphere of aquatic macrophytes, as the former rarely allows close contact to the entire belowground tissue at once and the latter rely on precise point measurements, which makes mapping the entire rhizosphere extremely tedious if not impossible. In addition, the optical nanoparticle-based sensors enable close spatial alignment of pH and O$_2$ concentration mapping thus facilitating co-localization of these important chemical parameters relative to particular plant/sediment and oxic/anoxic interfaces within the rhizosphere. However, at the current state, the present nanoparticle methodology only allows for O$_2$ and pH imaging in artificial sediments. The strengths of employing such reduced artificial sediment, as compared to natural sediment, encompass: (i) significantly improved visual assessment within the investigated rhizosphere, thus allowing for determination of the exact position of the entire below-ground tissue during imaging, which is a necessity when determining the effects of plant/sediment interactions on the rhizosphere biogeochemistry, and (ii) changes observed within the homogenous artificial sediment can be assigned to plant-mediated alterations, which can be difficult to conclude in highly heterogeneous natural sediment. Weaknesses of using an artificial sediment matrix, as compared to natural sediment, include: (i) a significantly reduced microbial abundance in the bulk sediment, and (ii) a potential lower sediment pH buffering capacity, which may lead to slightly overestimated responses. Moreover, a minor limitation of current ratiometric pH imaging is that high energy excitation light has to be used when exciting the pH sensitive indicator dyes, potentially causing artefacts in the pH images owing to, for example,
chlorophyll-derived red background luminescence. Further information on how to avoid/limit such potential artefacts in the pH images is available in the supporting information (Fig. S6). Nevertheless, nanoparticle-based imaging provides detailed information about the geochemical conditions and dynamics in the rhizosphere of aquatic macrophytes at high spatio-temporal resolution without the potential smearing effects seen with planar optodes and allows the first investigations of pH and O₂ dynamics in the entire seagrass rhizosphere in real-time and at all below-ground tissue/sediment interfaces. Nanoparticle-based imaging thus has the potential to further resolve important plant-sediment interactions, such as, for example, plant-derived sediment detoxification processes, in addition to, simply directing precise microsensor measurements to biogeochemical hotspots within natural sediment.

In conclusion, novel optical nanoparticle-based imaging revealed a pronounced spatio-temporal pH and O₂ microheterogeneity in the immediate rhizosphere of *Z. marina* L. Light stimulation of the leaf canopy and temperature elevation to the plants photosynthetically temperature optimum, i.e., from ~16 to 24°C, lead to higher oxidation capacity of the belowground tissue and higher pH levels in the immediate rhizoplane, where the temperature-induced stimulation seemed to predominate. Low rhizosphere pH levels correlated with the plant-derived oxic microniches. Patchy distributions of high rhizosphere pH levels were found on the tissue surface, and cross tissue pH microprofiles revealed enhanced pH levels at selected oxic/anoxic interfaces. We speculate that the higher pH levels on the tissue surface and at the oxic/anoxic interface may be due to a plant-derived stimulation of proton consuming microbial metabolisms such as sulfate reduction and excretion of alkaline substances. Protons produced or consumed during microbial metabolisms, in addition to plant-mediated allelochemicals and chemical re-oxidation of H₂S, thus seemed responsible for the photosynthesis/temperature-driven alterations of the geochemical microenvironment determined in the *Zostera marina* L. rhizosphere.
Acknowledgements

We would like to thank Egil Nielsen, University of Copenhagen (KU) for manufacturing the applied aquarium and experimental split flow chamber. We thank Sofie Lindegaard Jakobsen (KU) for technical assistance and Dr. Sergey Borisov (Graz University of Technology) for generously providing the used pH indicator. The study was funded by research grants from the Augustinus Foundation (KEB); Fab. P.A. Fiskers Fund (KEB); the Danish Council for Independent Research | Natural Sciences (MK); the Villum Foundation (MK, KK); and the Australian Research Council [ARC Linkage, LP 110200454] (MK).
References


Isaksen MF, Finster K. (1996). Sulphate reduction in the root zone of the seagrass *Zostera noltii* on


Marine Ecology Progress Series 69(1): 149-159.


Supplementary information

Figure S1: Luminescence spectra of the optical pH nanosensors in alkaline (pH 10; green) and acidic (pH 3; orange) solutions, showing a marked drop in luminescence in the yellow-orange-red wavelength interval (~550-675 nm) combined with an increase in the violet-blue-green wavelength interval (~430-530 nm) under acidic conditions. The nanoparticles were excited by a 405 nm LED and the spectra were recorded with a fiber-optic spectrometer (QE65000; oceanoptics.com).

