New insights on resource stoichiometry: assessing availability of carbon, nitrogen and phosphorus to bacterioplankton

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Abstract. Boreal lake and river ecosystems receive large quantities of organic nutrients and carbon (C) from their catchments. How bacterioplankton respond to these inputs is not well understood, in part because we base our understanding and predictions on 'total pools', yet we know little about the stoichiometry of bioavailable elements within the organic matter. We designed bioassays with the purpose ~~t~~of exhaust~~ing~~ the pools of readily bioavailable dissolved organic carbon (BDOC), bioavailable dissolved nitrogen (BDN) and bioavailable dissolved phosphorus (BDP) as fast as possible. Applying the method in four boreal lakes at base-~~-~~flow conditions yielded concentrations of bioavailable resources that ranged from 105-693 μg C L^{-1} for BDOC (2 % of <u>initial</u> total DOC), 24-288 μg N L^{-1} for BDN (31 % of <u>initial</u> total dissolved nitrogen) and 0.2-17 μg P L^{-1} for BDP (49 % of <u>initial</u> total dissolved phosphorus). Thus, relative bioavailability increased from carbon (C) to nitrogen (N) to phosphorus (P). We show that the main ~~part~~ <u>fraction</u> of bioavailable nutrient~~s~~ ~~resources~~ is organic, representing 80 % of BDN and 61% of BDP. In addition, we demonstrate that total C : N and C : P ratios are as much as 13-fold higher than C : N and C : P ratios for bioavailable resource fractions. Further, by applying additional bioavailability measurements to seven widely distributed rivers, we provide support for a general pattern of relatively high bioavailability of P and N in relation to C. Altogether, our findings underscore the ~~role of C as limiting factor~~<u>poor availability of C</u> for <u>support of</u> bacterial ~~growth~~ <u>me~~a~~tabolism</u> in boreal C-rich freshwaters, and suggest that these ecosystems are very sensitive to increased input of bioavailable DOC.

1 Introduction

Nutrient regulation of freshwater plankton productivity is central to the response of river and lake ecosystems to <u>changes in nutrient loading that result from</u> land use and climate change. By controlling phytoplankton primary production (PP) and bacterioplankton secondary production (BP), phosphorus (P) and nitrogen (N) are the two key macronutrients shaping aquatic ecosystems, with consequences for food web structure, biodiversity, and biogeochemical cycles (Jones, 1998). In addition to these nutrients, the supply of dissolved organic carbon <u>(DOC)</u> has strong effects on ecosystem functioning by

fueling BP and bacterial-based heterotrophic food chains (Dillon and Molot, 2005;Karlsson et al., 2012;Tranvik, 1998). While nutrient availability can be influenced by internal lake processes, the regulation of PP and BP in the majority of lakes worldwide is constrained by loading of inorganic and organic resources from the surrounding terrestrial landscape (Wetzel, 2001). In brown-water boreal lakes, nutrients bound to dissolved organic matter (DOM) (e.g., humic substances) often dominate inputs (Jansson, 1998). In such systems, terrestrial nutrient support of BP is of particular ecological and biogeochemical importance, as heterotrophic processes often greatly exceed autotrophy (Jansson et al., 2000).

While the importance of nutrient availability at the ecosystem level is evident, characterizations of the actual proportion of terrestrially-derived resources that can be readily used by aquatic microorganisms are difficult and attempts are rare. A variable fraction of C, N, and P of terrestrial origin is chemically bound in organic molecules that are typically too large to be directly taken up by microbes (Battin et al., 2008). The nature of the covalent bonds and the structure of organic compounds that hold N and P also differentially influence the bioavailability and turnover of associated nutrients (Vitousek et al., 2002). Such complexity makes it difficult to predict the potential for bacterial usage of these resources at ecologically relevant-scales (Bronk et al., 2007;Berggren et al., 2015;Helton et al., 2015). It is generally thought that the major fraction of ~~dissolved organic carbon (~~DOC~~)~~ originating from terrestrial soils is recalcitrant, yet bioavailability estimates from different lakes suggest that a variable proportion of DOC can be used by bacteria (e.g., 6-14 %; Tranvik, 1988). For dissolved organic nitrogen (DON), a summary of published assays suggests that anywhere from 2-75 % of the organic N pool may be bioavailable (Pellerin et al., 2006), with a range of 19-28 % reported for boreal streams during base-flow (Stepanauskas et al., 1999). Similarly, w~~W~~hile less studied, P bioavailability appears to be ~~equally~~ highly variable over space and time (Muscarella et al., 2014). For example, it has been shown that seasonal concentrations of bioavailable P ranged from 1 to 14 μg P L^{-1} in boreal headwater streams, representing from < 5% to nearly 50% of the total P pool (Jansson et al., 2012). Most studies on nutrient availability conducted in humic-rich waters have neglected this variability in bioavailability, focusing on either ~~on~~ total inputs (i.e., total N or total P) or on the turnover of specific fractions assumed to be bioavailable (e.g., dissolved inorganic nitrogen, DIN; molybdate reactive phosphorus, MRP). However, inorganic fractions may constitute only a small part of the total nutrient pools and can underestimate resource bioavailability in organic-rich waters with large pools of labile DON or dissolved organic phosphorus (DOP; Seitzinger et al., 2002).

These p~~P~~itfalls of assuming resource availability to bacterioplankton ~~and resource limitation~~ from total pools or inorganic fractions ~~to bacterioplankton~~ have prompted the suggestion that standardized bioavailability assays (~~re-~~growth bioassays) should be incorporated into the analytical toolbox of aquatic researchers (Lewis, 2011). Bioavailability represents an ~~These are~~ operationally defined resource, typically ~~bioavailability~~ measure~~ments~~d in assays ~~in which~~where a~~n~~ bacterial inoculum is added to a sterile-filtered water sample ~~solution~~ and the bacterial biomass is allowed to grow during a standardized incubation at a determinate temperature. ~~This~~ The ~~re-~~growth response is used to ~~assess how much~~quantify the resource~~s~~ that ~~were~~ was consumed during the incubation, which is a measure of bioavailability (sensu Berggren et al., 2015). Unfortunately results from the few different studies addressing bioavailable resource shares for bacterioplankton are difficult to compare since different methodological approaches are used (Berggren et al., 2015). For instance, studies of DOC bioavailability have

used methods that differ in terms of incubation length, temperature~~, inorganic nutrient concentrations~~, as well as ~~in the approach used to inoculate samples with microbial communities~~the choices of inoculum and inorganic growth media (del Giorgio and Davis, 2003). Similarly, as different techniques and assumptions have been applied to assess nutrient availability, results for N and P differ among studies and are generally not comparable as they often reflect variation in experimental factors rather than in the intrinsic molecular properties of the nutrients themselves. Thus, a standard and comparable method that can tackle the bioavailability of multiple elements to bacterioplankton is ~~still~~ missing.

Previous attempts to measure nutrient bioavailability of multiple elements have mainly been performed over very long time-scales (most data from 100-day incubations; see data review by Lonborg and Anton Alvarez-Salgado, 2012) and do not represent the pool that is immediately available for consumption. These l~~L~~ong-term assays have not been based on growth, but on ~~long term~~ changes in bulk nutrient concentrations in solution (Lonborg and Anton Alvarez-Salgado, 2012). However, d~~D~~uring long incubation periods various factors can interfere with the uptake of bioavailable resources, such as ~~for instance,~~ the dynamics of viruses and the development of toxic conditions that ~~can~~may ~~appear~~arise from repeated bacterial regeneration of resources (Cho et al., 1996). ~~These assays have not been based on re growth, but on long term changes in bulk nutrient concentrations in solution (Lonborg and Anton Alvarez Salgado, 2012).~~ To move the nutrient stoichiometry field forward, a promising option is to measure the uptake of nutrients through growth bioassays conducted at ~~more~~shorter ~~ecologically relevant~~meaningful timescales~~,~~. where ~~i.e. only longin which~~ the incubation length is reduced to a minimum and sufficient time for bacteria to take up most of the readily bioavailable pool ~~enough to exhaust the readily bioavailable nutrient pool during a few single days~~ (sensu Berggren et al., 2015). These~~Such~~ bioassays ~~will~~can ~~thus~~ increase our~~the~~ understanding of the direct controls o~~n bacterial metabolism by~~nf bioavailable nutrient pools~~ over the medium term pool~~.

Although growth bioassays have previously been applied to calculate bioavailability of single elements (Stepanauskas et al., 2002;Jansson et al., 2012;Stepanauskas et al., 2000), no ~~such~~ efforts to date have quantified the bioavailability of more than two elements simultaneously, so that the relative availability of multiple resources can be directly compared. In a recent review on bioavailability (Berggren et al., 2015), it was additionally suggested that nutrient bioavailability (as a fraction of the total pool) ~~actually may~~tends to increase from C to N and N to P in DOM-rich systems. While this hypothesis is generally consistent with our understanding of resource use in soils (Vitousek et al. 2002), it ~~has~~remain yet to be ~~accurately~~ systematically tested in surface waters.

