Effects of Epiphytic Biofilm Activity on the Photosynthetic Activity, pH and Inorganic Carbon Microenvironment of Seagrass Leaves (Zostera marina L.)

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Effects of Epiphytic Biofilm Activity on the Photosynthetic Activity, pH and Inorganic Carbon Microenvironment of Seagrass Leaves (Zostera marina L.)

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Epiphytic biofilms on seagrass leaves can lead to extreme microenvironmental conditions for the encapsulated leaf limiting both its photosynthesis and respiration. Yet, little is known about how the biological activity of the biofilm itself changes the seagrass phyllosphere microenvironment and dynamics. We used microsensors to measure O2 concentrations and pH gradients and calculate fluxes of O2, CO2 and bicarbonate (HCO3−) around seagrass leaves (Z. marina L.) covered with artificial, inactive biofilms and natural epiphytic biofilms. A sterilized seawater-agar matrix was used to make an artificial “inactive” biofilm on seagrass leaves with the same thickness as the natural leaf epiphytic biofilm, which impeded turbulent exchange of gases but did not have microbial activity. We compared the concentration profiles and fluxes of O2 and inorganic carbon of the “active” and “inactive” biofilms to investigate the effect of microbial activity and molecular diffusion in seagrass leaf biofilms. In light, the O2 flux of leaves with inactive biofilm was only 31% of the leaves with active biofilm, indicating that the photosynthesis of the microbial community in the biofilm makes up the majority of O2 production in the leaf microenvironment. During darkness, the O2 concentration profiles and O2 fluxes were almost identical in the “active” and “inactive” biofilms. The pH profiles showed the same trend with an increase in pH of ~1.0 in the “active” biofilms and ~0.3 pH units in the “inactive” biofilms in the light, and both showing a decrease of ~0.3 pH units in darkness compared to the bulk seawater. Our measurements thus demonstrate strong photosynthesis in the epiphyte layer driving phyllosphere basification and inorganic carbon limitation. The calculated CO2 concentration on the leaf surface decreased to 0.09 µmol L−1 in the epiphytic biofilm in the light compared to leaf surface CO2 concentrations of 13.8 µmol L−1 on bare seagrass leaves, and the CO2 influxes were only 3.0% and 5.4% of O2 effluxes for leaves with “active” and “inactive” biofilm, respectively. Calculations also showed that HCO3− influxes in light accounted for 91-97% of the total inorganic carbon influx to the seagrass leaf, although the HCO3− utilization via CO2 concentration mechanisms is energy-consuming. Besides increasing mass...
INTRODUCTION

Seagrass meadows are important ecosystems in aquatic environments (Terrados and Borum, 2004). They are found along temperate and tropical coastlines on all continents besides Antarctica, with a documented areal cover of ~125,000 km² globally and an estimated cover of >160,000 km² with moderate or high confidence (Unsworth and Cullen-Unsworth, 2017; McKenzie et al., 2020). Seagrass meadows are highly productive habitats that maintain high biological diversity of invertebrates, fish and marine mammals, and represent important nursery grounds for juvenile fish (Terrados and Borum, 2004). Seagrass meadows filter terrestrial runoff, reducing pollution and exceeding nutrients (Short and Short, 1984; Lemmens et al., 1996). As a rooted plant, seagrass decreases turbidity and hinders sediment erosion or suspension (Short and Short, 1984) and store substantial amounts of carbon into the sediment (Duarte et al., 2005; Fourquarean et al., 2012).

Therefore, seagrass meadows are a considerable carbon sink and play an important role in mitigating climate change (Cullen-Unsworth and Unsworth, 2018). Since 1980, seagrass meadows have been disappearing worldwide at a rate of ~110 km² yr⁻¹ mainly due to coastal development, poor water quality and climate change as the main threats (Waycott et al., 2009). Protection of seagrass meadows and better understanding of seagrass ecology are key to reversing this decreasing trend.

Coastal eutrophication is a major threat to seagrass ecosystems (Sand-Jensen, 1977; Borum, 1985; Drake et al., 2003). It stimulates epiphyte overgrowth on seagrass leaves affecting the leaf microenvironment, leading to extreme leaf physicochemical conditions for the plant (Ruesink, 2016; Brodersen et al., 2020a; Brodersen et al., 2020b). The epiphytic biofilm microenvironment can e.g., become hyperoxic and basified in the light and turn hypoxic or even anoxic in the dark (Noisette et al., 2020; Brodersen et al., 2020a; Brodersen et al., 2020b). Thick epiphytic biofilms can attenuate more than 50% of the incident photon irradiance of photosynthetically active radiation (PAR; 400–700 nm) and induce leaf surface warming of about 0.6°C, i.e., three times higher than bare leaves without epiphytes in the light (Noisette et al., 2020). Such dramatic microenvironmental changes in the leaf phyllosphere impact the seagrass function and fitness, as well as, the microbial metabolism of the biofilm (Sand-Jensen, 1977; Brodersen et al., 2015a).