Figure S2: Calibration of pH nanosensor luminescence. Ratio images, i.e., the ratio of red and blue channels extracted from the recorded RGB image, were quantified in small transparent glass vials with pH nanoparticle-containing agar buffered to defined pH levels spanning pH 4-10. The pH sensor nanoparticles were calibrated as follows. A solution of ~0.5% (w/w) agar-NaCl water (with a salinity of 34) was poured into small glass vials (3 mL volume). To adjust the pH value, 300 µL of a 100 mM buffer solution (citrate, phosphate or TRIS) were added. At a temperature of ~40°C, a small volume of the pH sensitive nanoparticles was added to a final concentration as used in the artificial sediment. After a short mixing step the agar was left to solidify. The glass vials were kept at constant temperature (16°C or 24°C) in a thermostated water bath and were then imaged with the ratiometric camera system using identical settings as for the seagrass sample. The ratio (red/blue channel) images were then correlated to the known pH values.
Vertical pH microprofiles in the bulk, artificial sediment containing pH sensitive nanoparticles measured with both a calibrated pH microelectrode (pH-50, tip diameter of ~50 µm; Unisense A/S, Aarhus, Denmark; Kühl & Revsbech, 2001) and the optical nanoparticle-based pH sensors. There was a high resemblance between pH microprofiles (and thereby the sediment pH levels) determined with the two different sensor types. The pH levels of the bulk, artificial sediment dropped rather rapidly in the first 0-5 mm depth from ~pH 7.5 at the seawater/sediment interface to ~pH 6 at the approximate position of the below-ground biomass, thus mimicking chemical settings in natural sediment (Burdige and Zimmerman, 2002; Stahl et al. 2006; Zhu et al. 2006) (further information about the casting procedure of the reduced, artificial sediment is provided in Brodersen et al. (2014)).

pH microelectrode measurements. The pH microelectrode was mounted on a micromanipulator (Unisense A/S, Denmark) and used in combination with a reference electrode (tip diameter of ~5 mm; Unisense A/S) immersed in the supporting water reservoir; both connected to a pH/mV-Meter (Unisense A/S). Before measurements commenced, the pH microelectrode was linearly calibrated from sensor readings in three pre-known pH buffers (pH 4, 7 and 9; linearly responding over the pH calibration range with a signal to pH ratio of 51 mV/pH unit) at experimental temperature and salinity. The microelectrode measurements were performed in the custom-made narrow split-flow chamber (Fig. 4.1), in the same area as the extracted vertical pH microprofiles obtained via the pH sensitive nanosensors, by manually handling the micromanipulator (increments of 1 mm). The artificial sediment surface was determined by manually moving the microsensor towards the seawater/sediment interface, while observing the microsensor tip and the sediment surface through a magnifying glass, as well as from signal readings.

Figure S3: Calibration curves for optical pH nanoparticle-based sensors at the two experimental temperatures 16 and 24 °C. Mean ratio values were fitted with a sigmoidal function ($r^2 = 0.99$ and 0.97, respectively). Error bars are ± SD (n=3).
When working with optical (luminescence-based) sensors several factors can lead to artefacts and consequently misinterpretation of the results. The following paragraph intends to create awareness of potential problems and gives direction for possible solutions.

**Intensity, ratiometric and lifetime based imaging:**

In general, three different types of luminescence imaging are used for readout of optical chemical sensors. The most error prone is simple luminescence intensity-based imaging, where the intensity of a pixel or region is correlated to the analyte concentration. This approach is affected by numerous potential artefacts such as fluctuations in the illumination source, uneven illumination and/or distribution of the luminescent indicator, interference from background illumination and bleaching of the sensor material. In order to overcome some of these potential artefacts, a ratiometric imaging approach (where the ratio between the luminescence intensity of an analyte-sensitive indicator dye and the luminescence intensity of an inert reference dye is correlated to the analyte concentration) can be used to correct for

---

**Figure S4:** pH microprofiles measured in the bulk, artificial sediment containing pH sensitive nanoparticles with a pH microelectrode (red symbols; mean ± SD; n=3) and with the optical nanoparticle-based sensors (black line). Y = 0 indicates the artificial sediment surface.

**Figure S5:** Calibration curves of optical O\(_2\) nanosensors measured at the two experimental temperatures (Blue = 16°C; Red = 24°C; mean red/green ratio values were fitted with an exponential decay function, \(r^2 = 0.99\) for both plots). The optical O\(_2\) nanoparticle-based sensors incorporated in the artificial, transparent sediment were calibrated as described in Koren et al. (2015). Error bars are ± SD, n=3.
uneven illumination or sensor distribution, and fluctuations in the illumination source. In terms of bleaching effects, a similar bleaching rate of the indicator and reference dyes is favourable; if one of them bleaches more easily than the other, this will lead to erroneous analyte concentrations. Background light and autofluorescence remains a problem in ratiometric imaging. Ratiometric imaging can be realized with relatively simple camera systems, like the SLR camera system used in this study (Larsen et al. 2011). Lifetime-based imaging, where the analyte-dependent change in the indicator luminescence decay time is monitored, is a very good and reliable alternative method but involves complicated and expensive instrumentation. Further details on this topic can be found in Meier et al. (2013).