In this study, we designed bioassays with the purpose t~~o~~f rapidly exhaust~~ing~~ the pools of readily available organic C, N and P, accessible to bacterioplankton in DOM-rich lakes. The bioassays were designed such that most of the nutrients were used within three days, although we measured the cumulative nutrient use ~~during~~up to seven days. We first calibrated our method by detecting the response (leucine incorporation) of nutrient-starved bacteria to known added amounts of bioavailable resources. We ~~further~~ then validated this bacterial response through comparison with common methods to detect bioavailability: lability incubations for DOC bioavailability (del Giorgio and Cole, 1998), ~~growth~~ cell production bioassays with N-~~-~~starved bacteria for N bioavailability (Stepanauskas et al., 2000) and measuring P content in bacterial growth cultures harvested on filter (Jansson et al., 2012). Specifically, by using this new bacterioplankton growth bioassay our study

~~aimed to ask~~addresses the questions: 1) How does the relative total bioavailability in DOM-rich surface waters differ between the elements, i.e. bioavailable dissolved organic carbon (BDOC) out of total DOC, bioavailable dissolved nitrogen (BDN) out of total N and bioavailable dissolved phosphorus (BDP) out of total P, respectively, and do these ~~shares~~ proportions vary seasonally?; 2) Are the organic bioavailable N and P pools larger than the corresponding inorganic pools?; 3) ~~Does the use~~By how much do ~~of~~ total C:N, C:P and N:P ~~yield~~ ratios ~~that are higher~~exceed ~~than the actual ratios between~~ bioavailable C:N, C:P and N:P~~.~~?~~:~~ This was tested by performing bacterial growth bioassays on four boreal lakes in northern Sweden with high DOM concentrations. In addition, we applied a simplified version of our new method to assess broad patterns in nutrient bioavailability across a larger cross-regional scale and climate gradient that comp~~rrom~~ises seven river systems with variable DOM concentrations.

2 Methods

2.1 Study area and sampling

We studied four lakes in northern boreal Sweden: Övre Björntjärnen, Lillsjöliden, Struptjärnen and Stortjärnen. All lakes are unproductive brown-water systems of similar size and morphology (Table 1). Lake catchments are dominated by coniferous forest (Scots Pine; *Pinus sylvestris* and Norway spruce; *Picea abies*) and wetlands (mires) in different proportions. The lakes are closely co-located (maximum distance 75 km) and influenced by similar climatic conditions. Average annual temperature, precipitation and runoff in this area are approximately 1.8 ˚C, 614 mm, and 311 mm, respectively (from 1981-2010; Laudon et al., 2013). Lake surface ice coverage extends from November to May; stratification occurs during late May/early June and mixing occurs after mid-September.

In addition to these lakes, we also sampled the outlet of seven Swedish rivers (Lyckebeån, Helge å, Nyköpingsån, Motala Ström, Torne älv, Töre älv, Öre älv) that drain into the Baltic Sea. River catchments are located between latitudes 55˚N and 65˚N, falling along a 1300 km north-south gradient, spanning a range of drainage areas of 440-34441 km^2, and with DOC concentrations from 5.6 to 23 mg L^{-1}. These rivers drain very different terrestrial environments from mountains, forests, and wetlands in the north to catchments with a significant fraction of agricultural land and urban development in the south (Sponseller et al., 2014). In addition, these systems are influenced by different climates, from sub-arctic in the north to temperate in the south. From north to south, average temperature, precipitation and ~~runoff~~ discharge respectively span from 1-8˚C, 631-824 mm, and 34-450 m^3/s (for 1999-2013; Swedish Meteorological and Hydrological Institute, SMHI).

Lake samples (2 L) were collected from 0.5 m depth at seven dates from September 2012 to September 2014 (Table 2). Samples were stored in acid washed 2 L high-density polyethylene bottles ~~and~~ or 4 L low-density polyethylene cubitainers (Thermo Scientific) in the dark at approximately 1 ˚C until arrival at the laboratory. River sampling was conducted once at the outlet of each river between June to July 2013 at 0.3 m depth, in the middle of the river or 7 m from the shore.

2.2 Determination of bioavailable C, N and P

To determine concentrations of BDOC, BDN and BDP we conducted growth bioassays in which limitation of either C, N or P was strongly induced by adding different combinations of bacterial growth media. Our growth bioassays were designed so that resource use efficiency was at its maximum and bacterial production would occur mostly within three days from the beginning of the experiment. The bacterial response to those bioassays was measured by leucine incorporation (Kirchman et al., 1985). The amount of leucine incorporated in each bioassay was then converted into concentrations of bioavailable resource based on experimentally determined standard growth curves (see detailed description below).

Bioassays were prepared immediately after or at latest within one to two weeks after sampling. To ensure proper conservation of the samples prior to the experiment, they were immediately filtered (Whatman GF/F) and stored in a climate~~-~~-controlled chamber at a temperature close to 1 °C. At the initiation of the experiment, 500 mL~~l~~ of each lake and river water sample was again filtered at 0.2 µm (suporCap 100, Gelman Sciences) and placed in a 1000 ~~-~~-mL~~l~~ Erlenmeyer flask. All bioassay samples were then inoculated with a standard bacterial community 2 % (v/v), ~~which~~ to ensure~~d~~ that differences in bacterial community composition did not influence resource bioavailability measurements (Martinez et al., 1996). The standard bacterial community consisted of a mixture of fresh unfiltered ~~stream and river~~ water from the ~~nearby~~ epilimnion and inlet of the lakes ~~the field sites~~ sampled at one occasion, which was maintained in the fridge at 4°C between experiment~~al runs~~s~~.~~. ~~This ensured~~ The water was amended 5% (v/v) with a modified (excluding C, N and P) bacterial medium ("L16"; Lindström, 1991) rich in micro-nutrients, trace metals and vitamins required for bacterial growth. The sample was then divided into three sub-volumes to which strong limitation of either C, N or P was induced by adding appropriate combinations of nutrients. C limitation was induced by adding N as NH_4NO_3 (final concentration 2000 µg N L^{-1}) and P as Na_2HPO_4 (200 µg P L^{-1}). N~~-~~-limiting conditions were created by adding C as $C_6H_{12}O_6$ (20000 µg C L^{-1}) and P as Na_2HPO_4 (200 µg P L^{-1}). P~~-~~-limiting conditions were created by adding C as $C_6H_{12}O_6$ (20000 µg C L^{-1}) and N as NH_4NO_3 (2000 µg N L^{-1}). Samples were then transferred into 1.5-mL~~l~~ Eppendorf tubes that ~~, which~~ were incubated in the dark at the standard temperature of 20°C, which is the most broadly applied temperature in bioavailability assessments of the literature (del Giorgio and Davis, 2003). For each bioassay incubation, leucine incorporation was measured at six time points (after 0, 1, 2, 2, 3 and 7 days) on five replicate samples each time. The inoculum added to our ~~bioassays~~ sample water represents an unknown addition of bioavailable C, N and P. To ensure that the amount of resource added through inoculation was insignificant, we analyzed five control bioassay replicates in which the only source of C, N or P was the amount of resource contained in the inoculum and thus the lake sample was replaced by Mili-Q water. All such control bioassays resulted in low amounts of leucine uptake (Fig. 1), which was then used to correct our estimates of resource bioavailability through subtraction (see supplementary material Table 2).~~.~~

To create standard curves for bacterial growth per unit limiting nutrient, sampled lake water from September 2012 was used to perform a bioassay following the approach described above but with varying concentration of target elements. For example, to a sub-volume that was induced to be C~~-~~-limited, $C_6H_{12}O_6$ was added to final concentrations of 330, 660, 1000,

1330, and 1500 µg C L^{-1} respectively. The response to each concentration was measured on ~~one to~~ triplicate samples and was used to construct the standard curve. The same procedure was applied to produce standard curves for N and P limited assays. NH$_4$NO$_3$ was added to concentrations of 105, 133, 205, 305, 405 µg N L^{-1}, and Na$_2$HPO$_4$ was added to concentrations of 15.5, 18.8, 20.5, 30.5, 40.5 µg P L^{-1} (see supplementary material Table 1). Standard curves for the rivers were based on the

5   same ~~approach~~ method but bacterial responses to each concentration were recorded one time.