In Danish waters, bacteria, diatoms and brown algae are commonly present in the epiphytic community with green algae mostly found in spring, and cyanobacteria and red algae mostly present in autumn (Wium-Andersen and Borum, 1984).

The activity of the microbial community in the epiphytic biofilm is an important factor for driving the dynamic nature of the seagrass phyllosphere, where epiphyte respiration and photosynthesis strongly affect the concentrations of essential solutes and gases within the seagrass leaf microenvironment, and microbes compete for dissolved inorganic carbon during the daytime and intensely consume oxygen at night, leading to an often negative relationship between the epiphyte biomass and seagrass photosynthesis (Drake et al., 2003; Brodersen et al., 2015a; Brodersen et al., 2020a; Brodersen et al., 2020b; Noisette et al., 2020). Furthermore, phytotoxic compounds such as nitric oxide (NO) can be produced in the epiphytic biofilm during phyllosphere anoxia via anaerobic biochemical processes that can be detrimental to the seagrass leaf and plant (Noisette et al., 2020).

Epiphytes can also obstruct the flow over the leaf surface, impede turbulent transport and slow down solute exchange between the seagrass leaf and the surrounding water via molecular diffusion (Koch, 1994). The epiphyte layer thickness together with the diffusive boundary layer (DBL) thickness above the epiphyte/seawater interface constitutes the total diffusion distance (TDD) (Jørgensen and Revsbech, 1985; Jørgensen and Des Marais, 1990). The TDD on leaves with epiphytes is much larger (often up to 10 times thicker) than on bare leaves, and therefore extends the molecular diffusion time and restricts nutrients and gas exchange between seagrass leaves and the surrounding water (Koch, 1994; Noisette et al., 2020). The limited molecular transport impedes the supplement of substrates and metabolic products accumulate within the biofilm, which exacerbate the negative impact of the epiphytic microbiota. In the light, high pH and O₂ levels in the leaf microenvironment reduce the leaf photosynthetic efficiency due to carbon limitation and enhanced photorespiration, which negatively affects the fitness of the seagrass plant (Brodersen et al., 2020a; Brodersen et al., 2020b).

Within the basified epiphytic biofilm, CO₂ is rapidly consumed due to abiotic (e.g., high-pH induced change in the carbon speciation towards HCO₃⁻ and CO₃²⁻) and biotic (e.g., photosynthetic DIC assimilation) factors, and the carbonate species mostly exist in the form of HCO₃⁻ (Brodersen et al., 2020a). Most seagrass are able to utilize HCO₃⁻ as an inorganic carbon source for leaf photosynthesis by using CO₂ concentration mechanisms (CCM), such as facilitated by carbonic anhydrase (CA). However, CCMs are energy-consuming processes that reduce the leaf photosynthetic efficiency (Koch et al., 2013). Furthermore, high O₂ and low CO₂ levels enhance RuBisCO oxygenase activity promoting photorespiration, which further reduces the photosynthetic efficiency of seagrass leaves (Brodersen et al., 2020a). However, to which extent the biological activity of the epiphytic biofilm contributes to the phyllosphere dynamics and affects photosynthesis and inorganic carbon availability for the seagrass leaf remains largely unknown.

Keywords: biofilm, carbon, pH, photosynthesis, seagrass
In this study, we used a pre-sterilized agar matrix to simulate an “inactive” epiphytic biofilm on seagrass leaves, which impeded mass transfer but did not have microbial activity. We compared the natural “active” and artificial “inactive” biofilm of similar thickness to distinguish between effects of epiphytic microbial activity and mass transfer impedance on seagrass leaf photosynthesis and respiration, as well as the inorganic carbon availability. We also performed comparative measurements on bare seagrass leaves and leaves with the epiphytic biofilm removed.

MATERIALS AND METHODS

Seagrass Sampling

Specimens of Zostera marina L. with and without epiphytic biofilms on leaves were collected from shallow coastal waters (<2 m depth) at Julebæk, North Zealand, Denmark (56°03’29.2’’N; 12°34’40.7’’E) during spring (March – May, 2021). The seagrass and the sediment around the roots were transported to the laboratory at the nearby Marine Biological Section (Helsingør, University of Copenhagen, Denmark) and were kept in reservoirs with constantly aerated water (20°C; salinity = 18) under a 12h:12h light/dark cycle (at a photon irradiance of ~200 µmol photons m⁻² s⁻¹) provided by metal-halide lamps, PAR=400-700 nm).

