Effects of terrigenous organic substrates and additional phosphorus on bacterioplankton metabolism and exoenzyme stoichiometry

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Abstract

1. Bamboo, as a pioneer vegetation, often forms forests on bare lands after catastrophic landslides. Compared to evergreen forest soil, bamboo forest soil is much more labile, with a higher percentage of microbially derived organic carbon (OC), lower molecular weight, and lower humic acid content. We hypothesised that different terrigenous organic matter (tOM) sources with varying lability and phosphorus (P) availability select for bacterioplankton with distinct metabolic pathways.

2. We incubated natural bacterioplankton assemblages with tOM leached from bamboo forest soil (BOM) and evergreen forest soil (EOM) and compared these to a lake water control. To test if microbial metabolism would be limited by OC or P availability of each tOM treatment, we used acetate as an extra labile OC source and phosphate as an inorganic P source. Bacterial metabolism was measured by analysing respiration via O₂ consumption and production via tritiated thymidine (TdR) assimilation.

3. Bacterioplankton metabolism is limited by the availability of P in BOM substrates. When using BOM, bacteria had higher enzymatic activities for phosphatase. The nutrients required for bacterial biomass seemed to be derived from organic matter. Under BOM treatment, bacterial production (BP) (0.92 ± 0.13 μg C L⁻¹ hr⁻¹) and cell specific TdR assimilation rates (0.015 ± 0.002 10⁻¹⁸ M TdR cell⁻¹ hr⁻¹) were low. Adding P enhanced BP (BOM +P 1.52 ± 0.31 and BOM +C+P 2.25 ± 0.37 μg C L⁻¹ hr⁻¹) while acetate addition had no significant effect on BOM treatment.

4. This indicated that the bacteria switched to using added inorganic P to respire a P-limited BOM substrate, which increased total BP and abundance, resulting in even more active respiration and lower growth efficiency. We also found higher activities for chitin-degrading enzyme β-N-acetylglucosaminidase, which is associated with N mining from aminosaccharides.
INTRODUCTION

An estimated 1.9 Pg soil-derived organic carbon (OC) is released from land to inland waters each year (Regnier et al., 2013). This complex terrigenous organic matter (tOM) accounts for 89% of the bulk dissolved OC in freshwater systems (Guillemette, McCallister, & del Giorgio, 2016). Terrigenous organic matter and primary production both influence aquatic ecosystem processes by providing varying degrees of carbon, nutrients, and energy (Tranvik, 1988), yet we are just starting to understand the importance of tOM quality for aquatic microbial metabolism. Heterotrophic bacterioplankton is a major player in the regulation of biogeochemical cycles of carbon and nutrients in lentic ecosystems. Lake bacteria can respire tOM (Karlsson, Jansson, & Jonsson, 2007; McCallister & del Giorgio, 2008), repackaging a portion of it into bacterial biomass (Kritzberg, Cole, Pace, Granéli, & Bade, 2004) and further transferring it into aquatic food webs (Berggren, Ström, et al., 2010; Guillemette et al., 2016). Different forest types can provide aquatic ecosystems with tOM of varying quality, some with more labile carbon sources or higher nutrient values than others, thus potentially impacting aquatic bacterial metabolism. However, past studies found that tOM from different woody forests had no effect on carbon-specific respiration due to a slight decoupling of catabolic and anabolic respiratory reactions or the inherent variability associated with respiration measurements (e.g. Lennon & Pfaff, 2005).

Our study compares the effects of tOM from woody and bamboo forests on the metabolism of aquatic bacteria. Belonging to the grass family, bamboo is an important forest ecosystem and natural resource in Asia, Africa, and South America. After an initial lag phase, bamboo forests can effectively accumulate around 2 Mg C ha\(^{-1}\) year\(^{-1}\) soil OC on bare lands after catastrophic landslides, whereas it would take several decades for evergreen woody forests to re-establish effective populations for soil OC sequestration (Schomakers et al., 2017). The chemical composition of soils differs between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016). Moreover, the ratio of microbially derived OC to ambient soil OC is especially high in bamboo forest soils compared to that of evergreen woody forests (Chang & Chiu, 2015), indicating that the former possesses higher levels of labile OC (Sparling, 1992). Although phosphatase activity appears to be similar between bamboo and woody forests: bamboo soils are characterised by a lower molecular weight and lower aromatic content OC pool compared to the high molecular weight (HMW) and high aromatic content OC of evergreen forest soils (Wang, Tian, & Chiu, 2016).
Determining how microbes allocate OC—i.e. how they balance molecule breakdown (e.g. for energy production) and build-up (e.g. for biomass production)—is particularly important for the understanding of organic matter (OM) turnover and fate (Schimel & Schaeffer, 2012). Bacteria have different strategies of resource use to cope with heterogeneous substrates, for instance, the selective use of labile OC (e.g. algal-derived source) for respiration and recalcitrant OC (e.g. lignin-derived source) for biomass production (Guillemette et al., 2016). Which pathways are selected depends on the chemical properties and accessibility of the consumed substrate in the environment (Russell, 2007) as well as the energy and stoichiometric requirements of cells (Vallino, Hopkinson, & Hobbie, 1996). Nutrient limitation and the trophic basis of bacterioplankton production can be understood by focusing on exoenzyme activities and their relationships with OM substrate availabilities or microbial production dynamics (Foreman, Franchini, & Sinsabaugh, 1998).