Integrated (cumulative) amounts of leucine incorporated by bacteria during lake or river bioassays over seven days were converted to concentrations of bioavailable element based on the slopes of the standard growth curves of either rivers or lakes, which describe how much leucine was incorporated per unit of bioavailable limiting element. For this conversion, the amount of incorporated leucine (given in nmol of leucine L^{-1} ~~per~~ for seven days) during each bioassay was divided by the

10   slope of the standard growth curve (nmol of leucine L^{-1} per mg of bioavailable nutrient L^{-1} for seven days). The resulting quotient represents the total amount of bioavailable nutrient taken up by bacterioplankton (mg L^{-1} for seven days; see supplementary material Table 3).~~).~~

2.3 Leucine incorporation

Measurements of protein synthesis were done using the method described by Smith and Azam (1992) and modified by

15   Karlsson et al. (2002). Accordingly, ^{3}H-leucine was added to sample water in Eppendorf tubes (specific activity varied between 60.5-115.8 Ci mmol^{-1}, Perkin Elmer) to a final concentration of 30-100 nmol L^{-1}. Additions of ^{3}H-leucine were dependent on bacterial activity tests performed prior to the experiments where different concentrations of ^{3}H-leucine identified the isotope saturation levels. Triplicate measurements were taken after 24 h, 48 h (we obtained six replicates at this time point), 72 h, 96 h and 168 h. Leucine incorporation into protein was determined by incubation for 1 h in the dark at 20

20   °C and incubations were terminated with trichloroacetic acid (TCA) additions of 5 % (w/v). A bacterial pellet was formed by centrifugation for 10 min at 14 000 rpm. The bacterial pellet was rinsed with 5 % TCA. After addition of 1.2 mL of scintillation cocktail (PerkinElmer) radioactivity was measured on a Wallac WinSpectral 1414 Scintillation counter (PerkinElmer). Incorporation of ^{3}H-leucine was calculated using an intracellular dilution factor of 2 (Smith and Azam, 1992). Leucine incorporation measurements were integrated for the six time points and summed into a single value that

25   represented the total amount of leucine incorporated for the seven-day period. Lastly, at time point 96 h, an extra vial was collected and used as a blank, pre-treated with TCA 5 % (w/v), followed by addition of leucine at a final concentration of 30 nmol L^{-1}.

2.4 Validation

We validated the bacterial ~~responses (~~ leucine uptake~~)~~ response to added amounts of BDOC, BDN and BDP (i.e., the slope of

30   the standard cuves) by ~~measuring~~ relating the measured leucine uptake ~~per unit ambient~~ to alternative estimates of bioavailable resource~~s measured~~ obtained with ~~alternative~~ independent methods. An alternative estimate of BDOC was obtained from measuring bacterial respiration (BR) during a lability incubation, which has been often applied in previous

~~aquatic research~~studies (del Giorgio and Cole, 1998; Jansson et al., 2000). The BR was determined by assessing decreases in dissolved oxygen concentrations ~~from~~ in ~~bioassays~~ water samples from lakes (n=13) and rivers (n=8). Sample water was prepared in parallel with, and in the same way as, the C bioassays described above. Volumes of 0.5 L were added to glass incubation bottles (in duplicate) which had sensor~~s~~ spots affixed to the inside surface. Oxygen concentrations were measured in the dark every 5 min for up to seven days with a FIBOX 3 (PreSens) that took optical readings from the outside of bioassay bottles. Estimates of BR were calculated from the averaged consumption of dissolved oxygen from the duplicate bottles by assuming a respiratory quotient of 1, which is a conservative value for unproductive lakes (Berggren et al., 2012).

Bioavailable N was assessed using an alternative method described by Stepanauskas et al. (2000) by counting the cells produced in growth bioassays with N-starved bacteria. For this test, two aliquots of 30 m~~L~~L were used for bioassays and one of them was amended with N-NH_4NO_3 to a final concentration of 0.405 mg N L^{-1}. Both incubations were performed at 20 ˚C degrees in the dark. Bacterial biomass was determined at the start of the incubation (t=0) and after three days (t=3) ~~after~~ when the bacterial growth had peaked (Fig. 1). Bacterial samples were fixed with 3 % (v/v) glutaraldehyde and kept at 5 ˚C until analysis. Analyses of bacterial cells were conducted on a flow cytometer (FACScan, Becton Dickinson) on samples stained with SYTO 13 and run with addition of beads as internal standard according to del Giorgio et al. (1996), using CellQuest Pro software. Bacterial cells were distinguished based on green fluorescence intensity and side scatter signals. Total bacterial abundance was calculated as the sum of the populations that were distinguished in the cytograms. The N content per bacterial cell was determined by dividing the amount of N added to the amended aliquot by the difference in bacterial abundance between the N-amended and the unamended aliquot. To obtain BDN; the calculated average N content per cell was multiplied by the number of bacterial cells that were produced in the bioassay without addition. ~~A more accurate method was used to To validate our~~We validated our estimates of leucine incorporation per unit bioavailable P by comparing it with the corresponding ratio in a completely independent boreal data set ~~bioavailability using a more accurate approach that directly measures P accumulation in bacterial cells~~ (Jansson et al. 2012). This independent data come from a freshwater study with near-identical bioassay conditions as in our P bioassays, with the major difference being that Jansson et al. (2012) used larger incubation volumes (> 700 mL) than we did when incubating in 1.5 mL Eppendorf tubes. Moreover, bioavailable P in the validation data was not assessed from bacterial growth data, but instead measured as P accumulation in bacterial cells harvested on filters. ~~Such an approach~~This is possible for P, ~~since~~because standard TP ~~instrumentation~~methods ~~allows to measure changes in P concentration with~~provide high analytical precision at the microgram level (molybdenum blue method) ~~and~~that can ~~thus~~resolve small changes in P concentration. ~~To do this~~Thus, ~~w~~We extracted the raw data from Jansson et al. (2012), where both cumulative leucine incorporation and ~~bioavailability~~bioavailable P ~~was~~were ~~assessed by an alternative approach from~~quantified during incubation of water from two northern Swedish streams sampled on six dates from late April to late October 2010. ~~, and in addition cumulative leucine incorporation during the bioassays was measured through the method described in this study. Hence,~~Here, ~~This alternative approach was used to determine in this study concentrations of bioavailable P was determined~~ as the difference in the particulate P (retained on a nominal cutoff of 0.2 μm

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2.5 Analytical methods and calculations

Lake ambient water chemistry was analyzed at the department of Ecology and Environmental Science at Umeå University. Sample water for determination of DOC and TDN was filtered through a pre-ignited (400 ˚C, 3 h) acid-rinsed Whatman GF/F filters. The filtered water was acidified with 1.2 M HCl and analyzed for DOC using a HACH-IL 550 TOC-TN. Filtered sample was analyzed for TDN also using a HACH-IL 550 TOC-TN, while determination of nitrate (NO_3^-) and ammonium (NH_4^+) was done according to the International Organization for Standardization (ISO) 13395-1996. Concentration of phosphate (PO_4-P, assumed to be represented by soluble reactive P) was determined from filtrates (GF/F) of water samples using the molybdate blue method Murphy and Riley (1962) and total phosphorus (TP) determined after oxidative hydrolysis with potassium persulfate (ISO 15861-1).

River DOC samples were filtered through a Whatman GF/F filter into a pre-acid washed 40 mL amber borosilicate vial, filled to the brim and tightly closed with silicon septa screw caps. Samples were kept cold in the fridge until analysis which took place at the G.G. Hatch Stable Isotope Laboratory, University of Ottawa. River samples for determination of (total dissolved nitrogen) TDN, NO_3^-, NH_4^+, TP and PO_4-P were frozen until analyses at the Evolutionary Biology Center, Uppsala University, following standard methods.

Our results provided estimates of total bioavailable resource pools. To calculate shares of bioavailable DON (BDON) and bioavailable DOP (BDOP), we subtracted the inorganic pools of DIN (NO_3^-, NH_4^+) and PO_4-P from the respective total bioavailable pools. Nutrient ratios were calculated in molar. We further calculated inorganic nutrient ratios of DIN to PO_4-P (DIN : PO_4-P).

2.6 Statistical analyses

Standard curves were fit by linear regressions using JMP 10 (SAS). Differences between the slopes of standard curves for each nutrient across lakes were tested by one-way analysis of variance (ANOVA, $p < 0.05$) in SPSS 22.0 (SPSS Inc., Chicago, IL, U.S.A.). Since there were no statistical differences between the slopes among the four lakes obtained for each respective resource (ANOVA, $p > 0.44$, $n = 20$), slopes were averaged for each nutrient across lakes. Differences between bioavailable resources results across lakes and for each lake across time were tested using the Kruskal-Wallis H test and Dunn's post-hoc test ($p < 0.05$) in SPSS. Differences between total and bioavailable resource ratios for the lakes were tested with dependent t-tests ($p = 0.05$) in SPSS. Previous work suggests that at higher DOM concentrations there is a greater discrepancy between bioavailable and total DOM fractions (Berggren et al., 2015). We therefore pooled the seven different rivers into two categories according to their DOC concentrations (3.7-23.0 mg C L^{-1}); this resulted in an ensemble of three rivers which had a DOC concentration higher than 10 mg C L^{-1} (rivers$_{>10\ mg\ C\ L}^{-1}$) and four rivers that had a DOC

concentration lower than 10 mg C L^{-1} (rivers$_{<10\ mg\ C\ L}$$^{-1}$). Differences between total and bioavailable river nutrient ratios for the two groups were tested with dependent t-tests (p = 0.05) in SPSS.