Experimental Procedures and Leaf Encapsulation in Agar Matrix

Seagrass leaf fragments (~8 cm long, cut near the tip from healthy seagrass leaves of similar age with no signs of deterioration) were bend into a slight U-shape, where the ends were mounted on black glass slides with black electrical tape. Microsensor measurements were conducted on the upper side of the leaf fragments (Figures 1A, B). Microprofiles of O₂ concentration and pH were first measured (see below) on leaves with epiphytes. Then the epiphytic community was carefully removed with a surgical razorblade, and gradients were measured on the epiphyte-removed (epi-removed) leaves. After these initial measurements, the leaves were briefly placed into a melting agar solution (1.5% w/w, 40°C) using filter-sterilized seawater (0.2 µm, with a salinity of 18%) to have the agar matrix covering the leaf surface. Briefly, this was done in a custom-made mold with removable glass slides as side walls, which controlled the volume of agar solution to precisely adjust the thickness of the agar matrix (Figure 1C). Small screws were positioned beneath both ends of the bare seagrass leaf fragments to bend the leaf slightly (Figure 1C), which create a small range of distance from the surface of the agar to the leaf surface, allowing us to perform measurements precisely where the agar encapsulation and thereby the TDD was as thick as the former epiphytic community. The outer limit of the DBL was determined as where the linear O₂ concentration gradient intercepts with the extrapolated bulk water O₂ concentration, and the TDD as the distance between the outer limit of the DBL and the seagrass leaf tissue surface. The depth profiles of O₂ concentration and pH were also measured on bare leaves without visible biofilm growing on the surface. The O₂ concentration and pH gradients were measured on each sample in darkness (i.e., 0 µmol photons m⁻² s⁻¹) and in light (230 µmol photons m⁻² s⁻¹). Before measuring each profile, leaf samples were exposed to dark/light conditions for more than 30 minutes until the pH or O₂ concentration at the leaf surface kept constant for minimum 5 minutes; to ensure steady state conditions while microprofiling. The natural epiphytic biofilm was defined as “active” and the artificial biofilm as “inactive” due to the sterilization processes (i.e., using filter-sterilized seawater and boiling the agarose solution) during the casting procedure.

Microsensor Calibration and Measurements

We used O₂ and pH microsensors to measure concentration gradients and calculate chemical fluxes around the seagrass leaves. A Clark-type O₂ microsensor with a tip size of ~25 µm (OX-25, Unisense A/S, Denmark) was linearly calibrated from signal readings in 100% air saturated seawater and anoxic water (using an alkaline ascorbate solution) at experimental temperature and salinity. A pH microelectrode with a tip size of ~100 µm (pH-100, Unisense A/S, Denmark) was calibrated using sensor potential readings against a reference electrode (REF-RM; Unisense A/S, Denmark) in commercial pH buffer solutions (pH 4, pH 7 and pH 10; Hach.com), at experimental salinity and temperature. The length of the sensitive pH glass in pH-100 is 150-250 µm, which limits the spatial resolution in the vertical direction to about 150-250 µm.

The microsensors and the reference electrode were connected to a multichannel microsensor meter (Unisense A/S, Denmark),
and the microsensors were mounted on a motorized micromanipulator system (Unisense A/S, Denmark). Both the multichannel microsensor system and the micromanipulator were connected to a PC, where data acquisition and sensor positioning was controlled via software (SensorSuite Profiler v3.2, Unisense A/S, Denmark). Microsensor positioning was relative to the seagrass leaf surface, as determined by manually handling the micromanipulator while observing the leaf surface and microsensor tip via a stereo microscope.

All microsensor measurement were performed on seagrass leaf fragments fixed onto black glass slides in a flow-chamber supplied with a constant flow (~1 cm s⁻¹) of aerated seawater (at 18°C and a salinity of 18) pumped from a supporting aquarium tank beneath the flow chamber (Figure 1B). Further information on the experimental setup and procedure can be found in Brodersen et al. (2014) and Noisette et al. (2020). Three or more technical replicates were measured at nearby position on each leaf fragment originating from 5 seagrass plants with natural epiphytic biofilm on the leaves and 4 seagrass plants without epiphytic biofilm; i.e., bare leaves (n = 12-15).

**Total Inorganic Carbon Measurements**

We used a calibrated total organic carbon analyzer (TOC-L; Shimadzu, Japan) to measure the total dissolved inorganic carbon concentration (DIC) in the bulk seawater around the seagrass leaves during the experiment (n=3). Samples were acidified and sparged to convert DIC into gaseous CO₂ that was quantified with a non-dispersive infrared detector (NDIR).

