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P-limitation drives changes in DOM production by aquatic bacteria

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ABSTRACT: Heterotrophic bacteria are key biogeochemical regulators in freshwater systems. Through both decomposition and production of organic matter, bacteria link multiple biogeochemical cycles together. While there has been a significant amount of work done on understanding the role of microbes in the aquatic carbon cycle, important linkages with other biogeochemical cycles will require more information about how organic matter transformations impact other nutrients, such as phosphorus. In this study, we conducted a culture-based laboratory experiment to examine the production of dissolved organic matter (DOM) by heterotrophic bacteria under varied nutrient conditions. In addition to quantifying the production of dissolved organic carbon (DOC), we also measured the production of dissolved organic phosphorus (DOP) and characterized the microbially produced organic matter using optical properties. Results demonstrated that measurable amounts of DOC and DOP were produced by heterotrophic bacteria under nutrient regimes ranging from carbon-limitation to strong phosphorus-limitation. Additionally, optical characterization of DOM revealed that the organic matter produced by bacteria grown under high phosphorus conditions was highly aromatic with similar optical properties to terrestrially derived organic matter. Overall, these findings suggest that heterotrophic bacteria can be important producers of organic matter in freshwaters and that continued trends of increased nutrient concentrations (eutrophication) may fundamentally change the composition of microbially produced organic matter in freshwater systems.

KEY WORDS: Phosphorus · Carbon · Heterotrophic bacteria · Dissolved organic matter · Microbes in biogeochemical cycling

1. INTRODUCTION

Heterotrophic bacteria are important regulators of multiple biogeochemical processes in aquatic ecosystems including the cycling of carbon (C) and phosphorus (P) (Cotner & Biddanda 2002, Cotner et al. 2010, Schlesinger et al. 2011, Jeyasingh et al. 2017). However, our understanding of how these key elemental cycles are linked in aquatic systems remains limited (Maranger et al. 2018). It is acknowledged that inland waters are biogeochemically active 'pipes' connecting terrestrial systems with the oceans, but this active pipe concept has traditionally only been applied to the processing of carbon in inland waters (Cole et al. 2007, Tranvik et al. 2009, Aufdenkampe et al. 2011). Recently, there has been a call to better understand how the freshwater pipe concept could be applied to macronutrient cycling in inland waters (Maranger et al. 2018). Developing our understanding of how freshwaters serve as active pipes for multiple elements requires an understanding of both the production and decomposition of organic matter.

Dissolved organic matter (DOM) is a major biogeochemically active carbon pool in freshwater systems (Tranvik 1988, Stets & Cotner 2008, Tranvik et al.

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2009, Catalán et al. 2016). To date, the bulk of the research conducted on DOM has focused on its production primarily by autotrophs and decomposition primarily by heterotrophs. However, heterotrophs not only consume DOM, but they also produce, or more precisely, transform it into various metabolites (Lechtenfeld et al. 2015). In marine systems, microbial production of dissolved organic carbon (DOC) can result in a pool of slow-degrading (or recalcitrant) carbon that can be exported to and buried in the deep oceans (Jiao et al. 2010, 2011, Lechtenfeld et al. 2015). This microbial carbon pump is now widely accepted as an important mechanism for storing carbon in the ocean. Microbial carbon production has also been presented as the dominant pathway for recalcitrant organic matter production in soils (Liang & Balser 2011, Cotrufo et al. 2015), resulting in soil DOM that has been heavily modified by microbial metabolism. While this pathway has been less explored in freshwater systems, it also appears to be an important control on DOM composition in freshwaters (Kawasaki & Benner 2006, Guillemette & del Giorgio 2012). Despite this known importance for global C cycling, the implications of microbial production of DOM on other nutrient cycles (such as dissolved organic phosphorus [DOP] production) are not well known.

The excretion of bacterial metabolites is an important mechanism for altering the composition and bioavailability of DOM (Romano et al. 2014, Lechtenfeld et al. 2015). The environmental conditions, such as availability of nutrients and the temperatures experienced by bacteria can greatly affect its quantity and characteristics. For example, bacterial production of phosphatase is strongly related to nutrient conditions (Cotner & Wetzel 1991, Romano et al. 2015). Recent work has shown that bacteria have several strategies for dealing with nutritional imbalance, including changing their biomass composition to more closely match the chemical composition of their resources (Makino et al. 2003, Mooshammer et al. 2014, Godwin & Cotner 2015a,b, Danger et al. 2016, Godwin et al. 2017). These stoichiometric strategies, ranging from strong elemental homeostasis to biomass composition flexibility, likely have important consequences for the composition of organic matter that is produced by heterotrophic bacteria, but how these different stoichiometric compositions impact organic matter transformations by heterotrophic bacteria remains unknown. In the present study, we explored the production of DOM by heterotrophic bacteria and determined how differing stoichiometric strategies impact the chemical composition of DOM produced. This was accomplished by growing bacterial strains that exhibited a range of biomass flexibility strategies under various conditions of nutrient limitation and assessing the composition of organic matter that was produced.

2. MATERIALS AND METHODS

2.1. Bacterial culturing media

WC Medium was prepared according the recipe in Guillard & Lorenzen (1972) with ultrapure water (Milli-Q System). Media was mixed in glassware that had been soaked in 10% hydrochloric acid for a minimum of 1 h and rinsed with ultrapure water to remove any trace phosphorus contamination. All chemical stocks used to make the media were American Chemical Society grade or equivalent. Glucose was added as the sole organic carbon substrate at a final concentration of 6.66 mM carbon. Nitrogen was supplied as sodium nitrate at a concentration of 1 mM, resulting in a media C:N molar ratio (6.6:1) equal to the Redfield ratio (Redfield 1958). Micronutrients, vitamins, and trace metals were supplied consistent with the recipe (Guillard & Lorenzen 1972). To manipulate the C:P of the media, phosphorus was added as potassium phosphate at 3 different levels: 0.067 mM P, 0.014 mM P, and 0.0067 mM P, resulting in media molar C:P ratios of 100:1, 500:1, and 1000:1, respectively. For each media C:P ratio, a single 10 l batch of media was made and filter sterilized into 1 l bottles to minimize batch effects within the experiment. The complete breakdown of media composition and stoichiometry is provided in Table S1 in Supplement 1 at www.int-res.com/articles/suppl/a085p035_supp/.

