

RESEARCH ARTICLE

Resource constraints highlight complex microbial interactions during lake biofilm development

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Abstract

1. This study evaluated how the availability of nutrients and organic carbon interact to influence the associations between autotrophic and heterotrophic micro-organisms during lake biofilm development. Considering that decomposers are often better competitors for nutrients than producers in aquatic environments, we hypothesized that heterotrophs would outcompete autotrophs for available nutrients unless heterotrophs were limited by organic carbon provided by autotrophs.
2. To test our hypothesis, we evaluated autotrophic (algae) and heterotrophic (fungi, bacteria) biomass in response to a factorial enrichment of nutrients (nitrogen and phosphorus in combination) and glucose using nutrient-diffusing substrates with either inorganic or organic discs in a subalpine lake. In the field, nutrient-diffusing substrates were exposed to either natural sunlight or placed under a darkened experimental canopy to evaluate the response of heterotrophs to nutrients and carbon subsidies in the presence or absence of algae. We expected that heterotrophs would be limited by organic carbon on inorganic substrates in the absence of autotrophic production (i.e., dark treatments), and that organic substrates would provide a carbon subsidy for heterotrophic metabolism.
3. Fungi were stimulated by nutrient enrichment on inorganic substrates in the presence of algae (light treatment), but not in the dark (without algae). The response of fungi to algal presence on inorganic substrates was similar in magnitude to the response of fungi to nutrients and glucose substrates incubated in the dark. In contrast to our expectations, elevated algal biomass did not stimulate heterotrophic bacteria in the presence of elevated nutrient levels on inorganic substrates, possibly owing to antagonistic interactions between bacteria and fungi.
4. The positive effect of nutrients on algal biomass was significantly reduced in favour of heterotrophs when nutrients were combined with glucose, suggesting that heterotrophs were able to outcompete algae for available nutrients in the absence of carbon limitation.
5. *Synthesis*. These results expand our understanding of how the availability of limiting resources governs the outcomes of complex interactions among micro-organisms in aquatic biofilms, and suggests that background levels of organic carbon

regulate producer and decomposer responses to nutrient availability during biofilm development.

KEYWORDS

alpine, bacteria, benthic algae, carbon subsidies, fungi, nutrients, producer–decomposer interactions, substrate

1 | INTRODUCTION

Biofilms are comprised of an assemblage of autotrophic and heterotrophic micro-organisms that interacts within a polysaccharide matrix in aquatic environments. The autotrophic component is comprised primarily of microalgae (including cyanobacteria), which acquire inorganic carbon to build organic compounds through photosynthesis. The heterotrophic component, mainly fungi and bacteria, use organic carbon fixed by algae as a source of energy within the surrounding biofilm (Kuehn, Francoeur, Findlay, & Neely, 2014; Miura & Urabe, 2017; Soares, Kritzberg, & Rousk, 2017; Wagner, Bengtsson, Findlay, Battin, & Ulseth, 2017). In such associations, where algae provide organic substrates to heterotrophs, algae are thought to benefit from the transformation of organic nutrients into inorganic forms (i.e., mineralization) during decomposition (Battin, Besemer, Bengtsson, Romani, & Packmann, 2016; Haack & McFeters, 1982). This mutually beneficial interaction is often credited for the close association between autotrophs and heterotrophs in biofilms (Rier & Stevenson, 2001; Wyatt & Turetsky, 2015) and forms the foundation for material cycling in shallow aquatic ecosystems.

The level of interplay between autotrophs and heterotrophs varies across aquatic ecosystems, and depends in part on the availability of nutrients within the environment. Although autotrophs and heterotrophs can have mutualistic metabolism, they require many of the same inorganic nutrients (Cotner & Wetzel, 1992), which are often limiting in aquatic ecosystems (Borchardt, 1996; Bracken et al., 2015; Elser et al., 2007). Compared to autotrophs, heterotrophs have a greater affinity for nutrient resources, especially phosphorus (Currie & Kalff, 1984), and can outcompete autotrophs in low phosphorus environments (Jansson, 1993; Joint et al., 2002; Rhee, 1972). Nevertheless, close associations between autotrophs and heterotrophs continue to occur in low nutrient environments (Scott, Back, Taylor, & King, 2008; Scott & Doyle, 2006), suggesting that energetic requirements for photosynthetic products prevent competitive exclusion by heterotrophs. Conversely, autotrophs and heterotrophs are often uncoupled in environments where energetic requirements are met by outside carbon sources owing to the ability for heterotrophs to outcompete autotrophs for available nutrients in the absence of carbon limitation (Bechtold, Marcarelli, Baxter, & Inouye, 2012; Klug, 2005; Stets & Cotner, 2008).

Despite the metabolic linkages between primary production and decomposition, these two processes are often evaluated independently in aquatic biofilms, with most studies focusing on autotrophic activities alone. This is particularly true for lakes, where

research on producer–decomposer interactions has focused overwhelmingly on planktonic assemblages and benthic environments have been considered mainly in regard to their exchange of nutrients with the pelagic zone (Vadeboncoeur, Vander Zanden, & Lodge, 2002). Relatively few studies have evaluated the effect of nutrient supply on heterotrophic activity in lake biofilms, despite the established role of decomposers in biofilm function in other aquatic environments (e.g., Battin, Kaplan, Newbold, & Hansen, 2003; Kalscheur, Rojas, Peterson, Kelly, & Gray, 2012). Furthermore, research aimed to simultaneously test for nutrient limitation of both autotrophs and heterotrophs in biofilms may have inadvertently favoured autotrophic production by limiting sampling to inorganic substrates (i.e., rocks), which selects for a largely autotrophic community (Johnson, Tank, & Dodds, 2009). Consequently, the involvement of nutrients in the association between producers and decomposers in aquatic biofilms is largely unknown, making it difficult to accurately predict how routes of energy flow vary among lake ecosystems or how an individual ecosystem may be altered by environmental change.