3 Results

5   The rate of leucine incorporation increased over time in most bioassays until day 2 (t = 2), before gradually decreasing until day 7 (t = 7; Fig. 1). In the bioassays that were performed with resource additions, the accumulated leucine incorporation over the 7-day period was proportional to the concentrations of bioavailable resource added (Fig. 2). The results rendered an average linear relationship describing amounts of leucine incorporated per bioavailable C, N and P (Fig. 2). Bioavailable resource concentration spanned from 104-692 µg C L^{-1}, 23-287 µg N L^{-1} and 0-16 µg P L^{-1} (Table 2).

10   Concentrations of BDOC did not differ among lakes (ANOVA, p > 0.61, n = 130). By contrast, the four lakes did vary in terms of average BDN and BDP (ANOVA, p < 0.05, n = 130). Lake Struptjärnen had on average the highest BDN and BDP concentrations (159 µg N L^{-1} ± 111 SE and 8 µg P L^{-1} ± 4 SE) and lake Lillsjöliden the lowest values (124 µg N L^{-1} ± 97 SE and 5 µg P L^{-1} ± 3 SE).

    There was a significant difference in bioavailable resource concentrations over time across the lakes (ANOVA, p < 0.05, n =

15 30-35; Table 2). In general, concentrations of BDOC across the lakes were highest in October 2012 (mean 356 µg C L^{-1} ± 84 SE) and lowest in August 2014 (mean 185 µg C L^{-1} ± 59 SE), with a 33 % difference in BDOC between maximum and minimum values during the studied period. Concentrations of BDN tended to be high in September 2012 (mean of 236 µg N L^{-1} ± 45 SE) and lowest in September 2014 (mean of 58 µg N L^{-1} ± 42 SE) and varied was 85 % between thehigher at maximum and thecompared to its minimum concentration. Concentrations of BDP were the highest in July 2014 (mean of 12

20 µg P L^{-1} ± 3 SE) and lowest in October 2012 (mean of 4 µg P L^{-1} ± 3.6 SE) and varied approximately 83 % throughout the studied period. There was no correlation between total and bioavailable element concentrations. Average fractions of bioavailable resources relative to the total pool were lowest for C, highest for P, and intermediate for N (Fig. 3). Organic forms were the major source of bioavailable resources to bacterioplankton and represented 80 % (± 13 SE) of the bioavailable N pool and 61 % (± 46 SE) of the bioavailable P pool (Fig. 3). The contribution of inorganic fractions was

25 therefore relatively more important for overall P than N bioavailability.

    Molar nutrient ratios calculated for the total pool of nutrients were significantly higher than ratios calculated with on basis ofin the bioavailable fraction (dependent t-test, p < 0.05, n = 26; Fig. 4). For example, the average ratio of total C : N was 55 (±9 SE) and was ca 13 times higher than C : N bioavailable ratio which averaged 4 (± 3 SE). Similarly, average C : P total ratio was 4774 (± 2135 SE) and was 12 times significantly higher than the average bioavailable C : P ratio 369 (± 915 SE).

30 However, there were no significant differences (dependent t-test, p > 0.474, n = 26) between total N : P ratios (average of 145 ± 386 SE) and bioavailable N : P ratios (average of 89 ± 44 SE), or between bioavailable N : P ratios and the DIN : PO$_4$-P ratio (mean of 29 ± 19 SE; dependent t-test, p > 0.134, n = 26).

The amounts of leucine incorporated per unit of bioavailable resource in our ~~re-~~growth bioassays (as determined by the slopes in Fig. 2) ~~was~~ were validated by extracting the same ratio from experiments performed using alternative bioassay methods (Fig. 5). The alternative bioassay methods were based on: 1) inferring BDOC from bacterial respiration; 2) calculating BDN from cell yields and; 3) analyzing BDP directly on the bacterial biomass (see methods). The growth responses (leucine incorporation) in our ~~re-~~growth bioassays overlapped with the growth responses obtained from experiments using the alternative methods. However, on average the growth response was slightly higher in our bioassays when compared to the alternative bioassays (Fig. 5).

For rivers, DOC appeared as the least bioavailable resource (in relation to the total pool) for both river groups: rivers$_{>10\,mg\,C\,L}^{-1}$ and rivers$_{<10\,mg\,C\,L}^{-1}$ (Table 3). In contrast, the BDN share was the most bioavailable with approximately half of the TN pool being bioavailable. Total nutrient ratios of C : N and C : P were statistically significantly higher (approximately 26 and 5-fold respectively) than the respective bioavailable resource ratios for rivers$_{>10\,mg\,C\,L}^{-1}$ (dependent t-test, $p < 0.05$, $n = 4$). We found no differences between total N : P ratio and bioavailable N : P ratios, n~~either~~or between each of these and DIN : PO$_4$-P ratios for both rivers$_{>10\,mg\,C\,L}^{-1}$ (dependent t-test, $p > 0.07$, $n = 4$) and rivers$_{<10\,mg\,C\,L}^{-1}$ (dependent t-test, $p > 0.10$, $n = 3$).

4 Discussion

4.1 Resource bioavailability as a driver of ecological patterns

Results from this study underscore ineffectiveness of total nutrient fractions as predictors of bioavailability in boreal freshwater ecosystems. ~~Surprisingly,~~ ~~I~~in these systems where absolute surface water DOC concentrations are large, C bioavailability was low~~est and was the strongest limiting factor for heterotrophic aquatic production~~ relative to N and P. This study not only reveals the ~~pervasive~~ likely control that C has on boreal heterotrophic aquatic ~~production~~ metabolism but also suggests that possible changes in C loading to the boreal water systems in the future may impact aquatic productivity and the turnover of nutrients. Northern catchments are thought to be particularly sensitive to ongoing climate change (Tetzlaff et al., 2013) and this refined understanding of bioavailable resource stoichiometry may be essential to forecast and mitigate aquatic ecosystem responses to these and other anthropogenic pressures at high latitudes.

4.2 Bioavailable concentrations of DOC, TDN and TDP in lakes

Our estimates, which reflect the ~~medium-term~~ resource pool readily available to bacterioplankton at any point in time, supported our expectations by showing that nutrient bioavailability (as percentage of the total pool), increased from BDOC to BDN and from BDN to BDP. The observed differences in N and P bioavailability match the overall trend reported for aquatic ecosystems in the literature (Berggren et al. 2015) and are generally consistent with our understanding of how these elements are bound to organic matter. Organic N tends to form covalent bonds directly to C and may be physically and chemically protected within complex, organic compounds that are resistant to decay (Schulten and Schnitzer 1998). Liberating this N is linked to organic matter depolymerization and C mineralization (Schimel and Bennett 2004), requiring

multiple exo-enzymatic steps that are energetically expensive (Sinsabaugh and Follstad 2011). By contrast, organic P is more often associated with ester bonds (C-O-P) that can be cleaved in a single enzymatic step independent of C mineralization (McGill and Cole 1981). In addition, other forms of inorganic P (e.g., orthophosphate) may be only loosely bound and exchanging with iron-humic complexes (Jones 1998). These binding properties are thought to govern differences in the relative rates of N and P cycling in soils (Vitousek et al. 2002) and our results suggest that the same factors may shape the relative bioavailability of these resources ~~also~~ in freshwater environments as well.

The method we describe here generated simultaneous bioavailability estimates for C, N, and P that were comparable to those from single--element bioassays reported elsewhere. Absolute concentrations of BDOC (100-690 µg C L^{-1}) were within the range of reported values for cedar bog wetlands (12-408 µg C L^{-1}; Wiegner and Seitzinger, 2004) and were at the lower end of values reported for rivers (108-180 µg C L^{-1}; Wiegner et al., 2006). Concentrations of BDN (30 - 320 µg N L^{-1}) were in agreement with bioavailable N concentrations reported for cedar bog wetlands (0-322 µg N L^{-1};Wiegner and Seitzinger, 2004). BDP (0-16 µg P L^{-1}) was comparable to values from a recent study on headwater streams during low flow (1-14 µg P L^{-1}; Jansson et al., 2012). In addition, organic forms dominated the total bioavailable N and P pool (80 % and 61 % respectively) in our four lakes, and 27 and 36 % of these organic pools were bioavailable, respectively. These results are in line with previous estimates and show that a large fraction of DON is available to bacterioplankton in diverse limnetic systems, e.g. in Baltic Sea rivers (30 %; Stepanauskas et al., 2002), in eastern US rivers (23 %; Wiegner et al., 2006) and in cedar bog wetland streams (33 %; Wiegner and Seitzinger, 2004). Published estimates of the share of BDOP (bioavailable dissolved organic phosphorus) relative to the total DOP pool, varied from 33-60 % in Baltic Sea brackish waters (Nausch and Nausch, 2007). Thus, our results agree with the results from previous studies and together they emphasize the importance of organic nutrient fractions in systems rich in organic matter, and also the bacterioplankton capacity to take up organic compounds.