**Planar optodes vs. nanoparticle-stained artificial sediments**

As this study utilizes nanoparticle-based sensors incorporated into transparent, artificial sediment we want to briefly discuss the benefits, but also potential artefacts, of this novel methodology in particular in comparison to the more commonly used planar sensor optode methods. Further details about the planar sensor optode method can be found in Santner et al. (2015). In brief, the use of planar optodes enables chemical imaging in the plane of an optical sensor foil. Analysis of complex structures like the below-ground tissue of seagrasses requires a close proximity of the planar optode to the tissue surface. This is often difficult, if not impossible, to achieve and can limit the part of the belowground tissue that can actually be analysed. In contrast, the nanoparticle method applied in this study enables simultaneous imaging of the entire below-ground tissue of seagrasses on a whole rhizosphere level (Koren et al. 2015).

An advantage of planar optodes is that an optical isolation can be applied on top of the sensor film. This protects the sensor from external light and protects the structure to be analyzed from the sensor excitation light (Glud et al. (1996)). The latter is particularly important when high energy light (e.g. UV light) is used for excitation as this can easily cause background fluorescence from biological samples. An optical isolation layer can obviously not be applied in case of the nanoparticle stained transparent, artificial sediment. This means that things like background fluorescence from the sample have to been taken into account when interpreting the images.

**Avoiding artefacts when working with intensity or ratiometric based imaging**

In general the following rules apply when trying to avoid potential artefacts

- Use an excitation light that will not cause autofluorescence in the biological sample.
  - If this is not possible, as the indicator requires a certain excitation wavelength, try to image the sample without the sensor particles to see how high the background is and subtract this if needed in the subsequent image analysis.
- Be aware of color-dependent scattering
  - Use as thin a layer of artificial sediment as possible, to reduce the light path, and use color-corrected optics
- Avoid background light
  - If not possible, e.g. if darkening of the room is not entirely possible; take an image with the excitation light source off. This “dark‘’ picture can then be subtracted from the images with the excitation light on. Nevertheless, it is advisable to get the surroundings as dark as possible.
- Bleaching: In case long-term light exposure is planned, it is recommended to test photostability prior to the measurements and account for potential photobleaching.
- Calibration: It is recommended to calibrate the sensor with the exact same conditions/settings as planned for the later measurements.

In the present study, all of the above-mentioned precautions were followed/secured during imaging. As UV light had to be used for pH imaging (e.g. Larsen et al. 2011), a few potential artefacts associated with the high energy excitation light were observed and are discussed below. All other above mentioned sources of potential artefacts could be excluded from the images.

**Artefacts seen in the pH images**

Artefacts are often easier to discover in the raw images of the different colour channels of the recorded RGB images.

As seen in Fig. S6, the blue colour channel appears to have a homogeneous intensity distribution (A), while the red channel shows some spots of high light (B). Especially in biological samples, this can e.g. be due to background fluorescence induced by the sensor excitation light. As the ratio of the two colour channels is used to calculate the pH image, such artefacts results in locally false pH readings. For example, Fig. S6 depicts 4 small areas with very high red luminescence (white arrows, B) associated with leaves and prophyllums, that are probably partly covered by epiphytes, where the blue excitation light induced chlorophyll-derived red background luminescence. In contrast to other regions that show dynamics in response to altered environmental conditions (such as at the rhizome; Fig. S6, A), the 4 high red luminescence areas remain unchanged.

All images were therefore interpreted with caution. Potential artefacts were thoughtfully analysed, as demonstrated in Fig. S6. Signals from regions potentially affected by artefacts (such as epiphyte-derived red background luminescence) were excluded from further analysis and marked on presented images by arrows, to ensure sufficient precaution was/are taken into account when interpreting the images. This is, as previously mentioned, a minor limitation of current pH imaging, as high energy excitation light has to be used when exciting the pH sensitive indicator/reference dyes.
Figure S6: Visualization of potential artefacts in the obtained pH images (images are from the 16°C treatment). The blue and red channel images are obtained by splitting the original RGB picture into its respective colour channels. The blue channel image (A) appears quite homogeneous in terms of intensity, while the red channel image (B) shows several high intensity regions. When merging the two channels (C) it can be seen that most of the picture appears in a homogeneous pink colour, while the hotspots in the red picture remain. This subsequently leads to very high apparent pH values at those spots as the ratio of red and blue channel leads to the final pH image (D). In contrast to other regions (e.g. low pH hotspot at the rhizome; A) those spots do not change over time and in response to the altered light levels and/or temperature. An additional artefact is presented by the region on top of the artificial sediment (e.g. square in the pH image; D). In this region the measured intensities are not due to the optical nanoparticle based sensors and only represent noise such as scattered light, wherefore this region has been excluded.
References


Chapter 12

Discussion and future directions

by

Mads Lichtenberg
The aim of this thesis was to investigate resource stratification on a microscale in dense aquatic photosynthetic systems such as macrophytes, biofilm and corals. With this, we asked the question if such densely pigmented systems conceptually display canopy interactions similar to terrestrial plants where resource stratification and high photosynthetic efficiencies can exist simultaneously. Living in suboptimal conditions for photosynthesis, and thus growth, puts pressure on an organism to employ structural and/or biochemical regulations which can counteract such suboptimal environments. In the following I have tried to conceptualize the regulating mechanisms seen in aquatic photosynthetic systems and compare them to mechanisms observed in terrestrial canopies.