**Microsensor Data Calculations**

Assuming that the carbonate system was in equilibrium and thus that pH and the calculated CO₂ and HCO₃⁻ profiles represent steady-state concentrations (De Beer et al., 1997; De Beer et al., 2000; Brodersen et al., 2020a), concentration profiles of CO₂ and HCO₃⁻ were calculated from the measured pH microprofiles and the measured DIC in the seawater (Dickson and Millero, 1987; Millero, 2010):

\[
\begin{align*}
\text{DIC} &= [\text{CO}_2] + [\text{HCO}_3^-] + [\text{CO}_3^{2-}], \\
[\text{HCO}_3^-] &= \frac{\text{DIC} \cdot k_1 \cdot [H^+]}{[H^+]^2 + k_1 \cdot [H^+] + k_2}, \\
[\text{CO}_2] &= \frac{\text{DIC} \cdot k_2}{[H^+]^2 + k_1 \cdot [H^+] + k_2},
\end{align*}
\]

where \(k_1\) is the equilibrium constant of CO₂ hydrolysis to bicarbonate and \(k_2\) is the equilibrium constant of bicarbonate to carbonate conversion at experimental temperature and salinity taken from Mehrbach et al. (1973), Dickson and Millero (1987), and Dickson (2010) \((k_1 = 9.952 \times 10^{-7}, k_2 = 5.482 \times 10^{-10}\), temperature of 18°C, salinity=18).

Fluxes of chemical species were calculated according to Fick’s first law of diffusion:

\[
J = -D \cdot \frac{\partial C}{\partial z},
\]

where \(D\) is the diffusion coefficient of either O₂, CO₂ and HCO₃⁻ in seawater at experimental temperature and salinity (tabulated values available at www.unisense.com), and \(\frac{\partial C}{\partial z}\) is the slope of the linear concentration gradient in the DBL.

**Data Analysis**

We conducted statistical analyses in SPSS (IBM SPSS Statistics 28.0.0). We used independent sample t-tests to assess (1) the differences of O₂ fluxes among the four treatments (i.e., leaf with epiphytes, epiphytes removed, leaf with agar encapsulation and bare leaf) and (2) the difference of carbon fluxes (i.e., CO₂ fluxes and HCO₃⁻ fluxes) of seagrass leaves with natural epiphytic biofilms and agar coatings (n=15). Furthermore, the confidence interval of the 95% confidence level was calculated using Student’s t-distribution to describe the range of our results.

**RESULT AND DISCUSSION**

Our experiments demonstrated that the presence and activity of epiphytic biofilms on seagrass leaves can induce major changes in the leaf phylosphere microenvironment during daytime, inducing hyperoxia and increased pH that limited CO₂ availability and reduced the photosynthetic activity and efficiency of the seagrass leaf.

**Phylosphere Diffusion Distance, O₂ and pH Dynamics**

The total diffusion distance (TDD = DBL + biofilm thickness) on leaves with epiphytes was 710 ± 91 µm, as compared to 105 ± 9 µm after removing the natural “active” epiphytic biofilm. The thickness of the agar matrix encapsulation and thus the TDD was the same as the thickness of the former epiphytic biofilm covered seagrass leaf. The DBL of bare leaves was 90 ± 6 µm thick. The biological variation in the TDDs and DBLs between the same type of leaf samples were mainly due to the respective thickness of the natural epiphytic biofilm and the surface roughness, respectively (Hurd, 2000; Brodersen et al., 2015a).

**Oxygen Dynamics**

Oxygen concentration profiles measurements on leaves with epiphytes showed that the oxygen concentration increased from 265 µmol L⁻¹ in the bulk 100% air saturated water to 552 µmol L⁻¹ at the leaf surface in the light, and decreased to 110 µmol L⁻¹ in the dark (Figure 2A and Table 1; n=5 biological replicates). Similar measurements on seagrass leaves covered with agar (i.e., “inactive” biofilm) showed a similar decrease in darkness, while the increase in oxygen concentration was much less in the light reaching 363 µmol L⁻¹ (Figure 2A and Table 1). A much smaller shift in oxygen concentration between dark and light conditions was measured on the surface of bare leaves and leaves with removed epiphytes surface (Figure 2B). The oxygen fluxes from the leaf surface into the active biofilm and from bare leaves to bulk water (i.e., 260 and 233 nmol cm⁻² h⁻¹, respectively) was 1.7 to 3.2-fold higher than fluxes from leaves after removal of epiphytes and bare seagrass leaves with agar in the light (Figure 6A; Tables 1 and 2). The oxygen uptake in the dark of bare leaves and leaves after removal of epiphytes was 1.3
to 1.6-fold higher in the dark (231 and 218 nmol cm\(^{-2}\) h\(^{-1}\), respectively), as compared to leaves with active or inactive epiphytic biofilms (Figure 6A; Tables 1 and 2). Such extreme diel changes in the leaf \(\text{O}_2\) microenvironment induced by epiphytic biofilm are similar to findings in recent studies (Brodersen et al., 2020a; Noisette et al., 2020).