2.2. Strain selection

A large field campaign was conducted in 2013 where water samples taken from lakes across the state of Minnesota, USA, were used to culture and isolate heterotrophic bacteria following the procedures outlined by Godwin & Cotner (2015b). Through these efforts, a culture repository of over 1000 unique bacterial strains isolated from freshwater systems was established. To quantify the variability in stoichiometric flexibility within this library, a sub-sample of ~135 strains was grown in continuous culture at 25% of their maximum growth rate at 2 media C:P levels (100:1 and 10 000:1) (described in Godwin & Cotner 2018). Biomass flexibility for these ~135 strains was calculated as the relative percentage increase in

biomass C:P when grown at high C:P conditions compared to the biomass C:P at low C:P using Eq. (1). Archival stocks of each strain were stored at −80°C in glycerol for future use. Eq. (1) shows the relative change in biomass stoichiometry at a molar media supply rate C:P expressed as a percent change from the biomass stoichiometry when grown under media conditions with a C:P of 100:1.

$$\frac{(\text{Biomass C:P at } 10\,000{:}1 - \text{Biomass C:P at } 100{:}1)}{\text{Biomass C:P at } 100{:}1} \times 100 \quad (1)$$

To select strains for this study, the ~135 strains described above were sorted by C:P biomass flexibility and split into quartiles. The 1st quartile (representing the lowest C:P flexibility values) was classified as inflexible, and the 4th quartile was classified as flexible. From these quartiles, we recovered 9 strains from the −80°C freezer and characterized the DOM they produced (5 inflexible strains and 4 flexible strains). A complete description of the strains is provided in Table S2 in Supplement 1.

2.3. Culturing bacteria

Once bacteria had been recovered from the −80°C freezer, a pair of starter cultures was generated for each strain by inoculating 20 µl of archival stock into 2 ml of WC media with a media C:P of 100:1. Resazurin was added as a respiratory indicator at a concentration of 20 µM to monitor the growth of bacteria in these starting cultures. Resazurin is a blue/purple-colored dye that is irreversibly reduced to the pink and highly fluorescent molecule resofurin, indicating active microbial growth (Sarker et al. 2007). Once resazurin indicated growth, these 2 ml starter cultures were used to inoculate duplicate 250 ml cultures of each strain by diluting the 2 ml starter with ~248 ml of fresh WC media (without resazurin) at a media C:P of 100:1. The cross-over of resazurin from the initial culture to the final culture would result in no more than ~0.024% of the organic carbon in the final culture being derived from residual resazurin (see Table S1 for this calculation). These cultures were incubated at room temperature (~22°C) on a tabletop shaker set at 150 rpm. Growth in 250 ml cultures was monitored using optical density (OD) readings, and cultures were harvested when OD readings plateaued, indicating stationary phase was achieved. This same process was repeated to grow cultures in WC media with a C:P ratio of 500:1 and 1000:1. Because the high C:P cultures contained less concentrated biomass (and therefore needed more volume filtered to measure the biomass), a final culture volume of 500 ml rather than 250 ml was used at this C:P ratio.

2.4. Collecting microbially produced DOM

Cells from cultures were collected onto pre-combusted, pre-weighed Whatman GF/F filters (0.7 µm nominal pore-size) to measure microbial biomass. Filters were then oven dried at 60°C for at least 24 h and weighed. Microbial biomass was calculated by subtracting the pre-weight of the filter from the post-weight after oven drying. Remaining media was filter-sterilized using a 0.22 µm pore-size polyethersulfone (PES) bottle top filter, and the residual media was collected in muffled amber glassware and stored at 4°C until analyzed (samples were analyzed within 2 wk of filtration).

2.5. Characterizing microbial DOM production

To characterize the chemical composition of the residual media, we measured dissolved nutrients and the specific-UV absorbance at wavelength 254 nm ($SUVA_{254}$). DOC and total dissolved nitrogen (TDN) were measured using a Shimadzu TOC-L model high temperature carbon-analyzer with a TNM-L module. To measure total dissolved phosphorus (TDP) and soluble reactive phosphorus (SRP), we used the molybdenum blue reaction with and without acid-persulfate digestion (Murphy & Riley 1962). To conservatively estimate DOP, we measured TDP and SRP in triplicate and subtracted the upper 95% confidence interval of the SRP measurement from the lower 95% confidence interval for TDP for each sample. To account for the amount of glucose that was not consumed during the incubation period, residual media glucose was measured using Amplex™ Red glucose/glucose oxidase assays (Invitrogen, catalog number A22189) according to the manufacturer's protocol. Microbially produced DOC was then calculated by subtracting the molar residual glucose measurement as carbon from the total DOC concentration. Absorbance at a wavelength of 254 nm was measured using a Cary 50 spectrophotometer. $SUVA_{254}$ values were calculated in 2 ways, both by dividing the absorbance at wavelength 254 nm by the total DOC produced during the incubation (the total residual DOC minus any residual glucose) and by dividing the absorbance by the total residual DOC value. These 2 approaches yielded nearly identical results (Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/a085p035_supp/), so we performed all

further analysis using only the $SUVA_{254}$ of the microbially produced DOM.