Complex tOM polymers often stimulate exoenzyme activities that catalyse the degradation of tOM into dimers and monomers, simple units that are then used in metabolic pathways (Chröst, 1990). The processes that determine metabolic rates are constrained by the chemical properties of the available organic substrates, such as humic content (Moran & Hodson, 1990), mean molecular weight (Weiss & Simon, 1999), the stoichiometry of growth-limiting nutrients (Hunt, Parry, & Hamilton-Taylor, 2000), and oxidation state (Vallino et al., 1996). To maintain biomass stoichiometry, microbial communities may adapt their foraging strategies to the available substrates (Sinsabaugh, Manzoni, Moorhead, & Richter, 2013). For instance, β-glucosidase (BG) activity would increase in response to cellobiose presence and decrease in response to glucose presence, and leucyl-aminopeptidase activity would increase in response to labile OC pool, constituting 45% of total bacterial consumption. Although labile OC is typically incorporated into biomass with low efficiencies (Linton & Stephenson, 1978), it potentially provides a significant carbon and energy source for bacteria (del Giorgio & Cole, 1998). In addition to substrate quality, the availability of inorganic nutrients is another key factor limiting bacterial growth (del Giorgio & Cole, 1998). Thus, we also hypothesised that (2) bacterioplankton using BOM substrate will have increased phosphatase activities compared to EOM in order to meet metabolic requirements.

Lennon and Pfaff (2005) found that dissolved organic phosphorus is a major driver of bacterioplankton productivity in different types of temperate deciduous–coniferous forest soil leachates. To investigate how bacteria use P in BOM treatments, we compared their metabolic activities in lake (control), BOM, and EOM substrates with and without additional inorganic phosphorus.

## 2 | METHODS

### 2.1 | Field sampling

Two batches of 10-day microcosm experiments were conducted using filtered lake water (LAKE) with bamboo forest A-horizon soil-derived OM (BOM, 26 October–5 November 2015) and evergreen forest soil (EOM, 9–19 November 2015). Surface water (depth c. 0.5 m) of the oligotrophic lake Lunz (Austria), was used after filtration (GF/C Whatman, 1 μm pore size, VWR international GmbH, pre-combusted at 450°C, 4 hr) to reduce particles and grazing pressure (Moran & Hodson, 1990). The collected water was acclimated inside a climate chamber (20°C) for 24 hr prior to the 10-day experiments.

The topsoil layer (0–10 cm) was collected from a bamboo forest that had established on a 1989 landslide scar, and from a reference evergreen woody forest in the Tsengwen reservoir catchment in Alishan Mountain Range, Taiwan (Schomakers et al., 2017). Currently, this area is low in agricultural activity (6.9% of the total area) but very landslide prone; 3% of the catchment area was bare-lands resulting from previous landslide events (J. C. Huang, personal communication, 2018). Soils were air-dried, gently ground, and homogenised with mortar and pestle to disintegrate the soil aggregates and sieved to 2 mm. To simulate the process of leaching during erosion and transport during landslide events, each type of soil (30 g) was soaked in 1 L autoclaved Milli-Q water and stirred for 48 hr (20°C, 650 rpm) to induce the leaching phase. The slurry was subsequently centrifuged and filtered (GF/F Whatman, 0.7 μm pore size, pre-combusted at 450°C, 4 hr); the leachates were freeze-dried (Freeze drier BenchTop6K v. Labor partner GmbH; Sun, Perdue, Meyer, & Weis, 1997) and stored at −80°C before use. Data showed no significant changes in the optical properties before and after freeze-drying of the soil leachates (Table S1).
2.2 | Experimental setup

Cylinder-shaped acrylic vessels (650 ml, Seitzberger GmbH) were used in the experiments and kept on a horizontal shaker (100 rpm) to guarantee well-mixed conditions and prevent the formation of biofilms and anoxic conditions. The incubation was conducted in the dark inside a 20°C-climate chamber (SimTech SKZ 020-s) to minimise the effect of primary production and photodegradation of dissolved OM. Approximately 1.5 mg freeze-dried BOM or EOM substrate was added and dissolved well into each incubation vessel filled with lake water; similar dissolved OC (DOC) concentrations were yielded on Day 0 in the two tOM treatments, which were significantly higher than that of the LAKE (Figure S1). LAKE-only control treatments (LAKEcontrol) from the two batch experiments were pooled for data analysis, because the water used in the two batch experiments was not significantly different in terms of physicochemical properties and microbiological activities (Table S2). The amendment of labile OC and P was conducted using acetate and phosphate, respectively, and the concentrations used were derived from previous studies focusing on stimulating microbial metabolic activities (e.g. Berggren, Laudon, et al., 2010; Ghosh & Leff, 2013; Guenet et al., 2014; Steen, Quigley, & Buchan, 2016) with some modifications according to previous typhoon field observations (Yeh et al., 2018). Table 1 shows the complete experimental setup with 3 OM substrates (LAKE, BOM, and EOM) and their four nutrient additions (control, +C, +P, and +C+P), which was designed to test the respective first and second hypotheses. Each of the batch experiments had a LAKEcontrol Nutrient addition to LAKE was conducted along with the EOM batch. In total, there were 13 different OM substrate × nutrient addition combinations, and each had three experimental replicates, resulting in n = 39. The +C treatments received 120 μl sodium acetate (C₂H₃NaO₂, 0.1 M, Sigma Aldrich) and the +P treatments received 1,300 μl sodium dihydrogen phosphate (NaH₂PO₄, 1 mM, Sigma Aldrich), resulting in 18.4 μM C and 2 μM P final concentration in the experiment vessels, respectively. The +C+P treatment received both the respective C and P amounts.

2.3 | Laboratory analyses

Samples were analysed on Day 0, 1, 2, 3, 4, 7, and 10, except for respiration which was measured only on Day 0, 4, 7, and 10. Water was filtered (GF/F Whatman, 0.7 μm pore size, VWR International GmbH, pre-combusted at 450°C, 4 hr) for solute analysis. Concentrations of ammonium, nitrite, nitrate, and phosphate were analysed on a continuous flow analyser FLOWSYS RA104 (Alliance Instr.) with a detection limit of 4, 1, 20, and 2 μg/L, respectively. Dissolved OC was measured on a portable TOC analyser (Sievers 900, GE) with a detection limit of 0.2 mg/L.