**pH Dynamics**

The pH gradients in the active and inactive epiphytic biofilms on seagrass leaves were caused by the balance between photosynthetic CO\(_2\) uptake and respiratory CO\(_2\) production, as well as the proton transport. The pH of the bulk seawater was 7.9, and increased towards the leaf surface in light reaching a pH maximum of pH 8.9 and 8.2 at the surface of seagrass leaves with an active and inactive biofilm, respectively, while pH decreased to a pH 7.6 in the dark (Figure 3A and Table 1). Such marked phyllosphere basification in the epiphytic biofilm micro-understory is similar to previous studies (Brodersen et al., 2020a). We found no significant changes in pH on bare seagrass leaves and leaves after removal of their epiphytes (Figure 3B). This can to some extent be explained by the spatial resolution of the pH microsensor, which is about 150-250 \(\mu\)m and thus larger than the thickness of the DBL on bare leaves. This also strongly limited our CO\(_2\) and HCO\(_3\)\(^{-}\) calculations for these leaf types (see below).

**Effect of Epiphytes on Dissolved Inorganic Carbon Availability**

We calculated CO\(_2\) and HCO\(_3\)\(^{-}\) concentrations from measured pH microprofiles and the total concentration of dissolved inorganic carbon (DIC) in the seawater (Figures 4A and 5A). The total DIC in the seawater was 15.1 ± 0.4 mg L\(^{-1}\) (or 1257.2 ± 35 µmol L\(^{-1}\)). The CO\(_2\) concentration decreased from 14.8 to 0.1 µmol L\(^{-1}\) at the leaf surface under the natural epiphytic biofilm in the light (Figure 4A), as compared to leaf surface CO\(_2\) concentrations of 9.7 µmol L\(^{-1}\) and 13.8 µmol L\(^{-1}\) on leaves with inactive biofilm and on epiphyte-removed leaves, respectively (Figure 4 and Table 1). Compared to the leaves without biofilms, the relatively low CO\(_2\) concentration at the leaf surface under inactive and active biofilms (i.e., 9.7 and 0.1 µmol L\(^{-1}\), respectively) indicate a limited CO\(_2\) diffusion limiting the photosynthetic performance in the presence of biofilms.
availability and supply for the seagrass leaves (Figure 4A and Table 1). In the inactive biofilm, the thicker TDD reduced the CO₂ availability by 29.9%, whereas in the active biofilm the CO₂ availability was reduced by 99.3% in the light (Figure 4A). Moreover, the contribution of CO₂ to the total inorganic carbon influx to the seagrass/epiphyte community also decreased from 12.6% at the epiphyte/water interface (i.e., active biofilm surface), to 8.6% at the leaf/agar interface (i.e., the leaf surface under inactive biofilm), to only 3.3% at the leaf/epiphyte interface (i.e., the leaf surface under the active biofilm). Thus, indicating strong CO₂ consumption by the epiphytic biofilm community markedly reducing the CO₂ availability for the underlying seagrass leaf. The low CO₂ availability at the seagrass leaf surface is thus likely a combined effect of (1) a high-pH induced change in the phyllosphere carbonate system speciation towards bicarbonate, (2) diffusion-limited CO₂ supply to the leaf surface due to thick total diffusion pathways induced by the epiphytic layer, and (3) CO₂ fixation by the photosynthetic organisms within the leaf epiphytic biofilm itself.

The HCO₃⁻ concentration decreased from 1235 ± 35 to 920 ± 37 μmol L⁻¹ through the active epiphytic biofilm in the light, as compared to a leaf surface HCO₃⁻ concentration of 1170 ± 19 μmol L⁻¹ under the inactive biofilm and 1264 ± 2 μmol L⁻¹ on the bare leaf surface (Figure 5 and Table 1). The HCO₃⁻ accounted for 91.4 and 96.7% of the total inorganic carbon flux into the seagrass leaf under the artificial inactive biofilm and active epiphytic biofilm, respectively (Figures 6B, D and Table 1). Furthermore, the total inorganic carbon flux (i.e., the sum of CO₂ and HCO₃⁻ fluxes) was 234 ± 8 nmol cm⁻² h⁻¹ on leaves with epiphytes, which was 4.6-fold higher compared to the flux on leaves with artificial inactive biofilm (p<0.001), and was consistent with the HCO₃⁻ influx was 4.9-fold higher than in leaves with inactive biofilm (p=0.001, Figures 6C, D and Tables 1, 2).