2.6. Data analysis

To test for the normality in the distributions of our dependent variables, we performed a Shapiro-Wilk test for normality. Several of our measurements were non-normally distributed, so we used non-parametric analyses (Kruskal-Wallis) to examine how stoichiometric flexibility and media composition impacted DOC and DOP production. To explore the interactions between stoichiometric flexibility and media level, we also completed pairwise comparisons for each outcome variable within each treatment level using a 2-sample Wilcoxon test. All statistical analysis was performed in R version 3.5.1 (https://www.R-project.org), and our analysis script is provided in Supplement 3 at www.int-res.com/articles/suppl/a085p035_supp/. Additionally, the complete raw data file is available in Table S3 in Supplement 1 with metadata provided in Table S4 in Supplement 1.

3. RESULTS

3.1. Quantifying microbially produced DOM

To quantify the production of microbially produced DOM, we measured DOC and DOP concentrations in cell-free media after microbial growth reached stationary phase. We also measured the amount of glucose remaining in the residual media to account for any of the starting carbon source that had not been consumed (Fig. 1). As expected, residual glucose was lowest when strains were grown at the lowest C:P (100:1) with less than 5% of the DOC in the residual media being glucose (Fig. 1). This efficient drawdown of glucose strongly supports the idea that organic C was limiting microbial growth in this treatment. In comparison, the residual DOC from strains grown under more P-limited conditions typically contained 10–20% of the DOC as glucose (Fig. 1).

Mean DOC production per unit biomass was higher under P-limited conditions compared to C-limited conditions (Fig. 2). DOC production per unit biomass at a media C:P of 100:1 ranged from 181 µM C mg^{-1} biomass to 910 µM C mg^{-1} biomass with a median value of 312 µM C mg^{-1} biomass. This contrasted with a range of 575–7460 µM C mg^{-1} (median of 2594) for cells at a C:P of 500:1 and 390–6635 µM C mg^{-1} biomass for cells at a C:P of 1000:1 (median of 1610) (Fig. 2). Across the

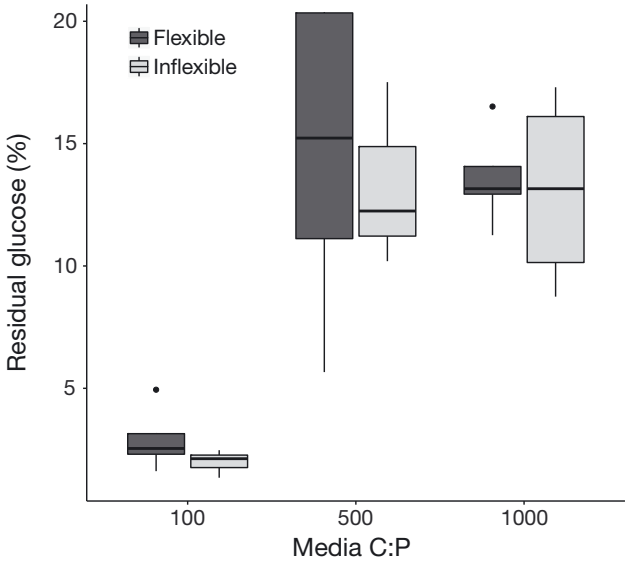

Fig. 1. Residual glucose concentration as a percentage of the residual dissolved organic carbon (DOC) pool. Residual glucose was significantly different across media type (Kruskal-Wallis; p = 0.0003); no significant difference between flexible and inflexible strains (Kruskal-Wallis, p = 0.57). Vertical bars represent minimum and maximum values; boundaries of each box represent the 1st and 3rd quartiles. Dark solid horizontal line is the median of each distribution; outliers (defined as outside 1.5 times the interquartile range above the 3rd quartile or below the 1st quartile) shown as single points

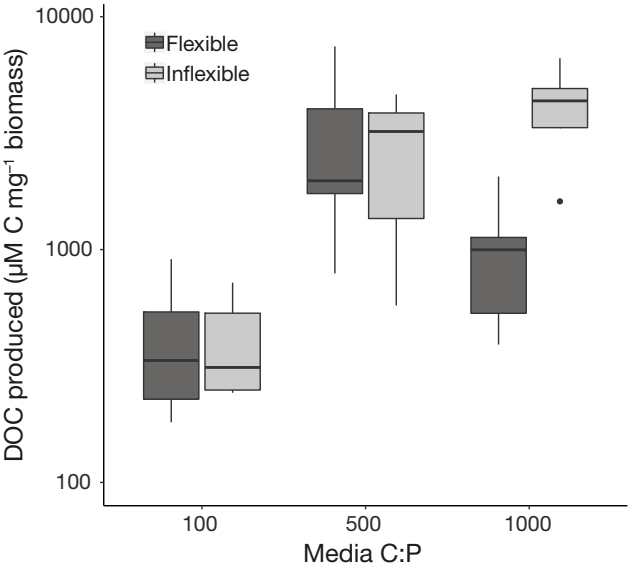

Fig. 2. Concentration of dissolved organic carbon (DOC) per unit biomass produced by flexible and inflexible strains (total DOC – residual glucose) grown at 3 unique media C:P ratios. DOC production per unit biomass was significantly different across media type (Kruskal-Wallis; p = 0.001); no significant difference between flexible and inflexible strains overall (Kruskal-Wallis, p = 0.64). Large and significant difference between flexible and inflexible strains specifically in the most P-limited conditions (Wilcoxon; p = 0.03). Bars, box boundaries, lines and outlier as defined in Fig. 1