Each sample was scanned for absorbance at 200–800 nm on a spectrophotometer (Hitachi U-2900) and for fluorescence properties on a fluorospectrometer (Hitachi F-7000) using a 1-cm quartz cuvette. Excitation–emission matrix (EEM) were generated via scanning the samples at excitation (Ex) wavelengths between 200–450 nm at 5 nm steps and emission (Em) wavelengths between 250–600 nm at 2 nm steps, at a scanning speed of 12,000 nm/min. Wavelength-dependent inefficiencies of the detection system where corrected using the manufacturer’s built-in correction methods. Deionised water EEMs were measured each day of analysis in triplicate, and the average subtracted to the sample EEM as a blank. Also, the area under the Raman peak (Em = 371–428 nm, Ex = 350 nm) was used to transform raw fluorescence data into Raman units (Lawaetz & Stedmon, 2009). Absorbance measurements were used to correct for inner-filter effects according to Lakowicz (2006). Parallel factor analysis was used to model the changes in optical properties of dissolved OM using the drEEM toolbox for MATLAB (Murphy, Stedmon, Graeber, & Bro, 2013) (The MathWorks, Inc.). Preliminary models were fit for 2–9 components, and the best model was chosen according to the following criteria: (1) modelled EEMs with pattern-less residuals; (2) chemically meaningful spectral loadings; and (3) split-half validation using for data splits, six combinations, and three validation tests (S4C6T3). Parallel factor analysis components were presented as percentages of the total fluorescence. Moreover, two indices were used, i.e. biological index (BIX, Em intensity at 280 nm divided by that at 365 nm), which is inversely related to aromaticity and average molecular weight (Peuravuori & Pihlaja, 1997). Non-filtered water samples were analysed for bacterial abundance (BA), bacterial respiration (BR), BP, and enzyme activity. For abundance analysis, samples were formaldehyde-fixed (2% final concentration, Sieczko & Peduzzi, 2014), shock-frozen in liquid nitrogen and stored at −80°C prior to further analysis. Stained microbial cells (SYTOX® Dead Cell Stains, Invitrogen, 2.5 μM final concentration) were counted by a flow cytometer (CytoFLEX, Beckman Coulter) and validated by direct count on an epifluorescence microscope (LSM 710, Zeiss); in the latter, 20 ocular fields and a minimum of 200 cells were counted for each sample.

The consumption rate of dissolved oxygen on OM substrate degradation was measured as a surrogate for BR rate following the method of Warkentin, Freese, Karsten, and Schumann (2007). Sub-samples from each experiment vessel were measured inside a clean 100-ml Schott bottle (Duran Group) equipped with a planar optode (Presens),
and were incubated in the same conditions as the experiment vessel. The dissolved oxygen concentration was measured at 0, 30, 60, and 90 min. Respiration rate (mg O₂ L⁻¹ hr⁻¹) was calculated as the slope of dissolved oxygen decrease over the 90-min incubation time, and the rate was converted to C units (µg C L⁻¹ hr⁻¹) assuming a respiratory quotient of 1 (del Giorgio & Cole, 1998; Smith & Prairie, 2004).

Bacterial production was measured by the incorporation rate of ³H-thymidine (³H-TdR) into the DNA of heterotrophic bacterioplankton cells, following the microcentrifugation method (Kirchman, 2001). Methyl-³H-thymidine with a specific activity of 80 Ci/mmol (Amersham Biosciences) was used as the radioactive tracer (20 nM final concentration) which showed a constant isotope 80 Ci/mmol (Amersham Biosciences) was used as the radioactive tracer (20 nM final concentration) which showed a constant isotope 80 Ci/mmol (Amersham Biosciences) was used as the radioactive tracer (20 nM final concentration) which showed a constant isotope 80 Ci/mmol (Amersham Biosciences) was used as the radioactive tracer (20 nM final concentration) which showed a constant isotope

2.4 Statistical methods

Repeated measures analysis of variance (rmANOVA) was performed in the software IBM SPSS (v.21, IBM corp.) to compare means of variables that are based on repeated observations. Bacterial metabolic parameters were dependent variables (DVs); sampling time (Day 0, 1, 2, 3, 4, 7, and 10) was a within-subjects factor; and OM substrate (LAKE, BOM, EOM) and nutrient addition (control, +C, +P, +C+P) were between-subject factors. The statistical design aimed to answer our research questions; i.e. (1) if the mean of BOMcontrol or EOMcontrol was significantly different from that of the LAKEcontrol; and (2) within each OM substrate, if the mean of the +C, +P, or +C+P treatment was significantly different from that of the control at different time points of the experiment. Levene’s test was used for testing if the error variance of the DV was equal across groups. If the assumption of sphericity was violated (Mauchly’s test), the degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Post-hoc tests were conducted using Bonferroni confidential interval adjustment. Main effects of the between-subjects variables (OM substrate or/and nutrient addition) were reported if there was no interaction between OM substrate and nutrient addition on the DV. Otherwise, the dataset was split according to between-subject variables and tested for simple main effect. Standardised major axis (SMA) and redundancy analysis (RDA) were analysed in R (version 3.5.2) and the software RSTUDIO (version