As a first consideration, it is worthwhile to explain what is meant by an ‘efficient photosynthetic system’. Generally, maximal light-use-efficiencies should occur if all phytoelements are subjected to the same irradiance, i.e. when phytoelements are situated in a homogenous light field with a low degree of self-shading (Terashima & Hikosaka, 1995). However, a completely homogenous light field with no self-shading is practically inexistent. Thus, optimal photosynthesis is achieved when all phytoelements operate at light-saturated (but not inhibited) rates according to their local light field, resulting in all phytoelements saturating simultaneously (Terashima & Hikosaka, 1995). Implicitly, this also means that either i) a homogenization of light must occur, or ii) individual phytoelements must be able to adjust their light absorbing properties and photosynthetic machinery to their respective light environments. Indeed, on a macroscale light is scattered and to some degree homogenized inside plant canopies, and leaves situated high in the canopy display higher photosynthetic capacities than leaves in the bottom – commonly referred to as sun- and shade leaves (Myers et al., 1997). Interestingly, strong similarities of these concepts are seen going from a macroscale toward the microscale, or leaf-scale, where light inside leaves can be redistributed by multiple scattering from intercellular airspaces effectively homogenizing the internal light field (Cui et al., 1991; Vogelmann, 1993). Concurrently, gradients in photosynthetic potential can be seen inside individual leaves, parallel to light gradients, where adaxial (top) layers generally display higher photosynthetic capacity than abaxial (bottom) layers (Vogelmann & Evans, 2002; Evans & Vogelmann, 2003). Thus, it seems that plant systems, from canopies to individual leaves, come close to optimal photosynthetic systems.

The homogenization of light in terrestrial systems, displaying canopy light extinction coefficients of ~0.5 (Zhang et al., 2014), stands in contrast to densely pigmented systems, such as photosynthetic biofilms, where heterogenous resource distribution is predominant and, although multiple scattering is present
in such systems (Kühl & Jørgensen, 1994) light is usually attenuated very rapidly with characteristic PAR attenuation coefficients of dense photosynthetic biofilms in the order of 10,000 times higher than in open ocean systems (Chapter 4 and 6; Michael et al. (2012)). Hence, alternative mechanisms must be employed to counteract this resource stratification.

In this thesis, we have demonstrated that, a strong stratification of resources exist on a microscale, across the investigated aquatic systems, with pronounced gradients of light-quantity and -quality as well as chemical parameters. The stratification of light leads to situations where a system can be highly light saturated near the surface while, less than 1 mm away the system is light sub-saturated and practically in shade. Despite the large phylogenetic difference between the investigated systems, certain aspects from these systems can be described conceptually similar to terrestrial plant canopies. In the following, a description of the individual regulating mechanisms will be given with examples from the investigated systems, and how these mechanisms can be seen as analogues to their terrestrial counterparts, where resource stratification and high photosynthetic efficiencies can exist simultaneously (Terashima & Inoue, 1985).

Redistribution of light is affected by tissue/community structure

Due to strong drag and shearing forces imposed by waves and currents, sessile aquatic macrophytes lack the ability to achieve complex 3D structural organisation like terrestrial canopy systems. In addition, aquatic macrophytes are not recognized to have specialized tissue structures which facilitate penetration of light as seen in higher terrestrial plants (Vogelmann, 1993; Vogelmann & Martin, 1993; Brodersen & Vogelmann, 2007; Egbert et al., 2008). In Phaeophyceae the tissue organization consists of outer absorbing cortex layers and a central transparent medulla layer. This organisation allows for photons which are not absorbed in the directly illuminated layer to be redistributed by scattering and transmission in the central medulla to the shaded cortex, in order to maximize photon absorption (Chapter 5 and 10). This strategy stands in contrast to terrestrial leaves where the complex structural organisation of individual leaves allows tissue configurations that serve to distribute photons internally and thereby maximize absorption.

However, there have been speculations that some green macroalgal species have tissue structures that facilitate a more even internal distribution of light. In Codium, water in the medullary tissue can be replaced by air which creates a highly reflecting surface at the base of the utricles, which has been
described to function as integrating spheres (Ramus, 1978). As a consequence, Codium displays high light-absorption in the outermost layers with high chloroplast concentration, but a nearly constant light availability in the central medullary tissue (Lassen et al., 1994). This internal homogenization of light creates a more even resource distribution and it was found that by illuminating a confined spot on the thallus, photosynthesis was stimulated >3 mm away from the directly illuminated area (Lichtenberg, unpubl.; Fig. 1). Thus, in the case where water is replaced by air in the medullary tissue, Codium can increase the internal scattering similar to what can be observed in some terrestrial leaves, where high scattering is caused by high refractive index mismatches between cells and the gas-filled intercellular spaces (Vogelmann, 1989).