For the CO₂ and HCO₃⁻ calculation, we assumed a constant DIC concentration and that the carbonate system was in equilibrium within the biofilm/agar. However, as the CO₂ was rapidly produced or consumed and the equilibrium between CO₂ and HCO₃⁻ is slow, the carbonate system may be out of equilibrium in the steep gradient along the biofilm (because of different diffusivities of CO₂ and HCO₃⁻; i.e., diffusion coefficient of 1.5274·10⁻⁵ cm² s⁻¹ for CO₂ and 0.9689·10⁻⁵ cm² s⁻¹ for HCO₃⁻ at experimental temperature and salinity), which could induce an error to the calculations (De Beer et al., 1997). Therefore, the pH measurements were first conducted when the pH signal was stable to minimize this uncertainty (e.g., De Beer et al., 2000; Brodersen et al., 2020a).

Epiphytic Microenvironment and Leaf Photosynthesis
The leaf epiphyte community showed higher net photosynthetic rates as compared to leaves with inactive biofilm, where the

### TABLE 2 | Results of the independent sample t-tests (p-value) on O₂ fluxes among our four treatments (i.e., leaves with epiphytes, epiphytes removed, leaves with agar encapsulation and bare leaves) and carbon fluxes (i.e., CO₂ fluxes and HCO₃⁻ fluxes) on leaves with epiphytes and leaves with agar encapsulation.

<table>
<thead>
<tr>
<th></th>
<th>O₂ flux</th>
<th></th>
<th>CO₂ flux</th>
<th></th>
<th>HCO₃⁻ flux</th>
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<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>epi</td>
<td>epi-removed + agar</td>
<td>0.008</td>
<td>0.287</td>
<td>epi-removed + agar</td>
<td>0.045</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>epi-removed</td>
<td>0.016</td>
<td>0.027</td>
<td>epi-removed</td>
<td>0.012</td>
<td>0.028</td>
</tr>
<tr>
<td>bare leaf</td>
<td>epi-removed</td>
<td>0.005</td>
<td>0.364</td>
<td>bare leaf</td>
<td>0.006</td>
<td>0.202</td>
</tr>
</tbody>
</table>

![Figure 3](image-url) | pH profiles measured on seagrass leaves with epiphytes (epi) and leaves where epiphytes were removed (epi-removed) and covered with agar (A), as well as on bare leaves and leaves where epiphytes were removed (B) in darkness (0 μmol photons m⁻² s⁻¹) and light (230 μmol photons m⁻² s⁻¹). The error bars indicate SEM; n=15 (replicates with epi, epi-removed and epi-removed + agar), n=12 (bare leaves). Note the different scale of the x- and y-axis between panels. Dashed line indicates the total diffusion distance (i.e., the surface of the leaf, biofilm or agar + the diffusive boundary layer).
oxygen flux of leaves with inactive biofilm was 31% of the leaf with active biofilm (p=0.008; Figure 6A and Tables 1, 2). The photosynthesis of the microbial community thus composed most of the oxygen production in the seagrass leaf microenvironment (about 69%); which might actually be underestimated as the epiphyte micro-understory is receiving less light of lower quality owing to shading effects and predominantly blue and red light absorption in the uppermost part of the natural epiphytic biofilm (Brodersen et al., 2015a; Brodersen and Kühl, 2022). The oxygen concentration profiles and oxygen flux were almost the same in active and inactive biofilm during darkness (p=0.287; Figures 2A, 6A and Tables 1, 2), indicating that the activity of the microbial community in the epiphytic biofilm contributed little to the respiration of the combined leaf/epiphyte community. The oxygen concentration gradient was thus mainly formed because of the effect of the TDD on molecular diffusion in our experiments. The apparent low dark respiration in the natural biofilm could be due to low bacterial biomass and/or that dark respiration often only accounts for ~10% of photosynthesis rates at light-saturation in microalgae (Geider & Osborne, 1989), and we note that other epiphytic biofilms might show a stronger effect on O2 availability for the seagrass leaf in darkness due to higher respiration rates.

The total inorganic carbon influxes across the seagrass leaves were of similar magnitude as the corresponding oxygen effluxes for leaves with epiphytes and leaves with inactive biofilm in the light (i.e., CO2/O2 flux ratios of about 0.90 and 0.63, respectively) (Figures 6A, B and Table 1). However, the CO2 influxes were
activity. Here, the O2 effluxes into seagrass leaf surfaces (A), the estimated carbon fluxes as the sum of CO2 and HCO3− fluxes at leaf surfaces (B), the estimated CO2 fluxes and HCO3− fluxes from leaf to epiphyte, from epiphyte to water and from leaf to agar (C, D, respectively) in darkness (0 μmol photons m−2 s−1, blue) and light (230 μmol photons m−2 s−1, orange). The error bars indicate the confidence interval of 95% confidence level according to the Student’s t distribution; n = 15 (replicates with epi, epi-removed and epi-removed + agar), n = 12 (bare leaves). Positive values denote efflux and negative values denote influx across the respective interface. Note in panel b, that it was not really possible to calculate the total carbon flux for leaves with the epiphytes removed and for the bare leaves, both with very thin DBLs, due to the limited spatial resolution of the pH microsensor (mean values are marked with red color code).