| Enzyme name | EC number | Substrate | Catalysis action | Reference |
|-------------|-----------|-----------|-----------------|-----------|
| β-glucosidase (BG) | EC 3.2.1.21 | 4-MUF-β-D-glucopyranoside | Hydrolysis of the β-glycosidic bonds; releases glucose | Mondini, Cayuela, Sanchez-Monéndero, Roig, and Brookes (2006) |
| Cellobiohydrolase (CBH) | EC 3.2.1.91 | 4-MUF-β-D-cellobioside | Hydrolysis of the non-reducing ends of the cellulose chain; releases cellobiose | Carreiro, Sinsabaugh, Repert, and Parkhurst (2000) |
| Alkaline phosphatase (AP) | EC 3.1.3.1 | 4-MUF-phosphate | Hydrolysis phospholipids and phosphosaccharides; releases phosphate ion | Mondini et al. (2006) |
| β-β-acetilglucosaminidase (NAG) | EC 3.2.1.52 | 4-MUF-N-acetyl-β-D-glucosaminide | Hydrolysis of aminosaccharides from chitin or similar molecules | Parham and Deng (2000) |
| Leucyl-aminopeptidase (LAP) | EC 3.4.11.1 | L-Leucine-AMC hydrochloride | Hydrolysis of peptide bonds adjacent to leucine and other amino acids | Carreiro et al. (2000) |

*aEnzyme commission number.
*bThe entry of N-acetyl-b-D-glucosaminidase was also listed as β-N-acetylhexosaminidase.

TABLE 2 Hydrolysis enzymes used in this study and the related catalytic reactions
The ANOVA-like permutation test was used to find out the most important explanatory variables in the RDA model depending on the values of Akaike information criterion; as well as the scaling between metabolic responses and OM properties. The residuals plots of the SMA regression can be found in Figure S2. Redundancy analysis was used to test the prediction ability of explanatory variables (OM substrate and nutrient addition) on response variables (metabolism) and was conducted in the vegan package (Oksanen et al., 2019). Automatic stepwise model building was used to find out the most important explanatory variables in the RDA model depending on the values of Akaike information criterion; insignificant contribution was excluded. The model was tested using the ANOVA-like permutation test anova.cca (Oksanen et al., 2019).

3 | RESULTS

3.1 | Terrigenous OM characterisation

Bamboo forest and evergreen forest A-horizon soil had similar pH (Table 3). However, evergreen forest soil had higher electrical conductivity, and higher percentage of soil OC and total N. The amount of total P (organic and inorganic) in the evergreen forest soil was almost twice as high as those of the bamboo forest soil.

3.2 | Quantity and quality of carbon and nutrient solutes

Dissolved OC concentrations of BOM\textsubscript{control} and EOM\textsubscript{control} were similar at Day 0 (Figure S1); both of their means were higher than LAKE\textsubscript{control} (rmANOVA, \( p < 0.001 \)). Nutrient addition had no effect on DOC concentration of any OM source. Nitrate-N concentration had similar means across different OM sources and nutrient additions. However, EOM had significantly higher ammonium-N concentration than BOM or LAKE regardless of nutrient addition (Table 4). Treatments + P and +C+P showed the greatest changes in phosphate concentrations in each OM substrate. However, EOM also had higher phosphate-P concentration than LAKE on Day 0, while BOM and LAKE had similar values (Figure S1).

Seven parallel factor analysis components (PCs) describing the quality of OM pools were identified from the model, and their spectral properties were compared with those of Stedmon and Markager (2005) (Table 5, Figure S3 and Table S3). PC2 and PC4, as well as PC1 and PC3 in Raman units were pooled for humic-like and fulvic acid-like OM pools, respectively; tryptophan-like PC5, tyrosine-like PC7, and an unknown free amino acid PC6 (Ex 300/ Em 338, Murphy, Ruiz, Dunsmuir, & Waite, 2006) were treated as the protein-like pool (Table 5). Organic substrate used in the experiment was the only factor influencing the percentage of each OM pool (Table 4). When comparing with LAKE\textsubscript{control}, BOM\textsubscript{control} yielded a higher percentage of the humic-like fluorophore pool, a lower fulvic-like pool, but a similar percentage of protein-like pool. EOM\textsubscript{control} had high humic and fulvic-like pools, but a lower protein-like pool than LAKE\textsubscript{control} (Table 4). As indicated by a low \( E_2' : E_3' \) and BIX, both BOM\textsubscript{control} and EOM\textsubscript{control} had higher mean molecular weight and higher recalcitrance than LAKE\textsubscript{control} (\( p < 0.001 \)); however, the molecular weight and recalcitrance of EOM was also higher than BOM. BOM\textsubscript{C+P} had significantly higher mean molecular weight than BOM\textsubscript{control}. Based on the SMA regression, the mean molecular weight increased with the fluorescence intensity of the humic-like fluorophore pool in EOM regardless of nutrient addition (\( n = 84, r^2 = 0.23, p < 0.001 \)), but this relationship was absent for BOM. Furthermore, both mean molecular weight and humic-pool had negative relationships with BIX in EOM substrate (\( n = 84, r^2 = 0.19, p < 0.001 \)), but this relationship was rather weak for BOM (\( n = 84, r^2 = 0.08, p = 0.007 \)).

3.3 | Bacterioplankton metabolism

Bacterial production was significantly higher in EOM\textsubscript{control} than LAKE\textsubscript{control} (\( p < 0.001 \), Figure 1a). The BP of +P and +C+P treatment of LAKE and BOM were significantly higher than those of LAKE\textsubscript{control} and BOM\textsubscript{control} respectively. EOM\textsubscript{control} had the highest cell-specific TdR assimilation rate, while BOM\textsubscript{control} had similar value as LAKE\textsubscript{control} (Figure 1b). However, all nutrient treatments of LAKE and BOM\textsubscript{C+P} showed higher cell-specific TdR assimilation rate compared to LAKE\textsubscript{control} and BOM\textsubscript{control} respectively. Bacterial production was positively related to BIX—the freshly released dissolved OM (SMA, \( r^2 = 0.38, p < 0.001 \), slope = 10.2), the fluorescence intensity of humic and fulvic-like pools (\( r^2 = 0.4 \) and 0.2, \( p < 0.001 \) and