Concentrations of BDOC, BDN and BDP varied seasonally in all lakes during the study period (Table 2). Major differences in BDOC were observed between mid-summer, when concentrations were lowest, and the end of the summer, when concentrations were high. Previous experimental work on boreal and arctic rivers has also shown minimal concentrations of BDOC during the summer season (Wickland et al., 2012). In addition, concentrations of BDOC tended to follow bulk DOC concentrations in boreal freshwater systems as suggested in Søndergaard and Middelboe (1995). ~~Because the design of our lake experiment controlled for most factors affecting bacterioplankton C uptake (i.e. temperature, bacterial communities, predation, hydrological conditions, inorganic nutrient concentrations, land use differences; del Giorgio and Davis, 2003), the variation in the amount of BDOC was most likely coupled to seasonal temperature fluctuations which influence soil microbial activity and consequently the quality of the exported organic C to surface waters (Kalbitz et al., 2000;Carlson et al., 2002).~~ By contrast, patterns of BDP concentrations opposed those of BDOC (Table 2): specifically, BDP peaked in mid-summer (July) and declined in the autumn. It has been shown elsewhere that bioavailable P concentrations in boreal streams can be 2-10 times higher during summer than during autumn (Jansson et al., 2012). This may be due to ~~the fac~~higher ~~air~~soil temperatures during summer which promote soil C metabolism and result in a higher export of P from soils to surface waters

when compared to that of C~~t that low BDOC concentrations in forests soils during summer lead to reduced uptake (i.e. reduce biotic demand for P) and consequently result in exports of DOM depleted in labile C and rich in bioavailable P~~ (Jansson et al., 2012).

Our results also supported the prediction that the bioavailable ratios of C : N and C : P would be considerably lower than counterparts based on total pools. A major implication of these differences is that ratios based on total pools grossly overestimate actual C availability. When such differences are large, the elemental ratios based on total pools can lead to incorrect predictions of resource limitation (Berggren et al. 2015). For example, in a recent study of two temperate estuaries, total resource stoichiometry predicted P limitation of bacterioplankton, while experimental evidence showed that C was the element constraining bacterial growth during base flow (Hitchcock and Mitrovic, 2013). Average DIN : PO_4-P ratios and particularly total TN : TP were however, closer to the average ratio of bioavailable TN : TP. Due to the high C recalcitrance, nutrient limitation predictions based on the ratio of total resource pools may be inadequate when C is included the ratio, but seem more promising when based on N and P.

Our results further show that while the median bulk stoichiometric ratio (3651C:71N:1P; Fig. 4) was 1-2 orders of magnitude higher than that expected from the Redfield ratio (106C:16N:1P; Anderson, 1995;Redfield, 1958), the median C : N : P of bioavailable resources (144C:29N:1P) was surprisingly comparable yet slightly above Redfield values (Fig. 4). There was, however, a wide variability in the bioavailable ratios among samples collected over space and time. ~~which~~ Such variance is consistent with another study that evaluated bacterial ~~biomass~~ stoichiometry ~~over~~ across a large number of lakes and showed that, while elemental stoichiometry varied among lakes in response to intrinsic and extrinsic factors, the overall mean ratio tended to converge with Redfield (Cotner et al., 2010). ~~It should be pointed out here that the C:N:P content of cells does not necessarily represent the relative rates of supply that are required for these elements, particularly given that relative C incorporation into biomass can be highly variable (also called bacterial growth efficiency; BGE). Considering the need for carbon to fuel respiration and build biomass, actual uptake ratios of C : N and C : P must take into account the fact that BGE in boreal waters can vary with the source and age of the terrestrial carbon from 0.06 to 0.50 (Berggren et al., 2007). Applying this reported possible range of BGE to our data suggests that the median bacterial demand for C : N and C : P varies between 7-58 and 290-2421, respectively. Considering that only a fraction of the bulk C, N and P was available for uptake (2 %, 31 % and 49 %; Fig. 3) the actual median C : N (3) and median C : P (166), was lower than these uptake ratios corrected for BGE, providing further support that C was limiting in all our samples.~~

4.3 Broad-scale riverine BDOC, BDN and BDP patterns

Broad-scale patterns of nutrient bioavailability at the river mouths did not differ between rivers$_{<10 \text{ mg C L}^{-1}}$ and rivers$_{>10 \text{ mg C L}^{-1}}$. Similar to what was observed in the lakes, DOC was the most recalcitrant nutrient considered. However, in contrast to our results from the lakes, TDN was the most bioavailable resource observed in the river mouths (Table 3). Although previous studies suggest that temperature differences across catchments can influence C : N ratios in streams and rivers through effects on terrestrial ecosystem properties (e.g., vegetation type) and soil development (Sponseller et al., 2014), our results

show a similar bioavailable resource stoichiometry at the outlet of all these rivers. Organic forms of N were a major source of bioavailability and dominated TDN, in agreement with estimates from other studies (Wiegner et al., 2006;Seitzinger and Sanders, 1997;Stepanauskas et al., 2002). Significant differences between total and bioavailable C : N and C : P ratios occurred only in rivers$_{>10\ mg\ C\ L}$$^{-1}$. ~~Whereas~~However, both rivers$_{>10\ mg\ C\ L}$$^{-1}$ and rivers$_{<10\ mg\ C\ L}$$^{-1}$ had no differences between total N : P, bioavailable N : P and DIN : PO_4^{3-} ratios. These results indicate that, similar to the ~~the lake results~~patterns observed in lakes, the use of bulk resource ratios ~~misinterprets~~ misrepresents resource bioavailability and limitation when: 1) C is part of the nutrient ratio: and 2) there is a high concentration of DOC in the water~~s~~.

~~Despite~~While Swedish rivers hav~~eing a~~substantial water renewal along ~~the~~ watercourses from the Scand~~es~~ to the Baltic Sea (Muller et al., 2013), at such ~~long timescales~~broad scales, several environmental factors may modify element bioavailability ~~in river waters~~through ~~the~~modification and differential uptake and re-mineralization of C, N, and P. For example, bacterial processing (Creed et al., 2015), ~~Creed et al. 2015 light~~photodegradation (Bushaw et al., 1996) and reactive oxygen (Gao and Zepp, 1998) ~~bioavailability impacting processes~~all may influence ~~OM~~organic matter degradation and changes in bioavailability over the long~~er~~ time scales encompassed by large river systems. ~~Nonetheless~~In this regard, it is interesting to note that our~~, the bioavailability~~ estimates of short-term macro-nutrient bioavailability ~~provided here for both~~were similar for lakes and rivers, which suggest that possible differences in ~~are notnot intended to representative of the~~ long-term ~~bioavailable pool in natural waters, but instead should be rather interpreted as bioavailable~~macro-~~element~~nutrient ~~estimates~~bioavailability across these very different sites did not seem to impact on the results determined under our specific laboratory conditions. The general pattern that we found across all sites was a relatively low bioavailability of C relative to that of N and P. This may suggest that C is more important as limiting factor for bacterial metabolism than previously thought. However, while ~~O~~our results ~~can not be~~ directly inform on the maximal pools of bioavailable macronutrients that can be readily consumed, the true exploitation of these resources in nature is ~~transferred to natural systems~~dependent ~~as~~on other (extrinsic) factors such as micro-element limitation, element co-limitation, and grazing pressures ~~may also influence potential element bioavailability~~. Thus, based on our result alone it is not possible to determine whether or not the in situ bacterial metabolism was limited by a specific macronutrient, although it appears more likely that C would be limiting than N or P.

4.4 Measuring bioavailability of C, N and P with leucine incorporation

The linear relationships obtained from standard growth curves relating leucine incorporation to bioavailable resource concentrations showed that incorporation over a 7-day period was significantly and positively related to the amount of resource added. The fact that these relationships were not statistically different among the lakes suggests that leucine incorporation was driven by the added resources rather than other factors that could have affected the experiment. For example, variations in lake pH could have impacted the amount of resources taken up in the bioassays (del Giorgio and Davis 2003; Li et al., 2012). ~~Because different methods were used, w~~Due to the lack of replication of~~We didcould not check~~

~~whether lake and~~ river standard curves, we could not test whether or not the standard curves of individual rivers were also similar to those of lakes.~~;~~ ~~h~~However, ~~our guess is~~we suspect that physical and chemical water sample properties may differentially influence leucine uptake in different systems. Our blank bioassays further confirmed the dependency between leucine incorporation and limiting resource concentration by showing that virtually no leucine incorporation occurred when

5 the limiting resource was lacking in the growth media.