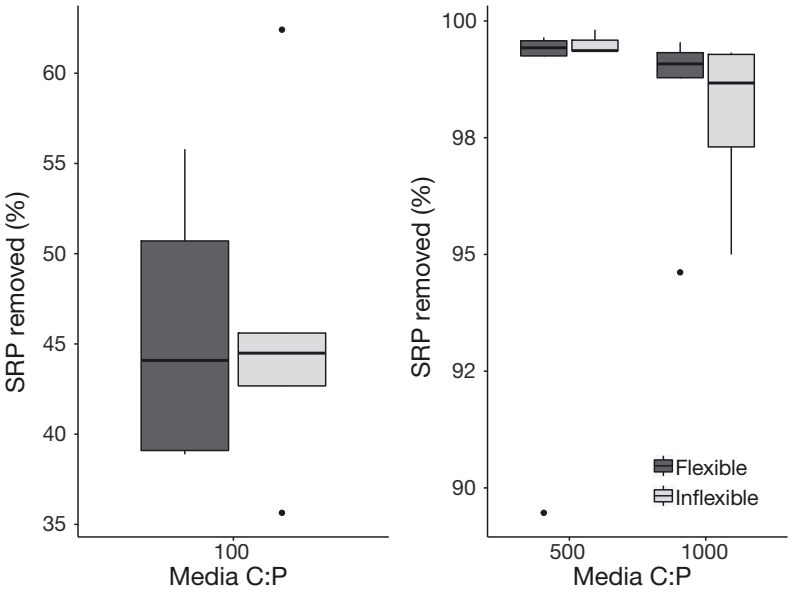

Fig. 3. Soluble reactive phosphorus (SRP) removal efficiency by flexible and inflexible bacterial strains grown at 3 different media C:P ratios. SRP removal efficiency significantly different across media type (Kruskal-Wallis; $p < 0.0001$); no significant difference between flexible and inflexible strains (Kruskal-Wallis, $p = 0.64$). Bars, box boundaries, lines and outliers as defined in Fig. 1

3 treatments, DOC production ranged from ~5 to ~76% of the original glucose pool. Media stoichiometry was a significant predictor of DOC production per unit biomass (Kruskal-Wallis, $p = 0.001$). Despite the fact that stoichiometric flexibility was not a significant predictor of per unit biomass DOC production ($p > 0.05$), flexible strains did have lower DOC production than inflexible strains under the most P-limited conditions (Fig. 2; Wilcoxon, $p = 0.03$), a pattern that warrants more thorough investigation in the future.

Phosphate levels in the residual media were highly impacted by the media C:P, with over 90% of the media SRP being removed in the 500:1 and 1000:1 media treatments compared to ~40–50% removal when the media C:P was 100:1 (Fig. 3). This again supports the idea that bacteria were experiencing C-limitation at the lowest media C:P and transitioned to P-limitation at the 2 higher C:P values. DOP production per unit biomass accumulation was strongly influenced by media starting conditions as well. DOP production was ~1–2 orders of magnitude larger under C-limited conditions compared to P-limited conditions (Fig. 4) but was detectable in all growth conditions (although not for all strains). Of the 27 total samples that were collected, 6 had DOP levels below detection (3 strains grown at 100:1 that were all inflexible and 3 strains grown at 500:1, 2 flexible and 1 inflexible). One inflexible strain only produced measurable DOP under the most phosphorus-limited condition, but all other strains had measurable DOP production for at least 2 media levels. For the strains that produced measurable DOP, values ~0.006 to 12.5% of the original phosphate pool (Fig. 5) were measured. DOP production per unit biomass was highly variable across treatments, and because of our relatively small sample size, neither media stoichiometry nor stoichiometric flexibility were significant predictors of per unit biomass DOP production. Similarly, DOP produced as a percentage of the original media SRP varied by orders of magnitude, and neither media stoichiometry nor stoichiometric flexibility were significant predictors of relative DOP production.

3.2. Optical characterization of microbially produced DOM

$SUVA_{254}$ is often used as an indicator of organic matter quality (Frey et al. 2016). All samples showed increased $SUVA_{254}$ values (in comparison to the $SUVA_{254}$ of the starting media) in the residual media, consistent with microbial production of

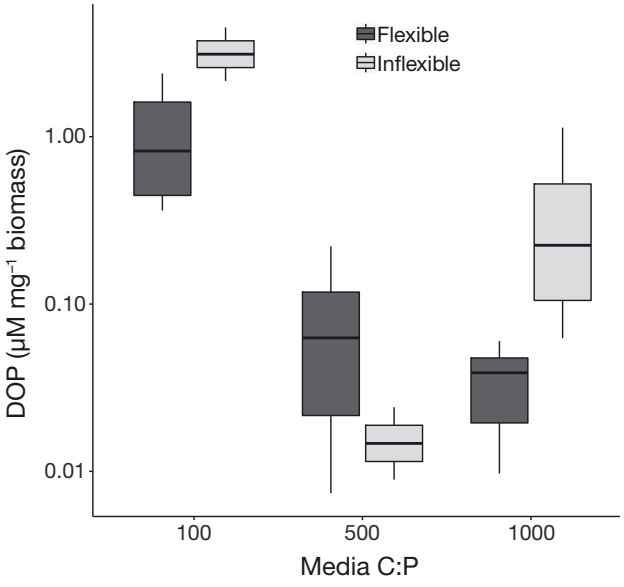

Fig. 4. Minimum amount of dissolved organic phosphorus (DOP) produced per unit biomass (log scale) over the incubation period. Sizeable differences in DOP production across media types and between flexible and inflexible strains, but large variation and small sample sizes result in only one significant difference between flexible and inflexible strains under the most P-limited conditions (Wilcoxon; $p = 0.01$). Bars, box boundaries, and lines as defined in Fig. 1

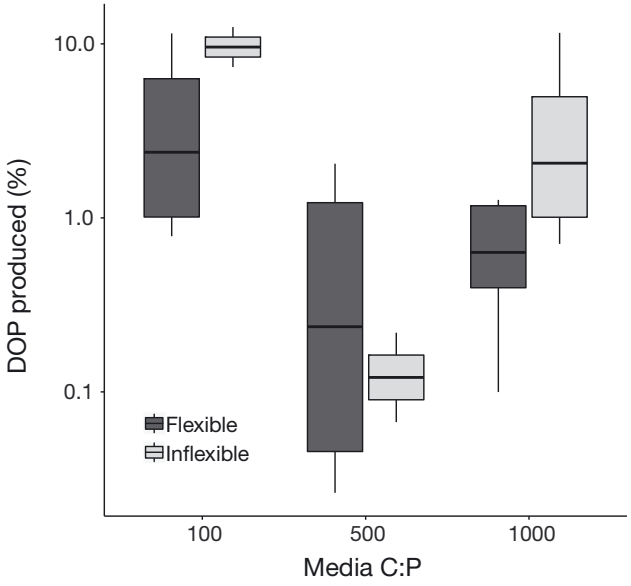

Fig. 5. Percentage dissolved organic phosphorus (DOP) production (log scale) relative to initial media phosphate concentration. High variability and limited replicates resulted in no significant difference in DOP production efficiency across media type or flexibility status. Bars, box boundaries, and lines as defined in Fig. 1