| Soil type                  | pH (H2O) | Electrical conductivity (µS/cm) | Organic carbon (%) | Total nitrogen (%) | Total phosphorus (µg/p) | Organic phosphorus (µg/p) | Inorganic phosphorus (µg/p) |
|---------------------------|----------|-------------------------------|-------------------|-------------------|-------------------------|----------------------------|-----------------------------|
| Bamboo forest A-horizon soil (BOM) | 4.9      | 108                           | 5                 | 0.3               | 446                     | 358.9                      | 87.1                        |
| Evergreen forest A-horizon soil (EOM) | 4.2      | 246                           | 9.41              | 0.74              | 836.8                   | 686                        | 150.8                       |
TABLE 4: Descriptive statistics of dissolved organic carbon (DOC), ammonium-N, nitrate-N, phosphate-P concentrations; humic-, fulvic acid-, and protein-like dissolved organic matter molecular weight index. Means ± 1 statistical differences (p<0.05, repeated measures ANOVA) among OM substrates and OC or P addition, respectively.

| Addition | DOC (mg/L) | Protein-like (%) | Fulvic-like (%) | Humic-like (%) | BIX | E3 | E2 | E1 |
|----------|------------|------------------|----------------|---------------|-----|----|----|----|
| OM       |            |                  |                |               |     |    |    |    |
| LAKE     | 2 ± 0.09   | 0.06 ± 0.11      | 3.49 ± 0.39    | 39.38 ± 0.52  | 0.43 | 0.01| 1.35| 0.21|
|           | C          | 2.04 ± 0.06      | 1.83 ± 0.09    | 33.49 ± 30.94 | 20.62 | 1.22| 0.96| 0.21|
|           | P          | 1.77 ± 0.06      | 2.33 ± 2.34    | 30.7 ± 31.2   | 0.96 | 0.02| 0.75| 0.01|
|           | +C         | 3.83 ± 0.32      | 2.21 ± 3.41    | 25.32 ± 39.43 | 2.92 | 1.48| 1.48| 0.75|
|           | +P         | 3.54 ± 0.36      | 2.69 ± 39.43   | 33.42 ± 30.4    | 0.96 | 0.02| 0.75| 0.01|
|           | +C+P       | 3.40 ± 0.21      | 3.76 ± 31.4    | 30.34 ± 35.83 | 0.96 | 0.02| 0.75| 0.01|
| BOM      | 3.86 ± 0.50 | 3.80 ± 41.46     | 3.86 ± 4.28    | 38.32 ± 42.68  | 1.48 | 1.48| 0.75| 0.01|
|           | C          | 3.83 ± 0.32      | 2.21 ± 3.41    | 25.32 ± 39.43 | 2.92 | 1.48| 1.48| 0.75|
|           | P          | 3.54 ± 0.36      | 2.69 ± 39.43   | 33.42 ± 30.4    | 0.96 | 0.02| 0.75| 0.01|
|           | +C         | 3.40 ± 0.21      | 3.76 ± 31.4    | 30.34 ± 35.83 | 0.96 | 0.02| 0.75| 0.01|
| EOM      | 3.48 ± 0.09 | 7.23 ± 23.72     | 3.67 ± 42.81   | 39.75 ± 37.73  | 0.96 | 0.02| 0.75| 0.01|
|           | C          | 3.48 ± 0.09      | 7.23 ± 23.72   | 3.67 ± 42.81   | 39.75 ± 37.73 | 0.96 | 0.02| 0.75| 0.01|
|           | P          | 3.59 ± 0.17      | 8.76 ± 15.04   | 39.75 ± 37.73  | 39.75 ± 37.73 | 0.96 | 0.02| 0.75| 0.01|
|           | +C         | 3.81 ± 0.15      | 8.76 ± 15.04   | 39.75 ± 37.73  | 39.75 ± 37.73 | 0.96 | 0.02| 0.75| 0.01|
|           | +P         | 3.81 ± 0.15      | 8.76 ± 15.04   | 39.75 ± 37.73  | 39.75 ± 37.73 | 0.96 | 0.02| 0.75| 0.01|

Relationships between hydrolysing exoenzyme activities

Using slopes of SMA regression, the scaling of MEF of C-hydrolysing enzymes MEF_{BG+CBH} versus MEF_{NAG} or MEF_{AP} between BOM and
EOM substrate was compared, as well as the difference between tOM substrate control and its nutrient additions. Significant relationships are presented in Table 6. Bamboo OM and EOM had significantly different slopes for MEF$_{BG+CBH}$ versus MEF$_{AP}$ and MEF$_{BG+CBH}$ versus MEF$_{NAG}$ relationships ($p = 0.008$ and <0.001, respectively) but similar slopes for MEF$_{BG+CBH}$ versus MEF$_{LAP}$ ($p = 0.901$). BOM yielded significantly higher slopes of both enzyme-scaling relationships than EOM. Nutrient addition only had effects on BOM substrate in terms of N and P acquisition scaling; i.e. the slope of MEF$_{BG+CBH}$ versus MEF$_{AP}$ for BOM$_{+C+P}$ was significantly lower than that of the BOM$_{control}$ and the slope of MEF$_{BG+CBH}$ versus MEF$_{NAG}$ was lower for BOM$_{+P}$ and BOM$_{+C+P}$ than for BOM$_{control}$.