In corals, the photosymbiotic algae in the genus Symbiodinium reside in the gastrodermal tissues of the cnidarian host. Traditionally, coral tissue has been regarded as a black, light absorbing box where incident light is absorbed to some degree by the Symbiodinium, while internal light interactions is primarily explained by scattering from the underlying CaCO₃ skeleton matrix (Enriquez et al., 2005; Marcelino et al., 2013). However, recent experimental evidence suggest that the tissue itself can influence the in hospite light environment, where multiple scattering between tissue layers can transfer light laterally; thus, homogenizing the internal radiance (Wangpraseurt et al., 2014). This lateral transfer of light is suspected to be an explaining factor in the high photosynthetic efficiencies seen in coral tissues (Chapter 2). In addition, corals have a wide range of regulating mechanisms that are able to modulate the internal light environment which will be discussed further below.

Microphytobenthic systems display differences in the internal light environment depending on the compactness and composition of the system (Chapter 4). Generally, a maximum in scalar irradiance can
be seen just below the surface due to a continuous flux of photons from above, while scattering in the uppermost layers result in a path-lengthening of photons and thus a longer residence time in these layers (Vogelmann, 1993; Kühl & Jørgensen, 1994). However, in dense photosynthetic biofilms, light is rapidly attenuated with depth due to high absorption by photosynthetic pigments, and the zone with sufficient light to sustain oxygenic photosynthesis is often less than 1 mm in thickness (Chapter 6). Such dense systems stand in contrast to e.g. coral reef sediments where photosynthetic algae and bacteria are loosely distributed in bright scattering CaCO₃ sediment particles. Scattering from these particles homogenizes light in the upper sediment layers by photon path-lengthening and thus creates a more even illumination of the photosynthetic organisms residing in these layers. This results in a more open, canopy-like organization with larger photic zones and higher areal photosynthetic rates compared to dense biofilms (Chapter 4).

**Dynamic modulation of internal light environment**

When the effect of incident irradiance exceeds the capacity for biochemical regulations in the photosynthetic machinery photodamage may occur. A strategy to avoid this over-excitation of the photosystems is to simply regulate the irradiance perceived by the photosynthetic cells. This can be achieved in a range of ways, where one mechanism is to simply migrate along a gradient of light. This movement is dichotomous, and can be defined to either maximize photon absorption in subsaturating light fluxes or minimize absorption during high light (Wagner & Grolig, 1992).

Such migratory behavior has been described in terrestrial leaves (Gorton et al., 1999; Wada et al., 2003) as well as in many aquatic green algae; especially in the Bryopsidales which have coenocytic cells where organelles can move around freely (Menzel & Elsner-Menzel, 1989). Most green algae possess xanthophyll cycle pigments that can dissipate excess light energy non-photochemically (Masojidek et al., 2004). However, recently it was reported that a functional xanthophyll cycle is absent in *Codium* (Cruz et al., 2015). This intertidal species is subjected to daily high fluctuations in the light environment and preliminary results suggest that chloroplast movement could play a role in *Codium*, possibly modulating the photon absorption dependent on the incident irradiance (Lichtenberg, unpubl.; Fig. 2).
Phototactic movement has also been described in many microphytobenthic systems, where motile algae and bacteria (e.g. gliding diatoms or cyanobacteria) can migrate to an optimal position where light is neither limiting nor inhibiting (Richardson & Castenholz, 1987; Serodio et al., 2006; Coelho et al., 2011; Cartaxana et al., 2016). The movement regulates the radiance experienced by individual cells but can also change the community light environment by altering the composition of pigments and by changing the proportion of biomass present in a given horizontal layer (Chapter 6). In leaves, chloroplast movement is restricted by cell walls and, therefore, chloroplasts move either to – or away from the periclinal walls to maximize light exposure or increase transmittance (Gorton et al., 1999). However, in microphytobenthic systems cells are not restricted by such borders and individual cells migrate toward their optimal light environment, which can change the photosynthetic efficiencies of individual cells as well as the overall radiative energy balance (Chapter 6). However, some systems are vertically restricted by chemical parameters where e.g. high concentrations of toxic compounds such as H₂S, or anoxic conditions can determine the spatial borders for the migration of oxygenic phototrophs (Chapter 6). The advantages of migration are obvious and can create a competitive advantage in highly dynamic and dense systems where large diel variations in irradiance and chemical micro-landscapes exist.