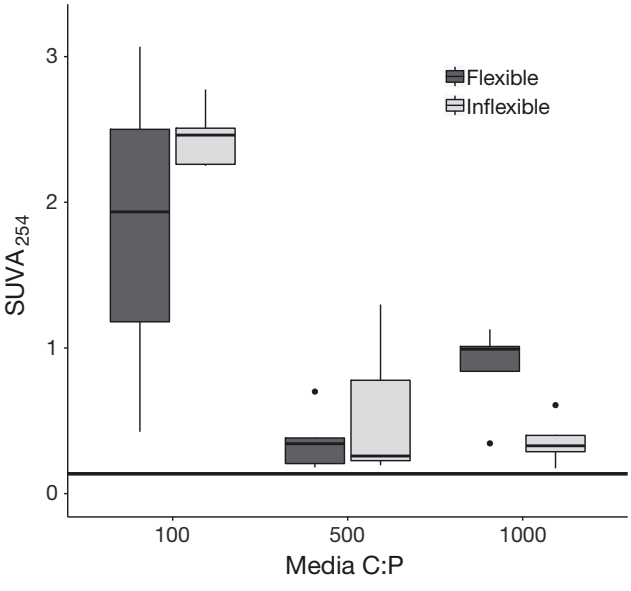

Fig. 6. Specific-UV-absorbance at wavelength 254 ($SUVA_{254}$, $l\ mg^{-1}\ l^{-1}$) for flexible and inflexible strains grown at 3 different media C:P ratios. $SUVA_{254}$ of the produced organic matter significantly different across media type (Kruskal-Wallis; p = 0.0006); no significant difference between flexible and inflexible strains overall (Kruskal-Wallis, p = 1.0). Large and significant difference between flexible and inflexible strains in the most P-limited conditions (Wilcoxon; p = 0.03). $SUVA_{254}$ value for the media prior to incubation is represented by the horizontal line at a value of 0.136. Bars, box boundaries, lines and outliers as defined in Fig. 1

aromatic carbon compounds (Fig. 6). Mean $SUVA_{254}$ values were significantly impacted by the media C:P (Kruskal-Wallis, p = 0.0006), with strains grown at 100:1 C:P having higher $SUVA_{254}$ values than strains grown at higher C:P. Biomass flexibility did not have a significant effect on the mean $SUVA_{254}$ of produced organic matter, but flexible strains did show much larger variation in $SUVA_{254}$ values compared to inflexible strains when grown at a media C:P of 100:1 (Fig. 6). In contrast, under more P-poor conditions, $SUVA_{254}$ values were much lower (typically less than 1) and much less variable.

3.3. Stoichiometry of microbially produced DOM

To examine how biomass flexibility and media conditions impacted the relative processing of C and P, we examined the stoichiometric ratios of biomass-normalized produced organic matter. Media type had a significant impact on the stoichiometry of produced organic matter (Kruskal-Wallis, p = 0.002) with bacteria growing under C-limiting conditions producing organic matter that was relatively P-rich (lower DOC:DOP) compared to cultures experiencing P-limitation (Fig. 7). Stoichiometric flexibility was not a significant predictor of the stoichiometry of the produced organic matter.

3.4. Biomass production by bacteria under different growth conditions

Microbial biomass varied widely across treatment types, making it difficult to discern any consistent patterns based on media type or stoichiometric flexibility. Nonetheless, flexible strains growing under C-limited conditions had the highest biomass accumulation, whereas inflexible strains growing under the most P-limited conditions had the lowest biomass (Fig. 8, Table 1). Under the most P-limited conditions, inflexible strains showed significantly less biomass accumulation than flexible strains (Wilcoxon; p = 0.02), and while a similar pattern existed under C-limited conditions (with flexible strains having higher median biomass than inflexible strains), the variability was much higher, resulting in no significant differences.

4. DISCUSSION

The present study discusses the implications of 3 key findings based on this work. Firstly, we demon-

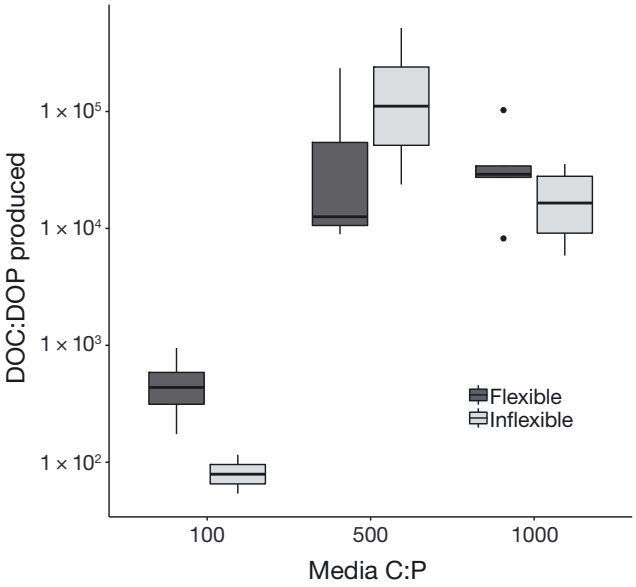

Fig. 7. Dissolved organic carbon (DOC):dissolved organic phosphorus (DOP) values (molar DOC per unit biomass divided by molar DOP per unit biomass) of microbially produced organic matter across all 3 media C:P levels for both flexible and inflexible strains, plotted on a log scale. DOC:DOP of the produced organic matter significantly different across media type (Kruskal-Wallis; p = 0.002); no significant difference between flexible and inflexible strains overall (Kruskal-Wallis, p = 0.88). Bars, box boundaries, lines and outliers as defined in Fig. 1