| TABLE 5 | Properties of the seven parallel factor analysis components (PCs) identified from the fluorescent dissolved organic matter of this study |
|------------------|-------------------------------------------------------|
| Components        | Stedmon and Markager (2005) | This study | Excitation and emission maxima | Origin | Properties                        |
| PC1               | PC4                      | Ex < 250/Em 448 | Terrestrial | Humic fluorophore group |
| PC3               | PC2                      | Ex < 250/Em 412 | Terrestrial | Humic fluorophore group |
| PC2               | PC3                      | Ex < 250/Em 504 | Terrestrial/autochthonous | Fulvic acid fluorophore group |
| PC4               | PC1                      | Ex < 250/Em 440 | Terrestrial/autochthonous | Fulvic acid fluorophore group |
| PC7               | PC5                      | Ex 280/Em 344 | Autochthonous | Tryptophan fluorophore (protein) |
| PC8               | PC7                      | Ex 275/Em 304 | Autochthonous | Tyrosine fluorophore (protein) |

**FIGURE 1** Bacterioplankton metabolism: (a) bacterial production; (b) cell-specific TdR assimilation; (c) bacterial respiration; (d) cell-specific O$_2$ consumption; (e) bacterial abundance; and (f) bacterial growth efficiency of Lake water (LAKE), bamboo and evergreen forest terrigenous organic matter (BOM and EOM, respectively). Each OM substrate had control, +C, +P, and +C+P nutrient addition treatments which were distinguished by bar colours shown in the legend. Letters mark the significant differences ($p < 0.05$) between tOM substrate and LAKE$_{control}$; asterisks mark the significant differences between nutrient addition and the respective OM substrate control (rmANOVA, Means ± 1 SEM, $n = 21$).
Standardised major axis regression between potential exoenzyme activity C:N versus C:P, denoted the relationship between activities of N- and P-hydrolase relative to C-acquisition (Figure 5), with OM substrate being the only factor regulating the slopes. Significantly different slopes for BOM and EOM (slope = 2.03 and 1.34, respectively, \( p = 0.002 \)) were observed; however, this SMA regression was not significant for LAKE substrate.

3.6 | Constrained RDA

The +C treatments were omitted from the analysis due to an insignificant contribution to the model. The constrained variables (BOM, EOM, and +P) explained 42% of total variance; the unconstrained variable accounted for 49.7% and Time accounted for 8.3% of total variance. The first and the second RDA axes accounted for 51.2 and 29% of total variance, respectively. The permutation test result of observed values was not different from that of the permutation on Time, implying there was a significant linear relationship between the response matrix and the explanatory matrix.

The first RDA axis was mainly attributed to the presence of tOM, separating BOM and EOM from LAKE (Figure 6). The second axis defined the effect of P concentration, separating the higher P content EOM and P-addition treatments from BOM. Both tOM substrates were associated with DOC concentration, N-hydrolysing enzymes, protein-like OM, and BA. However, EOM substrate was also associated with DIN concentration and humic-like OM pool, while BOM was related to P-hydrolysing enzyme. LAKE samples were only associated with BR. The +P addition was related to phosphate concentration, BP, and C-hydrolysing enzyme activity. In sum, tOM addition was the strongest factor controlling data ordination. In comparison to EOM, microbes using BOM as the main substrate were possibly subjected to dramatic changes when receiving additional P, as seen from the high variation between BOM and the +P treatment occupying opposite sides of the second axis.

4 | DISCUSSION

In this study, we investigated the effects of OC lability and P availability of tOM on aquatic bacterioplankton metabolic strategies. We found that the two tOM conditions induced different metabolic pathways. Differences in the enzymatic activities and their ratios show that this disparity is probably the result of nutrient availability rather than OC lability, and that the bacteria assemblages strategically targeted different organic compounds (e.g. Clinton et al., 2010).

4.1 | Effects of terrigenous substrates and additional labile OC on bacterioplankton metabolism

Substrate availability is a main factor that regulates bacterial growth efficiency—i.e. the percentage of carbon assimilated into biomass of the total amount of carbon consumed (Middelboe & Søndergaard, 1993). Bamboo forest soil OM had a lower percentage of N and P, which was associated with lower BP at the cellular level. However, this was compensated by higher cell abundance, ultimately resulting in a bacterial growth efficiency between that of LAKE and EOM. Soil studies have shown that labile carbon functional groups O-alkyl-C and carboxyl-C, which constitute carbohydrates or cellulose in fresh plant material (Solomon et al., 2010), are significantly higher in BOM than that of woody forests (Wang et al., 2016). However, the labile carbon did not translate to higher growth efficiencies. The BOM control treatment did not exhibit a higher degree of cell-specific TdR assimilation, cell-specific \( O_2 \) consumption rates, and bacterial growth efficiencies compared to that of LAKE control.

![Figure 2](image-url) Bacterial production versus humic-like OM pool. Bamboo (circles and dashed regression line, \( n = 84 \)) and evergreen forest terrigenous organic matter (triangles and solid line, \( n = 84 \)). Open symbols = samples without P addition; black symbols = samples with P addition.

![Figure 3](image-url) Bacterial growth efficiency versus bacterial abundance. Bamboo (light grey circles, \( n = 32 \)) and evergreen forest terrigenous organic matter (dark grey circles and solid regression line, \( n = 44 \)).
Bamboo OM might support increased BA but not production because the substrate has limited nutrition values, especially in P. This limitation can impact the regulation of bacterial physiology and metabolism. For instance, it has been shown that microbes may reduce the size of their DNA pool by maintaining fewer genome copies under P-limited conditions (Zerulla et al., 2014). We observed that BOM treatments induced exoenzymatic activities associated with polymer breakdown, which are often inversely correlated to bacterial growth efficiency (Middelboe & Søndergaard, 1993).