Figure 2: Possible light induced chloroplast movement in utricles of the siphonous green algae Codium fragile. The elongated cells (utricles) contain a central vacuole lined peripherally by cytoplasm containing a multitude of chloroplasts. Here three utricles are shown (oriented with the apical side upward) acclimated to either A) dark, B) low light (25 µmol photons m⁻² s⁻¹) or C) high light (400 µmol photons m⁻² s⁻¹) for 4 days before images were taken. Utricles were gently separated and imaged under a microscope (10x objective; IX-81, Olympus, Japan).
Photosymbiotic corals are spatially limited to habitats with appropriate light conditions. They inhabit a wide span of light-exposed habitats ranging from shallow reef flats where mid-day solar irradiance reaches >2000 µmol photons m⁻² s⁻¹ to shaded caves (Anthony & Hoegh-Guldberg, 2003) and >150 m deep waters (Bridge et al., 2013) in virtual darkness. Colonization of such a wide range of habitats is facilitated by the ability of corals to modulate and optimize their internal tissue light environment and thereby the light exposure of the zooxanthellae. The regulation of their internal light field serves to either filter out excess light that can be harmful to the algae, or to increase the photon flux reaching the algae in sun-exposed or shaded environments, respectively. There are several mechanisms by which the coral host regulates the light environment for its endosymbionts. The role of green fluorescent protein-like pigments (GFP) in coral photobiology has received growing attention, but at this point no universal function has been proved. Conversely, it seems that depending on species and in situ light environment, GFP influences tissue light environments in a multitude of ways and there is now evidence that it can function by i) screening out harmful UV and short-wavelength radiation (Salih et al., 2000; Smith et al., 2013), ii) converting short-wavelength radiation to longer wavelengths, which are more efficiently absorbed by the light-harvesting pigments of the symbiotic algae (Schlichter et al., 1986; Schlichter & Fricke, 1990) and, iii) increasing the scalar irradiance in the coral tissue due to wavelength dependent scattering and reflection from GFP-granules (Lyndby et al., 2016). In addition, there is also growing evidence that corals can modulate photon absorption by actively regulating surface reflection by tissue movement (Wangpraseurt et al., 2014; Wangpraseurt et al., 2016c). In low light, tissues are often in a relaxed state, while high light leads to tissue contraction (Levy et al., 2003) and it has been estimated that the tissue surface area varies with a factor of ~2 between the contracted vs. relaxed state (Wangpraseurt et al., 2016c). It is not unlikely that the role of GFP and tissue plasticity is tightly linked, as the structural organisation of GFP granules might change along with changes in the tissue contraction status. Thus, in high light the GFP granules could be organized to maximize reflection, while the dispersal of granules during low light would enhance the propagation and scattering of light in the tissue (Wangpraseurt et al., 2014; Lyndby et al., 2016).

Thus, corals display mechanisms that can regulate the internal light environment similarly to terrestrial leaves, where e.g. UV-absorbing pigments can screen out harmful radiation from sensitive cell compartments (Wagner et al., 2003). In addition, coral tissues can increase the internal scattering and modulate the absorptive properties by regulating surface reflection, which has the same effect as
regulating transmission by chloroplast movement in leaves (Gorton et al., 1999; Williams et al., 2003; Wangpraseurt et al., 2014; Wangpraseurt et al., 2016a).

Photoadaptation on a microscale

Photoadaptation to sun-exposed or shaded light environments in leaves usually occurs by regulating i) the chlorophyll content (Richardson et al., 1983), ii) the number and size of functional photosynthetic units (PSU) (Falkowski & Owens, 1980) and, iii) the chloroplast ultrastructure and the stacking of thylakoids (Lichtenthaler et al., 1981). Similarly, microalgae can photo-adapt to different light environments (Falkowski, 1980) and it was shown that the symbiotic dinoflagellate inhabiting coral tissues can adapt to a wide range of light intensities on a macroscale (Falkowski & Dubinsky, 1981), mainly by regulating Chl a content and the size of the functional PSU units. Migration as a response to light, as discussed above, is undoubtedly an important photo-protective mechanism but in immotile microphytobenthic communities it has been shown that upregulation of photoprotective xanthophylls are important (Cartaxana et al., 2016), and such different strategies for photoprotection is probably determined by the daily fluctuations in light environment and the associated metabolic costs (Murchie & Niyogi, 2011).

In this thesis we have shown that a strong stratification in the quantity- and quality of light exists on a microscale e.g. in dense photosynthetic biofilm where the light availability can change >25-fold over a vertical distance of 0.2 mm (Chapter 6). As microalgae can adapt to light environments on a macroscale (Richardson et al., 1983) it is also a possibility that photoadaptation can exist at a sub-millimeter scale in such dense systems (Jørgensen et al., 1987).