We used the leucine incorporation method as a proxy for bacterial growth and related it to bioavailable resource concentrations based on the premise that this process measures the rate of bacterial protein synthesis (Kirchman et al., 1985). Because proteins are large macromolecules within bacterial cells (approximately half of bacterial dry weight), they represent a substantial fraction of the resource uptake and its consequent conversion into biomass. Also, to carry out protein synthesis,

10 bacteria use both C and N; nitrogenous compounds are taken up from the growth medi~~a~~um to build proteins with energy obtained from C substrates. Phosphorus is also used in the process as it is crucial for controlling the adenosine triphosphate-adenosine diphosphate cycle, which provides energy for the intracellular molecular synthesis. Due to the critical role that these three elements play within protein synthesis, our results represent an unequivocal relationship between resource availability and the amount of protein synthe~~t~~sized. We measured resource bioavailability over a time period of seven days

15 and the major part of the resource pool was exhausted within three days (Fig. 1). In the context of ~~bioavailbility~~bioavailability assessments, seven days is a relatively short period and repeated bacterial regeneration of resources was in this way avoided (Cho et al., 1996). Although there may have been some resource recycling, our bioavailability estimates are automatically corrected for this artifact as these were calculated based on ~~a~~ standard curves for leucine incorporation per absolute unit of added bioavailable resource, constructed ~~relationship that was estimated~~ for the

20 exact same time period.

~~An important advantage of estimating nutrient bioavailability with our method is that uncertainties inherent to conversion factors (such as those used in bacterial production and flow cytometry) are avoided (Calvo Diaz and Moran, 2009). This is because our integrated leucine amounts are directly transformed into bioavailable resource units through the slope of a specific load growth relationship that is based on the growth of the exact same bacterial community exposed to a similar~~

25 ~~media. Still, T~~the design of our experiment could lead to possible sources of errors in estimates. For example, reference assays (standard curves) were performed at one occasion and used to interpret actual nutrient bioavailability at other occasions. This means that if BGE varied during the studied period it~~,~~ could result in differences in the amounts of leucine incorporated. We dealt with this possible shortcoming by designing our bioassays such that resource use efficiency would be maximized (by strongly inducing resource limitation; Jansson et al., 2006) and thus, possible variations in resource use

30 efficiency most likely did not play a substantial role on rates of leucine uptake (Fig. 5). In addition, the fact that glucose was used as reference source of C and energy in the calibration could lead to an overestimation of the standard C growth curves and possibly result in conservative estimates of bioavailable C. For example, glucose additions could have supported the part of the community with the fastest growth and therefore results may not compare to results from a community that was instead exposed to a natural substrate. Nonetheless, when comparing the amount of leucine incorporated by our standard

bacterial community per unit of bioavailable glucose with amounts of leucine incorporated ~~by different lake communities~~ per unit of natural bioavailable substrate (Fig. 5), we show that, on average, our growth response was only slightly higher than the growth response in experiments based on alternative bioassay methods (Fig.5). Thus, our resource bioavailable estimates presented here are most likely conservative but realistic.

5 Conclusion

Ongoing changes in the global C, N and P cycles have the capacity to modify the chemical conditions and nutrient balance of receiving waters (Finzi et al., 2011). Yet the effects of these changes on basal productivity and food webs of many inland waters remain difficult to predict. We ~~claim~~ suggest that to better forecast the impact of such changes, it is important that we refine how we consider and measure the stoichiometry of the main elements available to support aquatic production. This study contributes to our general understanding of resource dynamics in DOM-rich systems. Based on bioavailable resource ratios determined with a single approach, we show that resource bioavailability increases from C to N and N to P. P availability in these systems may, thus, be likely considerable higher than previously thought. This finding particularly calls into question whether results from most enrichment experiments done so far, which often show that P additions stimulate BP, are applicable to DOM-rich systems (Jansson et al., 2001). In addition, our findings reinforce the idea that despite boreal waters being DOM-rich, the C availability ~~still represents the major constraint to BP of~~in humic waters is extremely low. This means that expected future changes in the amount or character of C delivered to boreal surface waters will most likely drive changes in BP, which subsequently affects abiotic conditions, the biotic structure, and ecosystem functioning of freshwaters.

Competing interests

The authors have no conflict of interest to declare.

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Table 1. Descriptive lake data and concentrations of total dissolved nitrogen (TDN), nitrate (NO_3^-), ammonium (NH_4^+), total phosphorus (TP), phosphate (PO_4-P) and dissolved organic carbon (DOC) given as minimum and maximum values observed during the experimental period.

Variables	Övre Björntjärnen	Lillsjöliden	Struptjärnen	Stortjärnen
Location (latitude [N], longitude [E])	64°7'23.53"N, 18°46'43.04"E	63°50'41.71"N, 18°36'59.62"E	64° 1'22.62"N, 19°29'21.18"E	64°15'42.11"N, 19°45'44.73"E
Lake surface area (ha)	4.8	0.8	3.1	3.9
Maximal depth (m)	9.5	5.2	5.8	6.7
Total catchment area (ha)	284	25	79	82
Wetland coverage (%)	16	2	4	12
Forest coverage (%)	84	98	96	88
DOC (mg L^{-1})	18-29	13-19	19-25	19-27
TDN (µg L^{-1})	376-502	336-501	360-521	355-598
DIN (µg L^{-1})	5-35	10-40	3-43	4-35
TP (µg L^{-1})	8-25	4-15	8-25	7-15
PO_4-P (µg L^{-1})	1-8	0-4	0-3	0-2

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Table 2. (a) Bioavailable dissolved organic carbon, (b) bioavailable total nitrogen, and (c) bioavailable total phosphorus on seven sampling dates (columns). Values show means of five analytical replicates and standard deviations are provided within parentheses. Shared index letters within rows identify dates significantly different from each other (p < 0.05) which were determined by the Kruskal-Wallis h and Dunn's post-hoc test.

5

Lake	Sep 2012	Oct 2012	Jul 2013	Jun 2014	Jul 2014	Aug 2014	Sep 2014
a) BDOC, µg C L^{-1}							
Övre Björntjärnen	273^a		371^{bcd}	420^{aefg}	248^{be}	243^{cf}	216^{dg}
	(143)		(64)	(60)	(39)	(22)	(21)
Lillsjöliden	471	552^{ab}	334^{cde}	176^{ac}	205^d	215	176^{be}
	(435)	(338)	(49)	(36)	(7)	(17)	(36)
Struptjärnen	361^a		432^b	692^{acd}	337^e	178^c	107^{bde}
	(327)		(93)	(85)	(27)	(21)	(6)
Stortjärnen	319^a	428^b	283^c	301^d	213^e	104^{abcdf}	406^{ef}
	(210)	(228)	(49)	(35)	(15)	(8)	(130)
b) BDN, µg N L^{-1}							
Övre Björntjärnen	209^{ab}		74^c	61^a	84^d	73^e	23^{bcde}
	(13)		(13)	(6)	(14)	(5)	(1)
Lillsjöliden	287^{abc}	232^{def}	111^{gh}	33^{adg}	64^{be}	89^a	51^{cfh}
	(10)	(24)	(7)	(10)	(5)	(6)	(3)
Struptjärnen	259^{abc}		107^{ad}	220^e	273^{dfg}	60^{bf}	37^{ceg}
	(6)		(28)	(14)	28	(6)	(2)
Stortjärnen	188^{ab}	206^{cef}	82^{ac}	67^{be}	84^f	28^{acfg}	119^g
	(15)	(18)	(6)	(5)	(5)	(3)	(38)
c) BDP, µg P L^{-1}							
Övre Björntjärnen	9^{abc}		5^{ad}	5^{be}	9^{def}	7^g	3^{cfg}
	(1)		(0)	(0)	(1)	(0)	(0)
Lillsjöliden	3^{ab}	3^c	2^{def}	2^{cgh}	10^{acd}	7^{beg}	6^{fh}
	(0)	(0)	(0)	(0)	(2)	(1)	(0)
Struptjärnen	6^{ab}		6^c	9^{ad}	16^{bce}	7^e	4.^{de}
	(1)		(0)	(1)	(2)	(1)	(0)
Stortjärnen	0^{ab}	0^{cdef}	1^{gh}	10^{acg}	12^{bdh}	6^e	5^f
	(0)	(0)	(0)	(2)	(2)	(0)	(2)

10

Table 3. Resource bioavailability in relation to the total resource pool, shown as percent bioavailable dissolved organic carbon (BDOC), bioavailable dissolved nitrogen (BDN) and bioavailable dissolved phosphorus (BDP). The data is divided into two groups which show average results for rivers with more than 10 mg C L^{-1} (rivers$_{> 10 \text{ mg C L}}$$^{-1}$; $n = 3$) and rivers with less than 10 mg C L^{-1} (rivers$_{<10 \text{ mg C L}}$$^{-1}$; $n = 4$). Average element ratios of carbon to nitrogen (C : N), carbon to phosphorus (C : P), nitrogen to phosphorus (N : P) are calculated in molar for total (tot) and bioavailable resource fractions (bio). Ratios of dissolved inorganic nitrogen to phosphate (DIN : PO$_4$-P) are also provided. Standard deviations are given within parentheses.