The goal of this study was to evaluate how the availability of nutrients and organic carbon interacts to influence the balance between autotrophic and heterotrophic micro-organisms in natural lake biofilm communities. We hypothesized that heterotrophs would outcompete autotrophs for available nutrients unless heterotrophs were foremost limited by organic carbon provided by autotrophs or if nutrient levels surpassed their combined energetic requirements. To test our hypotheses, we evaluated autotrophic (microalgae) and heterotrophic (fungi, bacteria) biomass in response to a factorial enrichment of nutrients and glucose using nutrient-diffusing substrates (NDS) with either inorganic or organic discs in a subalpine lake. Our expectation was that the organic disc would act as a carbon subsidy for heterotrophs. Substrates were exposed to either light or dark conditions to evaluate the response of heterotrophs to nutrients and carbon subsidies in the presence and absence of algae, respectively. We predicted that: i) greater nutrient availability in light treatments would indirectly promote heterotrophic biomass on inorganic surfaces by increasing carbon subsidies available during periods of elevated autotrophic production, ii) nutrient enrichment would not promote heterotrophic biomass on inorganic surfaces in the absence of algae (i.e., in dark treatments) or in the absence of glucose enrichment owing to carbon limitation, and iii) carbon subsidies would promote heterotrophs in dark treatments and reduce producer–decomposer coupling in light treatments owing to the ability for heterotrophs to outcompete algae for available nutrients in the absence of carbon limitation.

2 | MATERIALS AND METHODS

2.1 | Site description

This study was conducted in the nearshore area of Castle Lake, an oligotrophic subalpine lake located within a protected glacial cirque basin in the Siskiyou Mountains of Northern California (41°13'N, 122°22'W; elevation = 1657 m). Castle Lake has a surface area of 0.2 km², approximately 54% of which is littoral zone with a depth of 3–5 m surrounding a basin with a maximum depth of 35 m (Axler & Reuter, 1996; Vander Zanden, Chandra, Park, Vadeboncoeur, & Goldman, 2006). The lake is ice covered from November to June (> 200 days) and receives nutrient inputs primarily from annual snowmelt and rainfall and water column, pooled mixed layer dissolved nutrient concentrations during the ice free season are typically <10 µg/L for nitrogen (NO₃-N + NO₂-N + NH₄-N) and phosphate-P (PO₄-P) (Higley, Carrick, Brett, Luecke, & Goldman, 2001; Müller-Solger, Brett, Luecke, Elser, & Goldman, 1997). The high transparency of the lake (Secchi depth ranges from 9 to 13 m) permits photosynthesis at depths up to 25 m (Axler & Reuter, 1996; Higley et al., 2001).

2.2 | Experimental design

We used a full factorial design with and without nutrients (N and P in combination), with and without glucose, and with and without sunlight (light and dark conditions, respectively) to examine the mechanisms driving interactions between algae, fungi, and bacteria on both inorganic and organic substrates. We used four planks as a base to replicate each treatment, resulting in an $n = 4$ for each treatment combination. We constructed NDS by filling 60 ml transparent plastic canisters (LA Container Inc., Yorba Linda, CA) with either agar (control treatment), agar + 0.5 M glucose (G treatment), agar + 0.5 M KNO₃ + 0.5 M KH₂PO₄ (NP treatment), or agar + all three (NP + G

treatment) (Rier & Stevenson, 2002; Tank, Reisinger, & Rosi, 2017). nutrient-diffusing substrates were covered with either a fritted glass disc (Tank et al., 2017) or a 1-mm thick untreated wood veneer disc (Tank & Dodds, 2003) to evaluate potential differences in biofilm development on inorganic (rock) and organic (wood) substrates, both of which occur on the lake bottom. Other studies have demonstrated that similar wood veneer discs provide a source of organic carbon to heterotrophs when used in combination with NDS (Tank & Dodds, 2003). Each disc was held in place by a tight-fitting cap with a 2.5-cm diameter circular hole cut to allow for biofilm growth.

nutrient-diffusing substrates were attached to the four planks and anchored to concrete blocks (Figure 1) submerged 25–30 cm below the surface along the south-facing shore, approximately 10 m from the shoreline. Each set of four NDS (i.e., subsamples of a single replicate) was positioned 30 cm apart and arranged so that each treatment was represented only once per plank (Figure 1). Light-transparent (L) NDS were positioned upright to receive ambient sunlight and dark (D) NDS were positioned upside-down and loosely skirted with dark polyester fabric that blocked >99% of incoming light to inhibit algal photosynthesis (Figure 1). The biofilm was allowed to colonize substrates for 20 days beginning 28 June 2017.