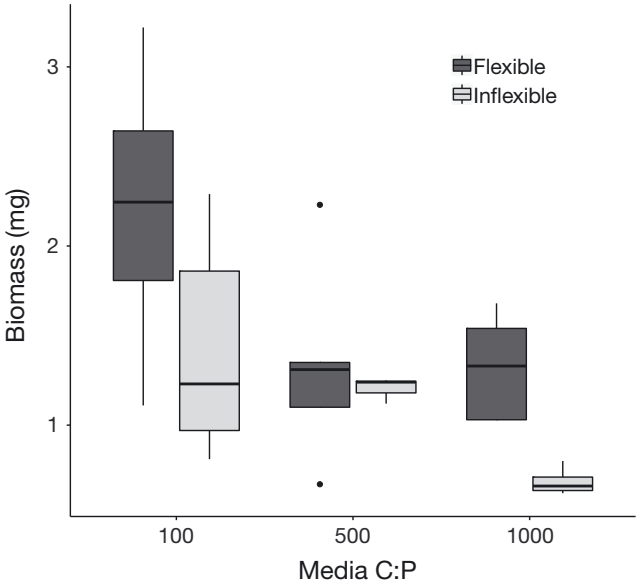

Fig. 8. Biomass production by flexible and inflexible strains across all 3 media C:P levels. Biomass accumulation was variable across media type but had a general trend of higher biomass accumulation at low C:P and lower biomass accumulation at high C:P. Flexible strains also tended to have higher biomass accumulation under all growth conditions, but only the most P-limited conditions resulted in statistically significant differences in biomass between flexible and inflexible strains (Wilcoxon; p = 0.014). Bars, box boundaries, lines and outliers as defined in Fig. 1

strated measurable amounts of DOC and DOP production by heterotrophic bacteria under nutrient conditions ranging from C-limitation to strong P-limitation. Secondly, optical characterization of microbially produced organic matter revealed that DOM produced by bacteria grown under C-limited conditions was highly aromatic, with $SUVA_{254}$ values as high as 3 l mg-C^{-1} m^{-1}, a value comparable to organic matter extracted from peatland soils (Hansen et al. 2016). This finding suggests that under C-limited conditions, microbial metabolism can produce DOM with similar optical properties to terrestrially derived

Table 1. Estimated (mean with range) relative allocation of carbon by both flexible and inflexible strains under high and low media C:P conditions. Biomass C was estimated as 50% of total dry mass; respired CO_2 was calculated by mass balance. Estimated growth efficiency (Est. growth eff.) was calculated by dividing the biomass C estimate by the total C drawdown (biomass+DOC+CO_2). All estimates represent median values. Carbon assimilation efficiency (C assimilation eff.) was estimated by dividing the total amount of biomass C by amount of glucose consumed during the incubation

Strain type	Media C:P	Biomass C (µM)	DOC (µM)	Residual glucose (µM)	Respired CO_2 (µM)	Est. growth eff. (%)	C assimilation eff. (%)
Flexible	100:1	1838 (925–2683)	1102 (370–2931)	46 (10–152)	3676 (894–5356)	38 (16–67)	28 (14–42)
Inflexible	100:1	1193 (675–1908)	507 (383–699)	10 (9–10)	4950 (4043–5593)	22 (11–40)	18 (10–29)
Flexible	1000:1	1102 (858–1400)	1281 (657–2740)	201 (104–201)	4077 (2114–5042)	20 (15–27)	17 (13–21)
Inflexible	1000:1	571 (517–667)	2821 (1288–4512)	406 (240–596)	2863 (887–4616)	9 (8–11)	9 (8–10)

organic matter, indicating that limitation status of the microbial community processing the organic matter may be as important a driver of $SUVA_{254}$ as the original source of the material, which is how these differences in values are typically interpreted. Lastly, stoichiometric flexibility of bacteria had variable effects on DOM production, but the effects were generally most pronounced under strong P-limitation.

While more work is needed to fully understand how the physiological growth strategies of different microbial taxa impact the production of DOM, the present study provides some important insights into this question. Overall, these findings have important implications for understanding the role of heterotrophic bacteria as significant producers of DOM in aquatic and terrestrial systems and lend insights into how we might expect this role to change under different nutrient conditions.

4.1. DOC and DOP production

We demonstrated that C-limited conditions result in low DOC production (Fig. 2) and high biomass accumulation (Fig. 8), suggesting that strains growing under C-limitation preferentially allocated available carbon to biomass or respiration rather than DOC. This observed tradeoff in C-allocation parallels previous work indicating that bacterial growth efficiency decreases as media C:P increases (Godwin et al. 2017). To explore this more fully, we examined the relative allocation of carbon into each potential pool (biomass, respiration, DOC, and residual glucose) by flexible and inflexible strains under high and low media C:P (Table 1). Lacking direct measurements of biomass C or respiratory C, we estimated these parameters assuming C was 50% of dry biomass (Bratbak & Dundas 1984) and then calculated respiratory C using a mass balance approach. We recognize that differences in C-allocation driven by the different nutrient treatments could impact the relative contribution of C to dry biomass, but previous work with strains in our lab (authors' unpubl. data) and others (Vrede et al. 2002) suggests that this change should be ~5% or less. This basic accounting showed that allocation of C to DOC was an important pathway under strong P-limitation for both flexible and inflexible strains and even exceeded respiratory C for inflexible strains at high C:P ratios (Table 1). Additionally, we estimated the carbon assimilation efficiency of each strain by normalizing the biomass accumulation of each strain to the amount of glucose consumed during incubation (Table 1). This calculation showed a pattern similar to the growth efficiency estimates, with flexible strains having higher C assimilation than inflexible strains and C assimilation being higher under C-limited conditions than under P-limited conditions (Table 1). This pattern supports the idea that there was preferential allocation of C to biomass under C-limited conditions and C-allocation to DOC became an important pathway under P-limitation.