Variable	rivers$_{>10 \text{ mg C L}}$$^{-1}$	rivers$_{<10 \text{ mg C L}}$$^{-1}$
BDOC (%)	2 (1)	3 (2)
BDN (%)	48 (16)	36 (20)
BDP (%)	20 (12)	31 (45)
C:N (bio)	1 (1)	2 (1)
C:N (total)	26 (5)	24 (13)
C:P (bio)	319 (287)	523 (795)
C:P (total)	1722 (378)	920 (93)
N:P (bio)	294 (353)	240 (251)
N:P (tot)	70 (27)	46 (21)
DIN: PO$_4$-P	88 (68)	2 (2)

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Figure caption

Figure 1: Leucine incorporation rates over the incubation time for a blank incubation and five spikes of C (spike 1=330, spike 2=660, spike 3=1000, spike 4=1330 and spike 5=1500 µg C L^{-1}), N (spike 1=105, spike 2=133, spike 3=205, spike 4=305 and spike 5=405 µg N L^{-1}) and P (blank, spike 1=15.5, spike 2=18.8, spike 3=20.5, spike 4=30.5 and spike 5=40.5 µg P L^{-1}).

5

Figure 2: Measurements of leucine incorporation in relation to additions of bioavailable C (as $C_6H_{12}O_6$), N (NH_4NO_3) and P (Na_2HPO_4). Regression equations for all points pooled together: bioavailable C= 784x + 384 ($R^2 = 0.74$, $p < 0.0001$; $n = 20$); bioavailable N= 2667x + 159 ($R^2 = 0.75$, $p < 0.0001$, $n = 20$); bioavailable P = 67575x - 110 ($R^2 = 0.80$, $p < 0.0001$, $n = 20$). Note that each individual regression line in the figure has a better fit than the average regression line.

10

Figure 3: Proportion of organic non-bioavailable, organic bioavailable and inorganic nutrient shares of dissolved organic carbon (DOC), total dissolved nitrogen (TDN) and total phosphorus (TP) for all lakes and all sampling occasions (n=26).

Figure 4: Bioavailable (bio) and total (tot) ratios (in molar) of carbon to nitrogen (C:N) and carbon to phosphorus (C:P) for all
15 **lakes and all sampling dates (n=26). Ratios of N:P are shown for total, bioavailable and inorganic (inorg) fractions. Different letters stand for significant differences (dependent t-test; $p < 0.05$; $n = 26$) among ratios. <u>Data shown as boxplots and includes mean as diamonds.</u>**

Figure 5: Log-scale boxplots incubation show leucine amounts per unit of bioavailable nutrient measured with validation methods:
20 **bacterial respiration (C), cytometry (N) and harvesting of cells in filters (P). Diamonds are average values for the validation methods and filled squares are average slope values for standard curves (same values as slopes in Fig. 2).**
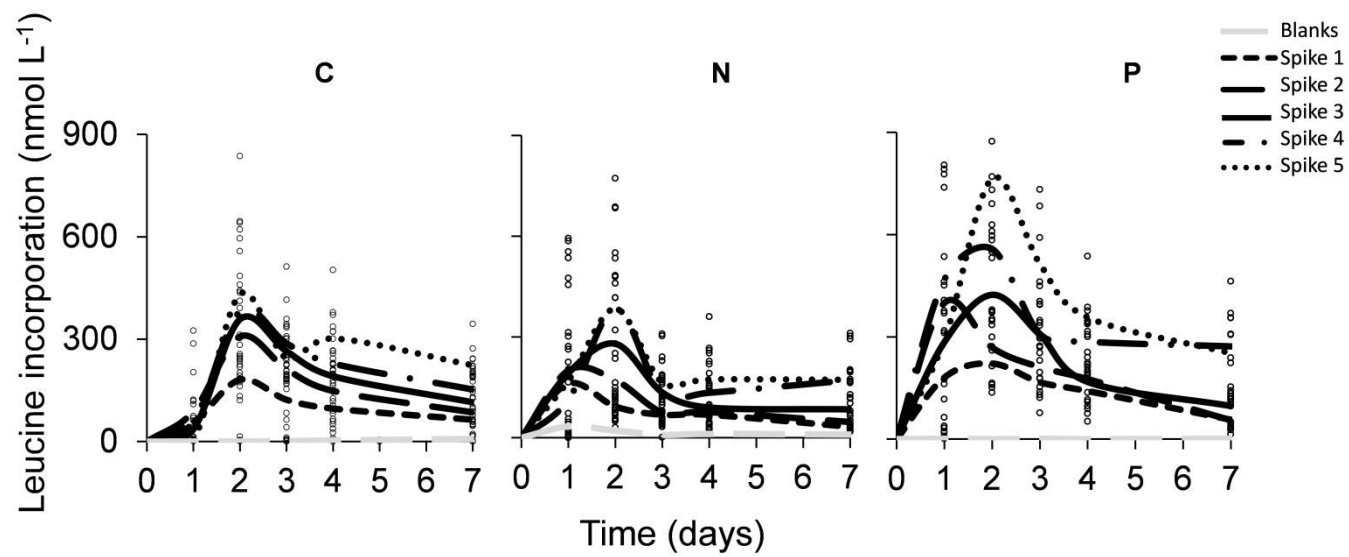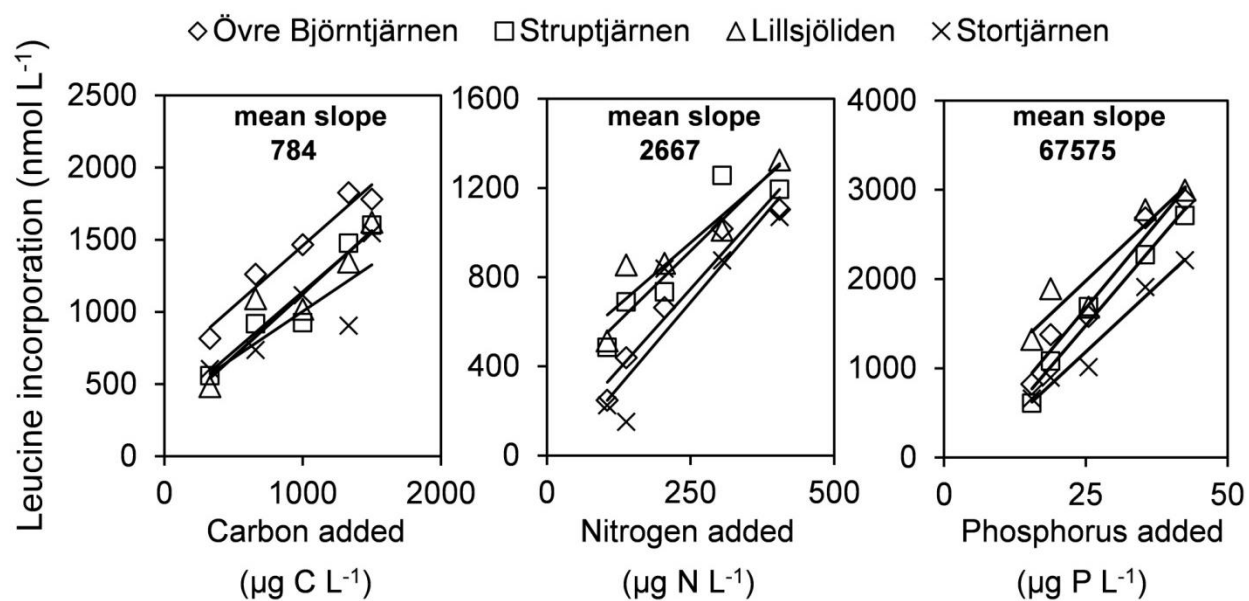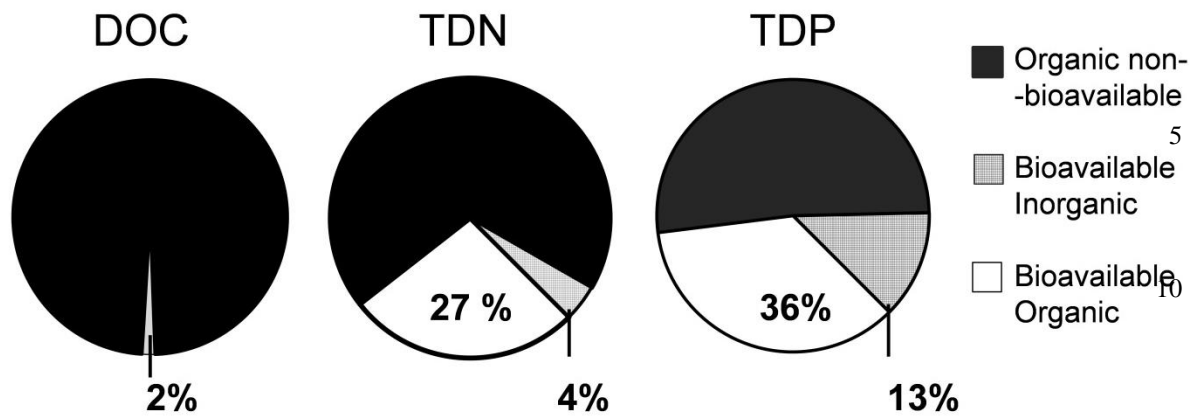
Figure 1.