In corals, the light environment has been shown to affect photoadaptation on a colony-scale, where shaded colonies display thinner tissues, higher photosynthetic efficiency per unit biomass, and lower dark respiration rates (Anthony & Hoegh-Guldberg, 2003). Such results are now also evident on a tissue-scale where different photosynthetic capacities of oral and aboral tissue layers have been hypothesized (Brodersen et al., 2014; Lichtenberg et al., 2016; Wangpraseurt et al., 2016b). Despite the internal homogenization of light, strong vertical light gradients can be observed in thick tissueed corals where light quantity can be reduced up to 15-fold over tissues of ~1 mm thickness (Wangpraseurt et al., 2012; Wangpraseurt et al., 2016b). Despite this large difference in light environment between oral and aboral tissue layers it was shown that net carbon assimilation was only reduced 6.5-fold in the aboral tissue,
indicating an enhanced light-harvesting efficiency or reduced respiration rate of the aboral layer (Wangpraseurt et al., 2016b). Supporting evidence for this vertical stratification of photophysiological activity was shown in Chapter 3, where higher PSII electron transport rates (ETR) were found in aboral, rather than oral tissue layers when corrected for the in hospite light availability. Thus, coral tissues exhibit differential photo-adaptation on a microscale, similar to leaf adaxial and abaxial photosynthetic capacities (Evans & Vogelmann, 2003). Additionally, adaptations to light quality could play a role and possibly explain the found results in Chapter 3, where higher photosynthetic efficiencies were seen in aboral tissue layers. In this study we used red actinic irradiance and, as the light field is red shifted as a function of tissue depth due to the efficient absorption of blue light by Chl a and coral host pigments, a differential adaptation to light quality/spectral composition could be possible. Similar adaptive responses to low visible-light quantities have now been shown in endolithic biofilm communities (Trampe & Kühl, 2016) and biofilms growing on the underside of the didemnid ascidian Lissoclinum patella (Behrendt et al., 2012; Kühl et al., 2012), where the main light harvesting pigment Chl a is almost entirely substituted with Chl d which has an absorption peak in the far-red region. Far-red light is poorly absorbed by the main light harvesting pigments Chl a, b, c and accessory pigments and thus penetrates deeper in such photosynthetic systems (Kühl & Fenchel, 2000; Wangpraseurt et al., 2012). Whether such adaptation in corals is achieved by regulating e.g. pigment composition in individual algae already present in the tissue, or by preferentially populating oral- and aboral tissue layers with algae displaying different photosynthetic capacities, e.g. from different clades, seem an interesting and important new question to study the differential photoadaptation in coral tissues. However, it appears that photosynthetic microniches do exist, and that acclimation to local light-quantity and –quality can occur on a submillimeter scale.

Technical advances and future directions

The studies in this thesis have provided novel information on microgradients of chemical parameters and light availability as well as spectral composition in a range of aquatic photosynthetic systems; but they have also been confined by certain methodological limitations. As an example, microsensors are powerful tools for examining the internal microenvironment and quantifying photosynthesis and respiration within biofilms, sediments, corals and macroalgae (Chapters 2-8), but are also restricted in their measurement characteristics as they practically represent 1-dimensional snapshots. Thus, such measurements are vulnerable in very heterogenous or dynamic systems. However, the investigation of structural heterogeneities of microscale chemical landscapes can be conducted very detailed, spatially and temporally, by extensive use of microsensors (e.g. exemplified in Chapter 8). This can be a very
cumbersome task as upscaling to 2D/3D/4D of these stand-alone point measurements consumes considerable amounts of time. However, recent methodological advances in optical chemical sensors have made it possible to map chemical landscapes over complex surface topographies such as coral tissues, biofilm or around surface associated structures as epiphytes or hyaline hairs. Such optical chemical sensors involves an indicator dye that absorbs light at specific wavelengths and quenches energy by luminescence as a function of analyte concentration (McDonagh et al., 2008) and, are usually immobilized in planar foils, on optical fibers or in particles. These techniques have allowed detailed characterization of coral tissue O$_2$ dynamics by magnetized microparticles (Fabricius-Dyg et al., 2012) and by spray-painting O$_2$ sensitive nanoparticles onto the surface of coral tissues (Koren et al., 2016). Furthermore, detailed characterization of the rhizosphere O$_2$ and pH dynamics around seagrass roots have been achieved by incorporating optical nano sensor particles in artificial sediment mimicking the natural chemical composition of the sediment (Koren et al., 2015; Brodersen et al., 2016).

Hitherto, the effect of surface structure on the flow morphology and mass transfer dynamics between submerged surfaces and the water column have been studied using microsensors in a range of systems (Jørgensen and Des Marais (1990); Shapiro et al. (2014); Chapters 7 & 8). With the use optical sensor particles, significant improvements in spatial and temporal measurements of boundary layer dynamics could be achieved. The use of such optical sensors have a great potential as they can be immobilized in a range of different ways to suit the respective system. Future approaches to describe such dynamics could be to e.g. suspend O$_2$ sensitive nanoparticles in seawater and investigate the effect of flow on O$_2$ concentration landscapes. By illuminating with laser sheets, spectrally tuned to the absorbance of the indicator dye, sequential 2D images could be acquired and be used to construct 3D images of surface O$_2$ concentrations in characteristic flow regimes.