2.3 | Sampling and analytical methods

At the end of the experiment, autotrophic biofilm accumulation on substrates was measured as chlorophyll *a* concentration (a measure of algal biomass) and total algal biovolume. We carefully removed one set of NDS discs with forceps and stored them frozen in 50 ml centrifuge tubes until chlorophyll *a* analysis. Chlorophyll *a* was extracted directly from substrates with 90% buffered ethanol in the dark for 24 hr and analysed with an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, CA) at 665 and 750 nm after acidification to correct for phaeophytin (APHA, 1998). From

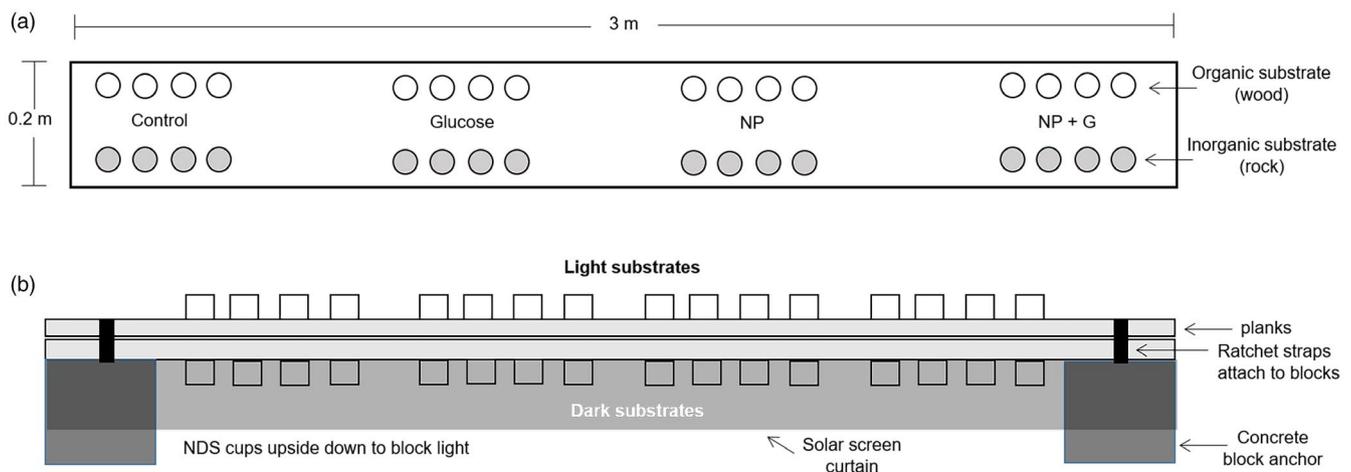


FIGURE 1 Schematic of experimental design viewed from the top (a) and from the side (b). nutrient-diffusing substrates (NDS) were attached to four planks and arranged so that each nutrient treatment was represented only once per plank. One plank represented a single replicate and four subsamples of each treatment. Half of the NDS within each treatment were covered with an inorganic fritted glass disc and half were covered with an organic wood veneer disc. Light-transparent NDS were positioned upright to receive ambient sunlight and dark NDS were positioned upside-down and loosely skirted with dark fabric that blocked >99% of incoming light to inhibit algae

a separate set of NDS discs, the biofilm was detached by vigorous scraping followed by brushing until no visible biofilm was present and the resulting material was preserved with 2% formalin for enumeration of algal cell density and total biovolume calculation. The preserved samples were homogenized with a vortex and an aliquot was pipetted into a Palmer-Maloney nanoplankton counting chamber and algal cell density was determined by counting ≥ 300 natural units per sample. Algal abundance was determined by dividing cell density by the product of the area sampled and the proportion of the sample counted (Lowe & Laliberte, 2017). Biovolume ($\mu\text{m}^3 \text{cm}^{-2}$ of substrate) was calculated by multiplying algal abundance by the estimated cell volume using geometric formulae provided by Hillebrand, Dürselen, Kirschtel, Pollinger, and Zohary (1999). To calculate total biofilm biomass, algal biovolume measurements were converted to units carbon using the equation $\text{pg C cell}^{-1} = 0.109 \times (\text{cell volume})^{0.991}$ according to Montagnes, Berges, Harrison, and Taylor (1994).

Fungal biomass associated with biofilm samples was estimated from concentrations of the fungal membrane sterol, ergosterol (Gessner, 2005). A separate set of NDS discs was placed in 50 ml centrifuge tubes and frozen in the field for fungal analysis. In the laboratory, frozen substrates were lyophilized to dryness, weighed and ergosterol extracted in alcoholic KOH (0.8% KOH in HPLC grade methanol, total extraction volume 10 ml) for 30 min. at 80°C. The resultant crude extract was partitioned into n-pentane, evaporated to dryness with nitrogen gas, and the dried ergosterol residues were quantified using high-performance liquid chromatography (HPLC) (Su, Kuehn, & Phipps, 2015). Fungal biomass was calculated using a conversion factor of 10 μg ergosterol/mg fungal carbon, assuming 43% carbon in fungal dry mass (Kuehn, 2016).