DOP production as a percentage of original media P was much lower than DOC production as a percentage of original media C, and the stoichiometry and optical properties of microbially produced DOM was strongly impacted by the media C:P, reflecting a trade-off between allocating carbon to biomass vs. DOP compounds. For example, inflexible strains in this study had lower biomass accumulation than flexible strains at both 1000:1 and 100:1 media C:P (Fig. 8) and produced higher amounts of DOP at these media C:P ratios (Fig. 4), indicating the tradeoff in C-allocation. This suggests that for inflexible strains, maintaining a uniform biomass composition (by excreting excess nutrients in DOM) was the strategy used rather than allocating additional C to biomass. This could be driven by a physiological constraint of inflexible strains, whereby excess DOM is excreted as DOC and DOP compounds. Alternatively, it may be adaptive for these strains to minimize their biomass to escape predation (Chrzanowski & Šimek 1990, Cotner et al. 1995, Langenheder & Jürgens 2001). In contrast, the flexible strains accumulated more C as biomass under imbalanced conditions by manipulating their biomass composition to more closely match their resources.

The relative DOC production values measured in this study under C-limitation were similar to those measured in previous work, which demonstrated that DOC conversion values ranged from 5–15% during long-term incubations in artificial seawater with a media C:P ratio of 106:1 (Ogawa et al. 2001, Koch et al. 2014, Lechtenfeld et al. 2015). In contrast, our values were higher than DOC production estimates of a single *Pseudovibrio* sp. grown in pure culture under phosphate-limitation (0.2–0.9%; Romano et al. 2014). However, it is important to note that Romano et al. (2014) measured production as solid-phase extractable organic matter for characterization using ultrahigh resolution mass spectrometry and therefore were only capturing a fraction of the total microbially produced DOM pool. The extraction efficiency was not directly reported by Romano et al. (2014), but typical values for this type of extraction are between 20 and 80%, with polar and low molecular weight mole-

cules being the hardest to extract (Raeke et al. 2016, Johnson et al. 2017). Finally, a recent study comparing the microbial production of carbon in both freshwater and marine samples found significant uptake and transformation of glucose by bacteria in both settings, supporting the potential importance of a microbial carbon pump in freshwater as well as marine systems (Daoud & Tremblay 2019). This similarity in DOC production efficiency among various marine microbial communities and the individual freshwater strains that we tested here suggested a high degree of similarity in the potential of freshwater bacteria to be significant producers of DOC as has been acknowledged in marine systems (Kawasaki & Benner 2006, Jiao et al. 2011, Lechtenfeld et al. 2015, Kujawinski et al. 2016).

In contrast to DOC, measurements of DOP production in controlled incubation experiments are sparse in the literature. One study that strongly paralleled this work in a marine setting (Lønborg et al. 2009) found a DOP production efficiency of 17%, which was comparable to the 12% we measured in our cultures grown at a C:P of 100:1. Several other studies have attempted to measure DOP production *in situ* in marine systems (Orrett & Karl 1987, Thingstad & Rassoulzadegan 1995, Yoshimura et al. 2014). These studies incorporated both phytoplankton and heterotrophic bacteria in their microbial pools, making it hard to make a direct comparison to our work, but at least one of these studies measured DOP production as ~5% of the SRP drawdown during an open ocean diatom bloom with most of the production being attributed to the autotrophic diatoms (Yoshimura et al. 2014). The data presented here suggest that freshwater bacteria can be similarly important producers of DOP.

In addition to overall amounts of DOC and DOP production, we also explored how nutrient conditions impacted the stoichiometry of produced organic matter. In this study, DOM produced under P-limitation was relatively P-poor compared to DOM produced under C-limitation (Fig. 7), and microbially produced DOM under all nutrient conditions was P-poor relative to the classic Redfield ratio of 106:1. In fact, the lowest values for DOC:DOP in our study were on the order of 10 000:1, similar to the upper bound of measured DOC:DOP values in lakes in the upper Midwest United States (Thompson & Cotner 2018). This comparison suggests that in natural systems, microbially produced DOM is likely a source of P-poor organic matter relative to the standing stock DOM. Given that the bioavailability of DOP is negatively associated with the DOC:DOP ratio (Thompson & Cotner 2018), we argue that DOP produced under P-limited conditions should be more resistant to further microbial processing and may exacerbate P-limitation. On the other hand, cultural eutrophication in freshwaters is likely shifting the experienced resource ratios of microbial communities towards C-limitation due to increased P loading, which should result in more P-rich organic matter production and, in turn, export more bioavailable DOP downstream in freshwaters.

It is worth noting that values for natural DOC:DOP pools in lakes (Thompson & Cotner 2018) are on the low end of what we measured in this study. This could be driven by the fact that in natural systems DOM is being produced by both autotrophic and heterotrophic organisms, which may be experiencing different resource limitation that is impacting the stoichiometry of DOM production. For example, if a large portion of the natural DOM pool is produced by autotrophs that are C-limited due to light limitation, one would expect lower DOC:DOP values. Alternatively, if bacterial production is a significant source of DOM in natural systems, the relatively low DOC:DOP values measured in the field compared to the cultures in this study would suggest that bacteria in natural systems may be more C-limited than previously thought. This would be consistent with previous findings that the experienced resource ratios by heterotrophic communities are lower than bulk chemistry estimates suggest (Thompson & Cotner 2018) and work suggesting that individual strains switch from carbon to phosphorus limitation at resource C:P values ~300–1000 (Godwin & Cotner 2015b). Taken together, these findings suggest that bacteria experience conditions that are C-poor relative to previous estimates and that C-limitation continues into much higher C:P values than the conical Redfield ratio of 106:1 would suggest. Also, it supports the view that bacteria in natural systems could be more strongly C-limited than current paradigms suggest.