Photosynthesis

Previously, the application of PAM instruments has been limited to the surface of the investigated systems, except from a few papers using the microfiber PAM system (Schreiber et al., 1996; Terashima et al., 2009; Oguchi et al., 2011). The analysis of ‘open’ or ‘closed’ reaction centers, i.e. F and F$_{m}$, retrieved with surface based PAM measurements is challenging in the way that the information obtained is the sum of signals detected from an unknown volume, where signals close to the detector contribute more than signals further away. Thus, the integrated measurements of F$_{v}$/F$_{m}$ can present complex signals derived from cells which in reality have been exposed to different levels of actinic light, while assuming
an equal illumination of cells, and thus conveying ambiguous indications of physiological status. Light dependent overestimations of integrated fluorescence up to 40% have been described in microphytobenthic assemblages (Serodio, 2004).

The invention of the microfiber PAM system should in principle have created a solution to this problem as the detection of signals is brought down to the microscale, usually around 30-50 µm around the fiber tip depending on the tip geometry and optical density of the investigated system (Kühl, 2005). With this system high spatial resolution mapping of chlorophyll fluorescence parameters can be carried out on surfaces and internally but will also reflect the heterogeneity of the system due to the small measurement volume.

Measurements of PSII electron transport rates (ETR) require knowledge on the mean effective PAR, on the absorption cross-section and on the balance between PSI and PSII photochemistry. The effective mean PAR can be measured directly using scalar irradiance fiber-optical microprobes but are difficult to perform in thin, cohesive tissues and will be biased by tissue compression due to the physical impact of the microprobe. Alternatively, the mean effective PAR can be calculated, but that requires detailed measurements of the angular radiance distribution (Kühl & Jørgensen, 1994) and information on the cell-size distribution, the scattering phase function and scattering- and absorption coefficients, which are usually not trivial parameters to determine (Privoznik et al., 1978; Berberoglu et al., 2009; Klughammer & Schreiber, 2015), albeit recent experimental and theoretical advances in biomedical optics allow detailed characterization of tissue optics using combinations of optical reflection spectroscopy, optical coherence tomography and Monte Carlo simulations (Wang et al., 1995; Wangpraseurt et al., 2016a; Wangpraseurt et al., 2016c).

In this thesis we demonstrated a novel approach where internal variable chlorophyll fluorescence measurements were combined with profiles of scalar irradiance, both measured with fiber optic microprobes, to correct the internal PSII electron transport rates to the actual light availability (Chapters 3 and 5). Furthermore, we developed an approach (Chapter 10) to locally estimate the amount of photons absorbed which is equivalent to the factor of light availability multiplied by the absorption factor in calculations of PSII electron transport rates (Schreiber, 2004). But most importantly, we could quantify the extent in which the effective PSII quantum yield was affected by tissue light gradients. This novel information was gained by applying actinic irradiance in different geometries to a cross section of an aquatic macrophyte while detecting variable chlorophyll fluorescence in tissue layers exposed to a
gradient of actinic irradiance, in order to show that even during high incident irradiances on the surface of a thallus, the PSII quantum yield of the lower shaded thallus side is practically unaffected and displays effective quantum yields similar to the dark adapted state (Chapter 10).

The system developed here is readily applicable to many other types of dense tissues. As an example, the role of scattering, e.g. by GFP granules, on light propagation and photosynthetic gradients in coral tissues seem an obvious next step, and could be studied either on cross sections of live coral or on tissue phantoms with known GFP concentration or with characteristic scattering coefficients (Palmer & Ramanujam, 2006).

The spectral flexibility of the supercontinuum laser system used in this study, allows the use of this method in literally any photosynthetic system by tailoring the spectrum and thereby e.g. the role of specific accessory pigments in light propagation and photosynthesis could be investigated. We also propose that the method could be combined with planar optodes or nanoparticle based optodes for pH or O2 in order to get detailed maps of photosynthetic efficiencies with associated chemical microenvironments around e.g. single chloroplasts or in dense tissues.

**Thesis conclusion**

In conclusion, we have shown across a range of phylogenetic different photosynthetic systems that strong resource stratification and high photosynthetic rates can exist simultaneously, similar to what has been shown in terrestrial canopies. We have shown that the optical properties and the structural organization of phytoelements are important traits affecting the photosynthetic efficiencies and that a range of different regulating mechanisms can be described conceptually similar to plant canopy interactions. Among these are i) regulations in the internal propagation of light by modulating tissue/community structure, ii) modulation of absorptive properties by active movement of phytoelements depending on the light field, and iii) indications of microscale photoadaptation to *in vivo* light gradients that can potentially increase whole tissue/community light utilization.
References


Bridge TCL, Hughes TP, Guinotte JM, Bongaerts P. 2013. Commentary: Call to protect all coral reefs. Nature Climate Change 3: 528-530


Cartaxana P, Cruz S, Gameiro C, Kühl M. 2016. Regulation of intertidal microphytobenthos photosynthesis over a diel emersion period is strongly affected by diatom migration patterns. Frontiers in Microbiology 7:872


Microscale Canopy Interactions in Aquatic Phototrophs