Bacterial biomass was determined from substrates by direct counts with epifluorescence microscopy. The biofilm was detached from a separate set of NDS discs as described above and the resulting slurry was preserved in a 2% formalin solution. Preserved samples were homogenized with a vortex and an aliquot of the sample was stained with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980). The stained aliquot was vacuum-filtered onto a 0.2 μm pore-size black filter (Osmonic, Livermore, CA) and the bacterial cell density was determined by enumerating ≥ 300 cells or 25 fields of view per filter at 1000 \times magnification using a Leica DM 4000 microscope with fluorescence (Leica Microsystems, Wetzlar, Germany). Bacterial biomass was calculated by a bacterial abundance/biomass conversion factor of 35 fg C cell⁻¹ (Theil-Nielsen & Søndergaard, 1998).

Water temperature (°C) and light (measured as Lux and converted to $\mu\text{mol photons/m}^2 \text{s}^{-1}$ photosynthetically active radiation (PAR) using a constant of 0.019 according to the manufactures specifications) were monitored in light-transparent and dark treatments with HOBOTEMP data loggers (Onset Computer Corporation, Cape Cod, MA) at 2-hr intervals for the duration of the experiment. At the beginning of the experiment, we filtered duplicate lake water samples through a 0.45- μm syringe-driven filter (Millipore Corporation, Bedford, MA) into sterile 120 ml nalgene® bottles and analysed

samples for NO_3 and PO_4 using a Dionex ICS-3000 ion chromatograph (Dionex Corporation, Sunnyvale, CA) and dissolved organic carbon (DOC) using a Shimadzu TOC-V carbon analyzer (Shimadzu Scientific Instruments, Columbia, MD). Dissolved oxygen (DO), pH and conductivity were measured at the start of the experiment with a Hach HQ model 40d multiprobe (Hach Company, Loveland, CO).

2.4 | Statistical analyses

Four-way general linear models (GLM) were used to evaluate the effects of nutrients (with, without), glucose (with, without), substrate type (inorganic, organic) and light (light-transparent, dark) on algal biomass (chlorophyll *a* and total biovolume) and bacterial biomass. Owing to unequal variance among treatment levels, a generalized linear model was used to evaluate the treatment effects on fungal biomass (Wilson & Grenfell, 1997). Total biofilm biomass ($\mu\text{g C cm}^{-2}$) was calculated by summing the carbon content of algae, bacteria and fungi within each treatment and differences in total biofilm biomass among treatments were evaluated with a four-way GLM. When GLM indicated significant differences among treatments ($p < 0.05$), *post hoc* least significant differences were used to make pairwise comparisons between factor levels. When necessary, data were $\log(x + 1)$ -transformed prior to analysis to correct for non-normal distribution and unequal variances among treatments. All statistical analyses were performed using SPSS 20 (SPSS, Chicago, IL).

3 | RESULTS

Nutrients had a stimulatory effect on algae, and the level of response depended on substrate composition and the presence or absence of glucose (Figure 2a,b). Algal biomass was significantly elevated by nutrient enrichment in the light-transparent treatments only (chlorophyll *a*: $F_{1,48} = 197.7$, $p < 0.0001$; total algal biovolume: $F_{1,48} = 228.9$, $p < 0.0001$), and the magnitude of algal biomass was greater on organic compared to inorganic substrates ($p < 0.0001$). Elevated levels of algal biomass were reflected in total biofilm biomass, which was greatest in the (NP)_L treatment ($F_{1,48} = 5.50$, $p = 0.02$) and twofold greater in organic compared to inorganic substrates ($p < 0.0001$; Figure 3). The positive effects of nutrients (NP)_L on algal biomass and total biofilm biomass were significantly reduced when nutrients were combined with glucose (NP + G)_L on both inorganic and organic substrates ($p \leq 0.03$; Figures 2a,b and 3a,b). There was no effect of substrate composition (inorganic, organic) or glucose on algal biomass in the absence of nutrient enrichment ($p > 0.05$) and algae were inhibited in dark treatments (chlorophyll *a* $\leq 0.05 \pm 0.02 \text{ mg/cm}^2$).

Fungi were elevated by nutrients in combination with carbon subsidies (wood, glucose) and by elevated algal biomass (Figure 2c). Fungi were stimulated by nutrients on organic substrates in both light (NP)_L and dark (NP)_D treatments ($p = 0.001$), and on inorganic substrates in the light (NP)_L (with elevated algae), but not in the dark (NP)_D (without algae). The effect of nutrients (NP)_L on fungal biomass was threefold greater in organic compared to inorganic