The EOM control treatment had significantly higher increase of BP and showed the highest mean molecular weight and yielded the highest percentage of humic- and fulvic acid-like pool but with the lowest percentage of protein-like compounds. The humic-like OM pool and mean molecular weight of EOM control were negatively correlated to biological index and BP. This agreed with the general model that the degree of substrate recalcitrance, in terms of microbial degradation, is positively correlated with the molecular weight of the OM pool (Saunders, 1976). This complex substrate induced
1983

the BG and leucyl-aminopeptidase activities in EOM control, the C and N released from the substrate were used for higher BP (Foreman et al., 1998). The metabolic activity of EOM control is in line with studies demonstrating that, under low concentrations of labile C, there is a bacterial preference for humic-like compounds over dissolved inorganic nitrogen as the former is a source of both C and N (Carlsson, Segatto, & Granéli, 1993; Ghosh & Leff, 2013).

When there is a surplus of carbon relative to available nutrients, bacteria often dispose of the excess carbon to help maintain intracellular stoichiometry. This is done by increasing respiration or by excreting extracellular polysaccharides or metabolites to dissipate extra energy (Decho, 1990; Hessen & Anderson, 2008; Linton, 1990). In this study, additional acetate was used to test the OC limitation when bacterioplankton uses the two tOM sources. Although not statistically significant, cell-specific $\text{O}_2$ consumption rates increased in both tOM substrates receiving +C or +C+P, whereas in LAKE conditions there was no such trend. This increase could potentially be attributed to an *priming effect*, that the added labile OC influenced the mineralisation rate of relatively recalcitrant tOM (Guenet et al., 2014). However, only the LAKE$_+C$...
treatment, not BOM\textsubscript{C} or EOM\textsubscript{C}, induced cell-specific TdR incorporation rates. Our result reflected imbalanced ambient inorganic nutrients in BOM\textsubscript{C} for bacteria to incorporate the added carbon. The added acetate was probably respired efficiently, leading to an 108% increase in respiration rate in BOM\textsubscript{C} without inducing other metabolic responses. However, this increase was not statistically significant due to large variability in respiration measurements. Although acetate provides a labile OC source, it is a relatively oxidised organic substrate poor in energy and may instead trigger the microbial community to maintain respiration or produce enzymes (del Giorgio & Cole, 1998).

By contrast, EOM\textsubscript{C} showed a slight reduction of BG and CBH activities by 21 and 29%, respectively, when compared to EOM\textsubscript{control}, suggesting that bacteria may have used acetate as a carbon source in addition to humic substrate. However, this result was statistically not significant. The experimental design could be a part of the reason. Following Berggren, Laudon, et al. (2010), who found that acetate is an important OC source for BP, we added similar concentrations of acetate to our treatments, but much lower than that of Steen et al. (2016). Furthermore, the amount of acetate added was probably proportionally too small compared to what was released from the IOM substrates, considering that 1 g of forest soil could form about 35–220 \( \mu \)g acetate per 24 hr in the aquatic context (Küsel & Drake, 1995).

Although discussions on microbial life cycle and community structure exceed the scope of this study, it merits further investigation. Bacterial growth efficiency tends to decrease as substrate quality (in terms of nutrient content) drops and population generation time increases (Middelboe & Søndergaard, 1993). On the one hand, HMW compounds might support taxa that can produce certain enzymes. This could reduce bacterial diversity upon HMW compound addition (Balmonte et al., 2019). On the other hand, however, some have suggested that humic substances may instead promote microbial diversity, as the enzymatic breakdown of complex substrates can support members in the community (Ghosh & Leff, 2013).

Overall, our result echoes the observation by Guillemette et al. (2016) that bacterioplankton use different metabolic pathways when using labile versus complex carbon. The more labile substrate was preferentially allocated to maintain respiration and exoenzyme production, whereas complex and recalcitrant IOM was preferentially allocated to BP and the carbon was repackaged into biomass. However, the acetate experiments suggested that nutrients might be the more important factor causing this disparity rather than OC lability between the two types of IOM. Indeed, the nutrition-rich EOM substrate induced greater bacterial growth efficiency regardless of further acetate or phosphorus treatments, with significantly higher TdR incorporation rates and lower \( O_2 \) consumption rates at a cellular level.

### 4.2 Effect of additional inorganic phosphorus on bacterial degradation of terrigenous substrates

The difference in soil IOM quality is derived from vegetation type. In particular, the P content of IOM is a major parameter influencing the production of freshwater bacterioplankton in the receiving waters (Lennon & Pfaff, 2005). Terrigenous OM contains organic P, which could alleviate P limitation of bacterioplankton production (Soares et al., 2018). The increase of BA is often the primary response when phosphorus-limited bacteria receive an input of P (Middelboe, Jørgensen, & Kroer, 1996; Steen et al., 2016). In our study, this was especially true for the EOM which showed significant increase in both BP and abundance with or without inorganic P addition. The organic P source in BOM\textsubscript{control} and EOM\textsubscript{control} increased the bacterial cell numbers (129 and 164\% higher than that of LAKE\textsubscript{control} on average, respectively). Under P-limitation, the uptake of C in BOM\textsubscript{control} and BOM\textsubscript{C} was allocated to respiration to maintain cellular functions such as membrane integrity, active transport systems, nutrient acquisition, and enzyme production (del Giorgio & Cole, 1998; Russell, 1991). Therefore, P-addition had a significant impact on the P-limited BOM substrate, which yielded significantly increased BA in BOM\textsubscript{P} and BOM\textsubscript{C+P} (35\% higher than that of BOM\textsubscript{control}) and an increased trend of BA. This suggested that the bacteria switched to use inorganic phosphate in these treatments, as bacteria production disassociated from the OM properties. Since inorganic nutrients are expensive energy sources, BR of OC is increased to meet the rising energy demands (Middelboe & Søndergaard, 1993). Subsequently, bacterial growth efficiency of BOM started to decline (uncoupling from BA) as it reached approximately 20\%. One factor that uncouples growth rate (population generation time) from bacterial growth efficiency is that bacteria may maximise growth at the expense of efficiency (Russell, 1991).