4.2. Optical characterization of DOM

Optical properties have long been used to characterize the composition of DOM and to infer the sources of production (McKnight et al. 2001, Stedmon et al. 2003, Hansen et al. 2016). One such optical property, $SUVA_{254}$, is strongly correlated to the aromaticity of DOM (Weishaar et al. 2003) as well as molecular weight (Chowdhury 2013) and has been used as an indicator of terrestrially derived organic matter, with

higher $SUVA_{254}$ values being associated with more terrestrial influence (Helms et al. 2008, Hansen et al. 2016). Therefore, $SUVA_{254}$ was used here as an optical characterization of the DOM produced by bacteria under different limitation conditions.

For context, $SUVA_{254}$ values for freshwater systems typically range from 1–6 l mg-C^{-1} m^{-1} (Hansen et al. 2016). In this study, the original media had a $SUVA_{254}$ value of 0.136 l mg-C^{-1} m^{-1} and increased over the incubation period in all cultures. DOM associated with algal production and aquatic plant leachates is typically assumed to have $SUVA_{254}$ values less than 1, whereas aged terrestrial organic matter typically has a value of 3 or higher (Pellerin et al. 2010, Hansen et al. 2016). We show that the microbial production of DOM from a single, non-aromatic carbon source could produce $SUVA_{254}$ values as high as 3, more similar to leachates from peatlands than from traditional autochthonous sources (Hansen et al. 2016). Importantly, we saw the highest values for $SUVA_{254}$ when bacteria were growing under C-limited conditions, consistent with the hypothesis that C-limitation should result in increased C-processing and leave behind more complex and less bioavailable C-substrates.

This finding has important implications for the use of $SUVA_{254}$ as a predictor of DOM source in freshwater, particularly in eutrophic lakes. The data presented here indicate that DOM with a relatively high $SUVA_{254}$ value does not necessarily originate in a terrestrial environment. Rather, this DOM may be produced by aquatic heterotrophic bacteria under C-limiting conditions. Therefore, $SUVA_{254}$ may be more indicative of nutrient limitation experienced by microbes processing organic matter than it is of the original organic matter source. Recent work has demonstrated that the vast majority of soil organic matter is highly processed by microbial communities before being exported to aquatic systems (Cotrufo et al. 2015), so the nutrient limitations of those microbial communities may impact the optical properties of this organic matter more than the original source. This suggests that the relatively high $SUVA_{254}$ values commonly associated with terrestrially derived organic matter may reflect persistently high C-demand in soil microbial communities (see Ekblad & Nordgren 2002, Demoling et al. 2007, Spohn & Kuzyakov 2013, Heuck et al. 2015) and/or aquatic communities rather than specific sources of organic matter production. Furthermore, the pattern of increased P-loading into soils and freshwater systems globally would tend to drive the C:P of microbial resources down, which should promote the production of high $SUVA_{254}$ DOM by bacteria.

4.3. Connecting stoichiometric flexibility to DOM production

We observed that resource stoichiometry is a strong control on the microbial production of DOM, both from a quantitative (Figs. 1, 4 & 5) and a qualitative perspective (Figs. 6 & 7). However, pinning down the effect of biomass flexibility and how this physiological strategy interacted with changing nutrient substrates proved challenging. There was a large amount of variation in DOM production within each of the 2 different stoichiometric strategies (flexible vs. inflexible). Nonetheless, differences occurred between flexible and inflexible strains, but only under strong P-limitation. For example, when P-limited, DOC production was lower in flexible strains than inflexible strains (Fig. 2) and biomass accumulation was higher for flexible strains than inflexible strains (Fig. 8), consistent with preferential allocation of C into biomass (as opposed to DOC or excess respiration) by flexible bacteria under P-limitation. This could be driven by a higher respiratory cost for flexible strains that was associated with obtaining P at low P conditions.

One important consideration that likely contributes to the high variability associated with stoichiometric strategies has to do with the nature of that classification. Stoichiometric flexibility is not an intrinsic characteristic of a particular strain; instead it is contextually dependent on the growth conditions (Godwin & Cotner 2018). Previous work has shown that relative growth rate and resource stoichiometry interactively control biomass flexibility (Godwin et al. 2017), and the batch culture approach used in our study allows bacteria to grow at maximum growth rate during the early phases of the incubation and variable relative growth rates later in the incubation period. Given that biomass flexibility seems to be maximized at low relative growth rates (Godwin et al. 2017), our experimental approach could have dampened the effect of biomass flexibility on DOM production. Repeating this basic design in a continuous culture, where relative growth rate can be controlled, may provide a better estimate of the effect of biomass flexibility by more fully activating the physiological response to nutrient imbalance.

In summary, our work lends important insights into the role of aquatic bacteria as producers of organic matter in freshwater systems and identifies key interactions between microbial physiology and nutrient conditions that may impact DOM production by heterotrophic bacteria. We demonstrate the potential for substantial DOM production by aquatic bacteria

under variable nutrient limitation conditions, including the production of highly aromatic compounds under high P conditions. Furthermore, this analysis suggests that interactions between biomass flexibility and nutrient conditions can control the efficiencies and nutritional composition of DOM production, particularly in relation to DOP. Finally, we demonstrated measurable amounts of DOP production by bacteria even under extremely P-limiting conditions, identifying a potential mechanism for the accumulation of low levels of DOP in oligotrophic systems. Taken together, these findings improve our understanding of the fundamental linkages between aquatic bacteria and DOM cycling and allow us to better predict how these linkages may change under future nutrient and carbon scenarios.

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