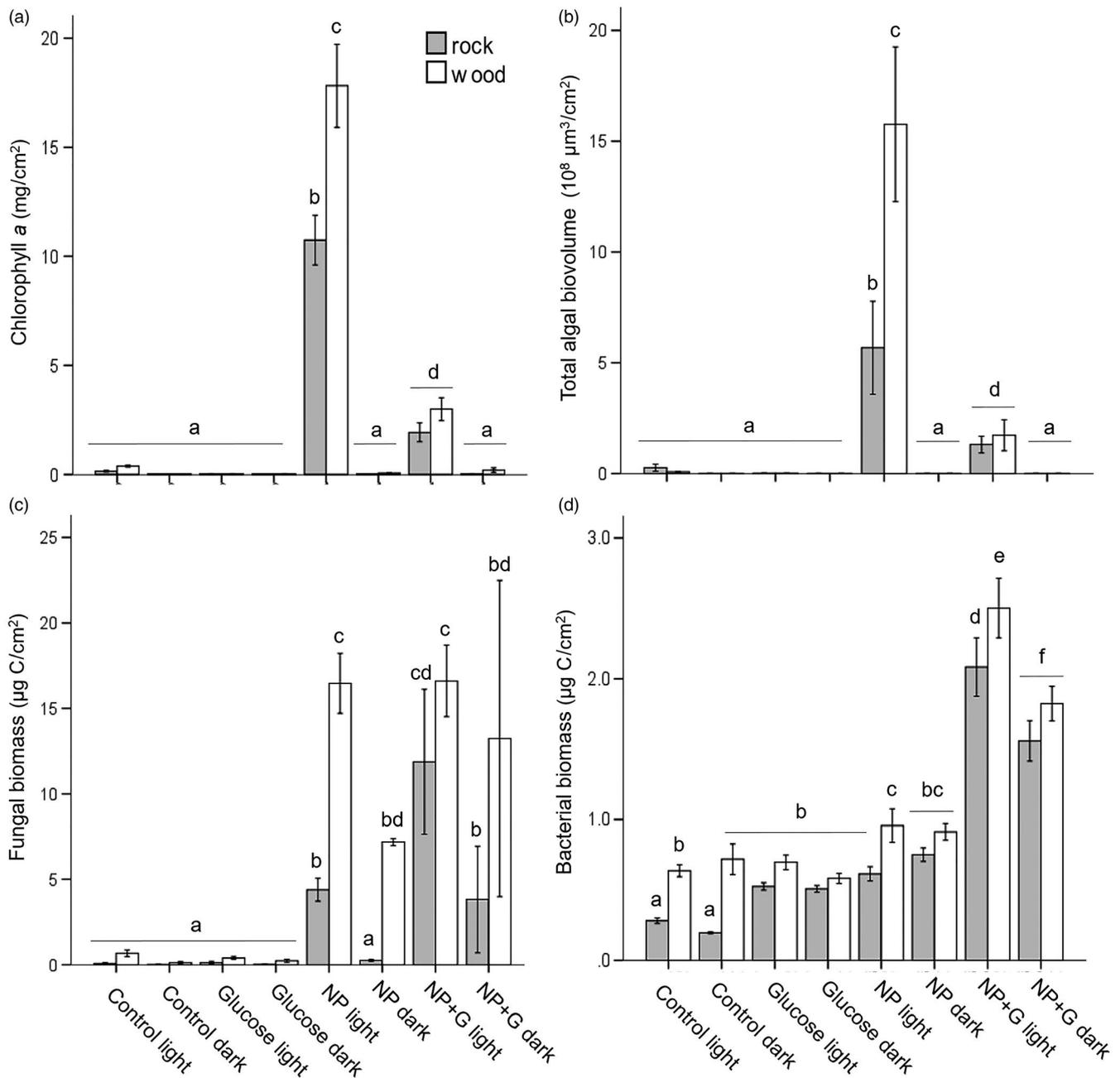


FIGURE 2 Mean ± 1 SE ($n = 4$) algal biomass measured as chlorophyll *a* (a), total algal biovolume (b), fungal biomass (c), and bacterial biomass (d) on nutrient-diffusing substrates enriched with either agar (control), glucose, nitrogen + phosphorus (NP), or a combination of all three (NP + G) in light and dark treatments on inorganic (rock) and organic (wood) substrates. Bars with the same letter are not significantly different among treatments ($\alpha = 0.05$)

substrates ($p = 0.001$; Figure 2c), resulting in a twofold increase in the fungal contribution to total biofilm biomass (Figure 3). A combination of nutrients and glucose promoted fungal biomass on inorganic substrates in the dark (NP + G)_D ($p = 0.03$), and the magnitude of the effect was similar to the (NP)_L treatment (with elevated algae). Organic substrates further increased fungal biomass compared to inorganic substrates in the (NP + G)_D treatment ($p = 0.04$), but not in the (NP + G)_L treatment ($p = 0.19$). There was no effect of substrate composition (inorganic, organic) or glucose enrichment on fungal biomass in the absence of nutrient enrichment ($p = 0.91$).

Heterotrophic bacteria were stimulated by nutrients, organic (wood) substrates and glucose, but not in the presence of elevated algae (Figure 2d). Bacterial biomass was significantly greater on organic substrates compared to inorganic substrates in the control ($p \leq 0.02$). Glucose enrichment alone also stimulated bacterial biomass on inorganic substrates compared to the control ($p = 0.04$), but there was no additional effect of glucose on organic substrates compared to the control ($p \geq 0.35$). Bacterial biomass had the greatest influence on total biofilm biomass in control and glucose treatments on both inorganic and organic substrates, where total