Enzyme ratios of C:N and C:P are indicators of environmental CNP stoichiometry. The differences in slopes of the production-specific exoenzyme activities—\( MEF_{BG+CBH}/MEF_{NAG+LAP} \) versus \( MEF_{BG+CBH}/MEF_{AP} \) relationships indicate differences in the stoichiometry of N and P acquisition relative to C availability (Sinsabaugh et al., 2010). Scaling relationships for exoenzyme activities normalised to productivity showed that the bacteria potentially acquired more units of C per acquired P when using BOM\textsubscript{control}. This value dropped after BOM received external P input. Moreover, the ratio between NAG and LAP signals the relative importance of the cell wall-derived aminosaccharides such as chitin as a N source (Sinsabaugh et al., 2010). In our study, BOM\textsubscript{control} and BOM\textsubscript{C} had a ratio of 0.5, which was 18.5 times higher than that of EOM\textsubscript{control} and EOM\textsubscript{C} (ratio = 0.027). This shows that the breakdown of peptidoglycan in bacterial cell walls (Benner & Kaiser, 2003) or fungal chitin (Gooday, 1990) was relatively important in BOM. Bacterial breakdown of aminosaccharides in turn generated sources of N such as small oligosaccharides and N-acetylglucosamine, which can be directly incorporated into bacterial cells (Wetzel, 1991). This was supported by the significant positive correlation between BP and the fresh released OM pool indicated by BIX in BOM\textsubscript{control} and BOM\textsubscript{C} treatments. Moreover, bacteria probably used the released fresh dissolved OM to produce humic-like bacterial substances (e.g. Guillemette & del Giorgio, 2012), as the fluorescence intensity of humic-like pool of these treatments increased along the BP gradient. The
concentration of N-acetylglucosamine also promotes the chitinase gene expression of chitinolytic bacteria (Delpin & Goodman, 2009), potentially leading to the rapid turnover of this specific bacterial assemblage that preferentially uses labile BOM (e.g. Crump, Kling, Bahr, & Hobbie, 2003). The chitinolytic degradation process could have generated deacetylated cellulose-like molecules (Beier & Bertilsson, 2013) and contributed to the simultaneous increase of CBH activity in the BOMcontrol. However, the advantage of using aminosaccharides to compensate N requirement decreased after BOM treatments received inorganic P (NAG to LAP ratio = 0.1).

EOM, by contrast, provided 87.6% more dissolved organic P than BOM. Using this organic P source may have allowed bacteria to also utilise more recalcitrant carbon molecules (Benner, Lay, K&nees, & Hodson, 1988), like humic substances, probably through enhanced exoenzyme production (Sinsabaugh, Findlay, Franchini, & Fischer, 1997). However, phosphatase activity often negatively correlates to inorganic P concentration (Clinton et al., 2010), as seen in the decreased phosphatase activity in EOM treatment and all +P treatments regardless of tOM source. As the bacteria in EOMcontrol and EOM+P also consumed the added inorganic phosphate for production, it is likely that the P demand in these treatments increased. As the cell number and production in EOMcontrol and EOM+P remained similar to that of EOMcontrol, the rising P-demand was probably related to the need to synthesise more phosphorus-rich molecules (Yao et al., 2016).

The RDA sums up the interactive effect between tOM, additional acetate, and phosphate on aquatic bacterioplankton metabolism. We argue that EOM provided a nutrient- and humic-rich tOM substrate, which had a higher capacity to maintain metabolic direction towards bacteria production and yield higher bacterial growth efficiency. This was similar to the results of previous experiments that added aged humic material to bacterioplankton assemblages (e.g. Eiler, Langenheder, Bertilsson, & Tranvik, 2003; Lennon & Pfaff, 2005). Humic-rich EOM stimulated BG activity to produce available OC. However, with the acetate concentration we added in this experiment, labile OC proved to be a statistically insignificant term in the RDA model. Both LAKE and BOM allocated most resources into activities related to molecular breakdown and were more influenced by additional phosphorus. bamboo OM provided a labile OC source that enhanced N- and P-hydrolysing enzyme activities. Once they received additional P, bacterioplankton dramatically changed either their metabolic strategy or community structure to maintain their stoichiometric requirements.

5 | CONCLUSION

Our results show that BOM and EOM provide bacterioplankton with substrates of different OM quality and P content, inducing distinct metabolic pathways (BP or respiration). These results have several important implications for future research.

We showed that bacterioplankton in BOM treatments invested in cell numbers and exoenzyme activities that hydrolyse aminosaccharides, which could be associated with the breakdown of microbial detritus (e.g. cell walls) in the tOM pool. The metabolism of microbially-derived carbon may thus unlock carbon sequestered in microbial biomass of terrigenous carbon sources in aquatic systems. Future research can further investigate whether microbially-derived or plant-derived compounds were consumed for bacterial growth or respiration with, for instance, tracer techniques.

Another implication is that aquatic systems dominated by BOM inputs are likely to increase their bacterial cell numbers, produce exoenzymes, and respire more C after receiving abundant terrigenous phosphorus eroded seasonally during heavy rainfall events in steep riparian zones (Lee et al., 2013). Furthermore, as tillage and N fertilisers are commonly used in bamboo forests for enhancing vegetation biomass production (He & Ye, 1987), N residues entering the aquatic systems will be likely to change the nutrient acquisition strategy of aquatic microbial assemblages. This is because there is interdependence between the acquisition of organic N and P in microbial communities in BOM as opposed to EOM substrates. We thus advise future research to focus on the effect of N manipulation on aquatic metabolism using BOM as the main substrate.

Finally, as tOM substrates originating from different vegetation types can induce distinct bacterioplankton metabolic pathways, future model-building should take tOM quality into consideration when estimating soil carbon turnover rates along a terrestrial-aquatic continuum.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
Further data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION** Additional supporting information may be found online in the Supporting Information section.