FIGURE 6 | Measured O2 fluxes at seagrass leaf surfaces (A), the estimated carbon fluxes as the sum of CO2 and HCO3− fluxes at leaf surfaces (B), the estimated CO2 fluxes and HCO3− fluxes from leaf to epiphyte, from epiphyte to water and from leaf to agar (C, D, respectively) in darkness (0 μmol photons m−2 s−1, blue) and light (230 μmol photons m−2 s−1, orange). The error bars indicate the confidence interval of 95% confidence level according to the Student’s t distribution; n = 15 (replicates with epi, epi-removed and epi-removed + agar), n = 12 (bare leaves). Positive values denote efflux and negative values denote influx across the respective interface. Note in panel b, that it was not really possible to calculate the total carbon flux for leaves with the epiphytes removed and for the bare leaves, both with very thin DBLs, due to the limited spatial resolution of the pH microsensor (mean values are marked with red color code).

only 3.0% and 5.4% of oxygen effluxes for leaves with active epiphytic biofilm and leaves with inactive biofilm, respectively (Figures 6A, C and Table 1); i.e., similar to findings in a previous study on Z. marina leaves with epiphytic biofilms (Brodersen et al., 2020a) and other marine organisms like symbiont-bearing foraminifera (Köhler-Rink and Kühl, 2005) and corals (De Beer et al., 2000). Some of this discrepancy can be explained by (1) that O2 acts as an alternative electron acceptor to CO2 in photosynthesis via photorespiration in Z. marina plants (Buapet and Björk, 2016), and/or (2) that the O2/CO2 ratio at the site of RuBisCo differs from the leaf surface concentrations and fluxes due to internal carbon concentration mechanisms involving enhanced local enzymatic dehydration of HCO3− to CO2 facilitated by CA, (Köhler-Rink and Kühl, 2005; De Beer et al., 2000), as well as CO2 supply mechanism likely involving the aerenchyma. The leaf or leaf/epiphyte community thus consumed CO2 rapidly in light, and especially the natural active biofilm strongly impeded CO2 supply from the bulk seawater to the underlying leaf, causing low CO2 concentration and low CO2 influxes into seagrass leaves with epiphytes. Such DIC competition between plants and epiphytic biofilms is similar to other aquatic plants (e.g., Jones et al., 2002; Wijewardene et al., 2022) and makes seagrass highly dependent on HCO3− utilization. The low CO2 availability limits seagrass leaf photosynthesis (Figure 6A) when comparing the O2 production of epiphyte-removed leaves and the epiphyte-removed and agar encapsulated (i.e., inactive biofilm) leaves, which both excluded microbial activity. Here, the O2 efflux on epiphyte-removed leaves was 1.7-fold higher than from the leaves with inactive biofilms (p = 0.016; Figure 6A and Tables 1, 2), which can be attributed to the reduced-CO2 leaf microenvironment. Furthermore, the O2 efflux of epiphyte-removed leaves was only 58% compared to natural bare leaves (p = 0.005; Figure 6A and Tables 1, 2) indicating that leaves under epiphytic biofilms were of lower fitness and health; although the respiration was of similar order of magnitude (p = 0.364; Figure 6A and Tables 1, 2).