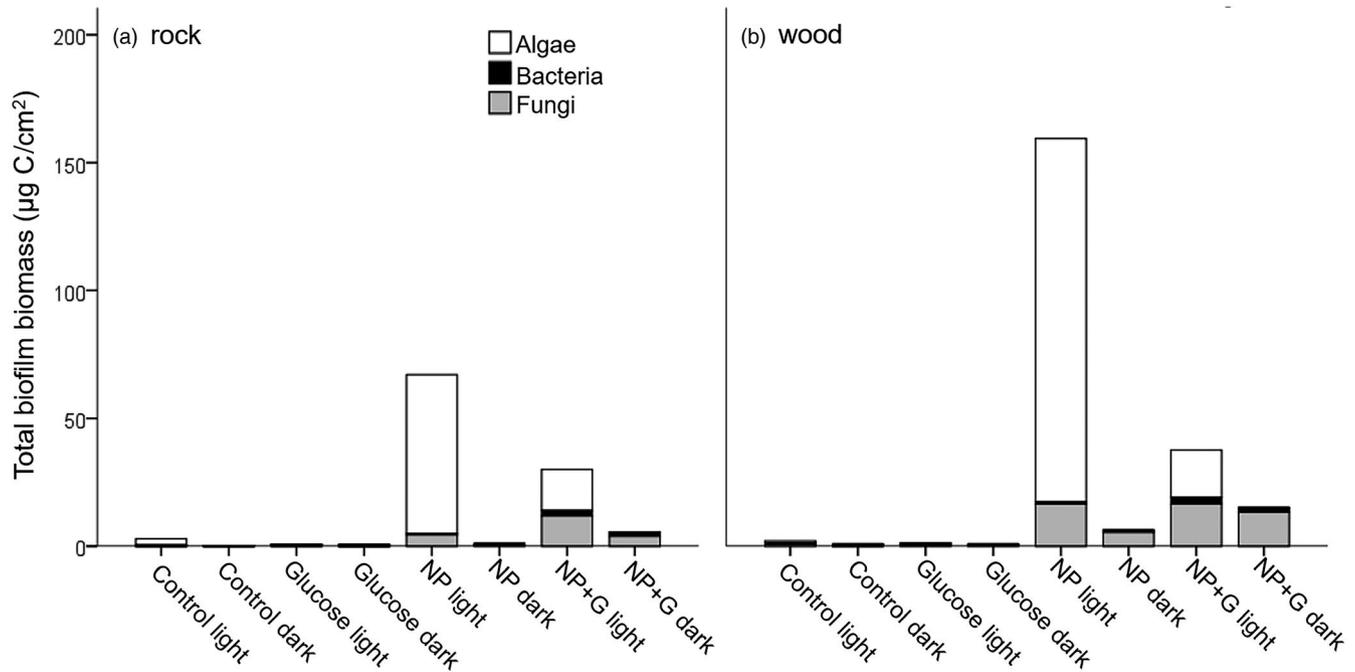


FIGURE 3 Total biofilm biomass and composition on nutrient-diffusing substrates enriched with either agar (control), glucose, nitrogen + phosphorus (NP), or a combination of all three (NP + G) in light and dark treatments on rock (a) and wood (b) substrates

biofilm biomass was restricted by low nutrient availability (Figure 3). Bacterial biomass was elevated by nutrient enrichment on both inorganic and organic substrates ($p = 0.02$), and the response was similar between the (NP)_L (with algae) and (NP)_D (without algae) treatments. Organic substrates further increased bacterial biomass compared to inorganic substrates in the (NP)_L treatment ($p = 0.02$), but not in the (NP)_D treatment ($p = 0.27$). A combination of nutrients and glucose (NP + G) elevated bacteria compared to the individual [(G), (NP)] resource amendments ($p = 0.002$), and the magnitude of the effect was significantly greater in the (NP + G)_L treatment compared to the (NP + G)_D treatment ($p \leq 0.001$). There was an additional substrate effect in the (NP + G)_L treatment shown by greater bacterial biomass on organic compared to inorganic substrates ($p = 0.005$), but not in the (NP + G)_D treatment ($p = 0.07$).

Mean (\pm SE) PAR ($\mu\text{mol photons/m}^2 \text{s}^{-1}$) values were 273.1 ± 22.5 (range 64.9–1019.6) in the light treatment and $< 1.19 \pm 0.03$ in the dark treatment during daylight hours ($p < 0.0001$) and daily water temperature ($^{\circ}\text{C}$) was similar between light (22.2 ± 0.16) and dark (21.8 ± 0.17) treatments ($p > 0.05$). Dissolved oxygen was 6.42 ± 0.04 mg/L and pH was neutral (7.06 ± 0.06). Dissolved nutrient concentrations (NO_3 and PO_4) were 25.6 ± 8.24 $\mu\text{g/L}$ and 3.53 ± 0.27 $\mu\text{g/L}$, respectively, and DOC was 6.80 ± 0.11 mg/L.

4 | DISCUSSION

Microbial biofilms play a key role in aquatic ecosystem functioning through their contribution to nutrient uptake and retention, primary production and community respiration, and as an energetic base of the food web (Battin et al., 2016; Goldsborough, McDougal,

& North, 2005; Vander Zanden et al., 2006). Many of these functions are governed by microscale interactions that involve the exchange of resources among micro-organisms, including species of algae, bacteria, and fungi, which occur in close proximity within the biofilm matrix (Battin et al., 2007). Compared to our understanding of plankton community dynamics (e.g., Seymour, Amin, Raina, & Stocker, 2017), we know far less about the factors that regulate the associations among micro-organisms within aquatic biofilms, especially as it relates to resource availability. Further, inferences from pelagic studies are of limited value for benthic habitats given the potential importance of substrate type for resource availability and supply of nutrients through groundwater. By enriching inorganic and organic substrates with nutrients and glucose in a factorial design with and without autotrophic production (using light and dark treatments, respectively), we were able to evaluate the independent and interactive factors governing biofilm development in the nearshore area of an oligotrophic, clear water subalpine lake. We found biofilm development to be regulated by complex microbial interactions that ranged from cooperative to competitive depending on availability of carbon and nutrients within the environment.

Nutrient limitation of benthic algae growing on hard surfaces has been well documented across a range of freshwater ecosystems (Borchardt, 1996), including lakes (Fairchild, Lowe, & Richardson, 1985; Rodusky, Steinman, East, Sharfstein, & Meeker, 2001). Owing in part to their undeveloped watersheds that receive minimum nutrient inputs, nutrient limitation of benthic algae is a common feature of alpine lakes (Lepori & Robin, 2014; Maberly, King, Dent, Jones, & Gibson, 2002). Previous research on nutrient limitation of algal production in Castle Lake has focused primarily on the pelagic zone (but see Higley et al., 2001), with most studies showing

that phytoplankton are limited by a combination of nitrogen and phosphorus (Elser & George, 1993; Elser et al., 1995). A whole lake manipulation using labelled ammonium nitrate shows that biofilms account for 90 and 73% of nitrogen depletion and uptake (Axler & Reuter, 1996), indicating that biofilms play a strong role in governing water column nutrient availability. Our goal in this study was not to determine which nutrient(s) limit autotrophic production within the lake biofilm, but instead, to manipulate algae in a way that would allow us to evaluate relationships among biofilm components. Our results show that algal biomass was significantly elevated in the presence of nitrogen and phosphorus enrichment in light-transparent treatments, but not in dark treatments (Figure 2). This allowed us to use light as an independent variable to evaluate how heterotrophic micro-organisms respond to elevated nutrient levels in both the presence and absence of autotrophic production.

Our expectation was that heterotrophs would be limited by organic carbon on inorganic substrates in the absence of autotrophic production (i.e., dark treatments), and that organic substrates would provide a carbon subsidy for heterotrophic metabolism. Our results support this hypothesis, showing that both fungi and bacteria were significantly elevated on organic substrates compared to inorganic substrates in dark treatments (Figure 2). A key difference between fungi and bacteria was that bacteria were stimulated on organic substrates in the absence of nutrient enrichment whereas fungi were elevated only when organic substrates were supplemented with nutrients. Owing to minimum water nutrient content and to the high lignin to nitrogen ratios of woody debris (Melillo, Naiman, Aber, & Eshleman, 1983), nutrient limitation of fungi has been widely reported in other benthic ecosystems (Tank & Dodds, 2003; Tank & Webster, 1998). In streams with forest canopy for example, elevated nutrient availability (especially N) tends to stimulate fungal biomass on organic substrata (Suberkropp, 1995), resulting in an overall increase in organic carbon decomposition within the stream (Suberkropp & Chauvet, 1995). Although bacteria were stimulated on organic substrates without nutrients, further increases in bacterial biomass on organic substrates with nutrients suggest that bacteria may require supplements from the water column to prevent secondary limitation by nutrients on woody debris (e.g., Hoellein, Tank, Kelly, & Rosi-Marshall, 2010).

It is well established that primary producers provide resources to, and exchange resources with, heterotrophic micro-organisms in aquatic environments (Battin et al., 2016). As a consequence of this interaction, heterotrophic production is often coupled with algal production across a range of freshwater ecosystems (DeColibus et al., 2017; Rier & Stevenson, 2001; Scott et al., 2008). For the most part, previous studies have focused on the association between algae and heterotrophic bacteria, especially in lakes (e.g., Danger, Leflaive, Oumarou, Ten-Hage, & Lacroix, 2007; Durán, Medina-Sánchez, Herrera, & Carrillo, 2016; Medina-Sánchez, Villar-Argaiz, & Carrillo, 2004; Stets & Cotner, 2008), where bacteria are the foremost decomposer within the pelagic environment. Our study adds to a growing pool of literature showing that benthic algae, which grow in close association with

a diverse community of heterotrophic micro-organisms (Battin et al., 2007), can stimulate fungal activity within aquatic biofilms (Kuehn et al., 2014; Miura & Urabe, 2017; Soares et al., 2017). We found that fungi were stimulated by nutrient enrichment in the presence of natural light (with elevated algae), but not in the dark, without algae. The magnitude of this effect is similar to that reported by Kuehn et al., (2014) when fungal biomass was assayed under similar light and dark treatments in a wetland detrital-periphyton complex. Further, we found that the positive response of fungi to elevated algae on inorganic substrates (in the light) was similar in magnitude to their response to nutrients in the presence of glucose enrichment (in the dark). This result indicates that fungi were limited by labile organic carbon in the presence of elevated nutrient levels on inorganic substrates and this constraint was alleviated by algae. Interestingly, the positive effect of algae was approximately 15-fold greater on inorganic substrates than organic substrates (i.e., light vs. dark), suggesting that the association between algae and fungi becomes more favoured on inorganic substrates and perhaps in lakes with low levels of dissolved organic carbon. A reciprocal exchange of resources (e.g., CO₂ supply from fungi) may have in turn promoted greater levels of algal biomass on organic substrates where fungi were more abundant compared to inorganic substrates, underscoring the importance of producer-decomposer co-dependency for the development of biofilms in shallow aquatic ecosystems.