The activity of epiphytic biofilm thus results in dramatic changes in the seagrass leaf microenvironment, as O2 concentration and pH increased in light, and most DIC (73.2-98.2%) facilitated by CA existed in the form of HCO3− within the basified phyllosphere. Most seagrasses including Zostera marina L. are able to utilize HCO3− as source of inorganic carbon in photosynthesis (Koch et al., 2013), and our results showed that the HCO3− influx was 29.5-fold higher than the CO2 influx in light, accounting for 96.7% of the total inorganic carbon influx to the leaf surface under the active biofilm (Figures 6B, C and Table 1). However, the direct uptake of HCO3− and intracellular conversion of HCO3− to CO2 or extracellular conversion by extracellular carbonic anhydrase are all energy-consuming CO2 concentration mechanisms (CCM), reducing the efficiency of carbon assimilation and energy storage (Lucas, 1985; Larkum et al., 1989; Larsson and Axelsson, 1999; Hellblom et al., 2001; Beer et al., 2002; Hellblom and Axelsson, 2003). Another energy-expensive potential CCM is using proton pumps to locally acidify the leaf surface, which for seagrasses have been suggested several times in previous studies (e.g., Beer et al., 2002; Borum et al.,
Here, the hypothesis is that acid zones on the leaf surface of marine plants increase the CO₂ availability for photosynthesis-driven carbon fixation through local stimulation of CO₂ formation, via low-pH driven conversion of HCO₃⁻ to CO₂, within the leaf diffusive boundary layers by proton (H⁺) extrusion (Beer et al., 2002). However, our detailed pH measurements in high spatiotemporal resolution (~0.1 mm) within the leaf DBL did not show any indications of a general acidification of the seagrass leaf DBL as a possible CO₂ concentration mechanism (Figure 3); even not in the pH measurements on leaves with agar that artificially increased the TDD and thereby allowed for several point measurements with the pH microsensor within the artificially increased proton diffusion pathway. However, if such leaf surface acidification mechanism exists, as indicated by utilizing pH buffers in previous studies (Larkum et al., 2017), it must be very localized within separated leaf micro-acid zones. In darkness, the respiration of seagrass leaves and epiphytic biofilm lead to hypoxic conditions on the leaf surface driven by the DBL and TDD decreasing the diffusive O₂ supply from the surrounding water to the plant. Such decreased availability of oxygen makes seagrass more susceptible to suffocation and H₂S intrusion from the surrounding sediment due to restricted intra-diffusional O₂ transport to below-ground tissues via the aerenchyma (Brodersen et al., 2015b). Under global warming, such inadequate internal tissue and rhizosphere aeration may be further aggravated owing to temperature-induced enhanced epiphyte and leaf respiration.

In a previous study of epiphytic biofilms on Z. marina L., the pH reached 9.6 in a very dense and thick epiphytic biofilm (1.2 mm) in the light (300 μmol photons m⁻² s⁻¹), and CO₂ microsensor measurements showed that the CO₂ concentration decreased to 0 μmol L⁻¹ ~0.2 mm above the leaf surface, which is consistent with our measurements and calculations (Brodersen et al., 2020a). High O₂ and low CO₂ concentrations on the leaf surface reduce the O₂ efflux and CO₂ influx, causing O₂ accumulation intracellularly and low intracellular CO₂ concentrations, which impede leaf photosynthesis and enhance photorespiration (Mass et al., 2010), thus representing a threat to seagrass photosynthetic performance and overall health (Sand-Jensen et al., 1992; Raven et al., 2014; Buapet and Björk, 2016). Furthermore, a previous study demonstrated reduced seagrass gross photosynthetic rates at high pH, high O₂ and low DIC seawater, with a maximum decrease in gross photosynthetic rate of 75% detected in Zostera marina; thus, further supporting our finding that the basified leaf microenvironment induced by epiphytic biofilms in the light has strong negative impacts on seagrass leaf photosynthesis (Buapet and Björk, 2016). Combined with increased respiration under global warming, such epiphyte-induced reduced leaf photosynthesis may lead to a negative oxygen and carbon balance in exposed seagrass plants; putting key ecosystem services at risk of ceasing. Eutrophication stimulates epiphytic biofilm overgrowth of seagrass leaves (Borum, 1985), where a previous study has shown 3 to 5-fold higher epiphyte biomass in nitrogen and phosphorus-enriched seawater compared to ambient seawater (Jaschinski and Sommer, 2008). Coastal eutrophication is therefore likely to put more stress on exposed seagrass meadows. However, it is important to mention that epiphytes can also be beneficial to the plant hosts. Microorganisms, can e.g., facilitate uptake of dissolved organic nitrogen by seagrass leaves (Tarquinio et al., 2018).

In conclusion, we found that microbial activity in epiphytic biofilms mostly affected the seagrass leaf chemical microenvironment in the light: causing (1) high O₂ and pH conditions and (2) strong consumption of inorganic carbon, which leads to low carbon availability for the seagrass plant. Furthermore, leaves with the epiphytes removed produced less oxygen than the bare leaf and thus exhibited lower photosynthetic capacity. In the basified phyllosphere, the relatively low CO₂ concentration and thus intensified demand for energy-requiring HCO₃⁻ utilization limited leaf photosynthesis, and the high O₂ and low CO₂ concentration in the cell will increase photorespiration and further reduce the photosynthetic efficiency. In eutrophic coastal waters, the overgrowth of seagrass leaves with epiphytic biofilm resulting in thicker TDD and strong microbial activity can thus lead to intense diurnal changes in the seagrass leaf chemical microenvironment that can have negative impacts on seagrass performance and therefore is a potential hazard for seagrass fitness and vital ecosystem function.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

KB and MK designed experiments and provided essential infrastructure. QZ performed the experiments and analyzed the data (supervised by KB). QZ wrote the manuscript with editorial help from all co-authors. All authors have given approval to the final version of the manuscript.

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