In contrast to our expectations, elevated algal biomass did not stimulate heterotrophic bacteria on inorganic substrates, resulting in an uncoupling between algae and bacteria in the presence of elevated nutrient availability. This finding is in contrast to previous studies where algae have been shown to support the metabolic activities of heterotrophic bacteria (Kuehn et al., 2014; Seymour et al., 2017; Wyatt & Turetsky, 2015), with coupling observed across a range of aquatic environments (DeColibus et al., 2017; Durán et al., 2016; Rier & Stevenson, 2001; Scott et al., 2008). Although we can assume that algae and bacteria were both competing for the same inorganic nutrients (Bratbak & Thingstad, 1985), the uncoupling did not appear to be the result of competitive exclusion as there was no additional increase in bacteria on inorganic substrates in the presence of elevated nutrient levels in the dark (i.e., in the absence of algae). Considering that some nutrient uptake systems in bacteria are energetically expensive (Jansson, 1993; Teixeira de Mattos & Neijssel, 1997), exudates released by algae may not have been adequate to alleviate carbon limitation of bacteria, possibly owing a low yield (i.e., percent extracellular release) in the presence of adequate nutrient supply (Wyatt, Tellez, Woodke, Bidner, & Davison, 2014). Alternatively, the subdued response of heterotrophic bacteria in the presence of elevated algal biomass may have been the result of competitive interactions with fungi, which has been demonstrated in other studies (Mille-Lindblom, Fischer, & Tranvik, 2006; Wohl & McArthur, 2001), though most were conducted in the absence of autotrophic production. Interestingly, heterotrophic bacterial biomass was more elevated when nutrients were supplemented with glucose

in light treatments than in dark treatments, suggesting that algae may facilitate heterotrophic bacteria through other mechanisms, perhaps by increasing the surface area for colonization within the biofilm (Rier & Stevenson, 2001).

The positive effects of nutrient enrichment on autotrophic production were significantly reduced with glucose enrichment, suggesting that heterotrophic micro-organisms were able to outcompete algae for available nutrients in the absence of carbon limitation. Although autotrophs and heterotrophs are mutualists through nutrient cycling, they are also competitors for inorganic nutrients (Bratbak & Thingstad, 1985; Danger et al., 2007). Heterotrophs typically have a greater affinity for nutrients than algae (Currie & Kalff, 1984) and can outcompete algae in conditions of low nutrient availability (Cotner & Wetzel, 1992; Jansson, 1993). The dependence of heterotrophs on the photosynthetic products from algae is therefore considered an important condition allowing for the stable co-existence of both groups (Daufresne & Loreau, 2001). Our results support this hypothesis, showing that heterotrophs were able to outcompete algae for available nutrients when energetic requirements for organic carbon were met by outside sources. Similar examples of uncoupling between autotrophs and heterotrophs have been widely reported in pelagic environments when low levels of nutrient availability coincide with elevated levels of organic carbon (Klug, 2005; Stets & Cotner, 2008). The ability of heterotrophs to outcompete algae in our study suggests that nutrient levels were not elevated enough to meet the energetic requirements of both groups in the presence of glucose. Given that changes in total biofilm biomass were largely driven by the presence or absence of autotrophic production, the 37%–41% reduction in the autotrophic component of the biofilm in the presence of heterotrophic competitors resulted in an overall decrease in total biofilm biomass. It is important to note that algae were not outcompeted by heterotrophs in the presence of nutrient enrichment on wood substrates (without glucose), suggesting that organic carbon quality is an important factor governing competitive outcomes between autotrophs and heterotrophs for nutrients during biofilm development (Hoellein et al., 2010; Johnson et al., 2009).

Aquatic biofilms are comprised of an assemblage of algae, bacteria and fungi that interact in complex ways, yet most studies to evaluate resource limitation in benthic environments have been conducted by monitoring a single response variable, usually autotrophic production. Although this approach allows investigators to evaluate the net outcome of community interactions (e.g., the level of autotrophic biomass), it does not provide insight into the mechanisms by which they occur. Our understanding of planktonic dynamics suggests the interplay between autotrophic and heterotrophic members of aquatic biofilms should be influenced by the availability of limiting resources, especially nutrients and organic carbon (e.g., Stets & Cotner, 2008). Our results support this hypothesis, showing that the availability of organic carbon and nutrients interacts to mitigate associations between autotrophic and heterotrophic micro-organisms during biofilm development. We were able to identify a range of fundamental ecological relationships that were governed by

resource availability within the lake biofilm, including: 1) commensalism between algae and fungi in the presence of elevated nutrient availability, 2) antagonistic interactions between bacteria and fungi in the presence of elevated algae, and 3) competition between autotrophs and heterotrophs for nutrients when energetic requirements for carbon were met by outside sources. These findings document the complex nature of biofilm dynamics in aquatic ecosystems and suggest that certain aspects of lake biofilm ecology may be particularly susceptible to environmental change, especially perturbations such as nutrient inputs that can disrupt the exchange of resources between autotrophs and heterotrophs.

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AUTHORS' CONTRIBUTIONS

S.C., S.P.B., S.S., A.R.R., and K.H.W. conceived the ideas and designed methodology and experimental approaches; RCS collected the data and enumerated algae; K.A.K. conducted the fungal analysis; M.N.S. enumerated heterotrophic bacteria; A.R.R. analysed the data; K.H.W. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.c7g30cp> (Wyatt et al., 2019).

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