Figure 2.

DOC **TDN** **TDP**

■ Organic non-
-bioavailable

▨ Bioavailable
Inorganic

□ Bioavailable
Organic

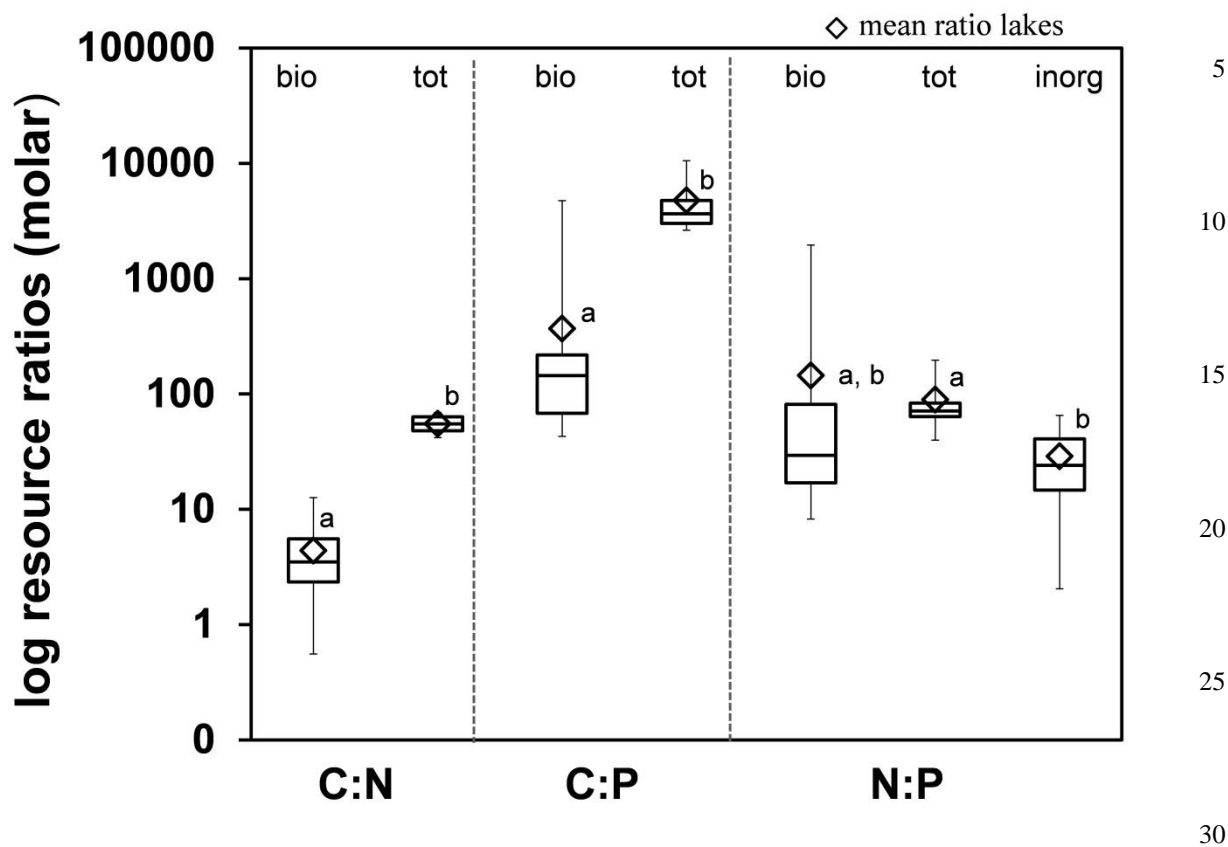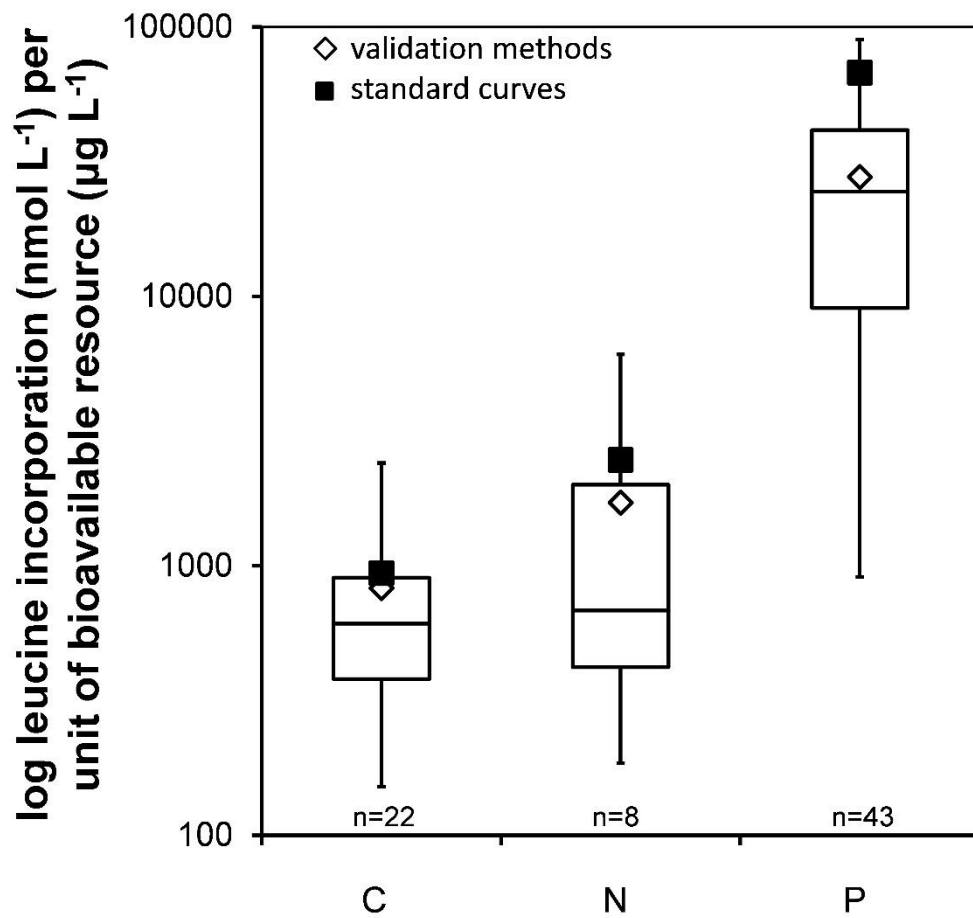2%   4%   13%

27 %   36%

Figure 3.

Figure 4.

Figure 5.

Interactive comment on "New insights on resource stoichiometry: assessing availability of carbon, nitrogen and phosphorus to bacterioplankton" by Ana Soares et al.

5   anaralvessoares@gmail.com

Response to Referee 1

We thank Referee 1 for constructive and relevant comments to the manuscript and for helping us to improve it. We
10 addressed all comments below.

General comment Referee 1:" The manuscript by Soares and others is a novel and important contribution to this topic. In
particular, their innovative experimental approach offers an answer to the question: what resource stoichiometry to bacteria
15 actually experience in situ, given that not all measurable forms are bio-available? The work was thoughtfully designed and
executed and will be of interest to the readership of Biogeosciences.
Two areas require attention from the authors. First, the conclusion that C is limiting is
not adequately supported by the manuscript in its present form (see below). Second,
the uncertainties in bioavailable concentrations must be made more clear. Aside from
20 these two areas, the paper is strong and the other comments are minor/clarification."

Referee comment 1:" Page 1 Line 24. What is the evidence for this in the present study? Although the resource
stoichiometry derived from their results suggests that C will likely be limiting before N or P, this does not automatically
mean that C is limiting. That extension of
25 resource stoichiometry is applicable only if 1) the bacteria are resource-limited and not
under top-down control; 2) the only potentially limiting resources are C, N, or P; and 3)
the system is presumed to be at steady state resembling a chemostat."

Authors' comment: We agree with the Referee and acknowledge that we do not present direct evidence showing C
30 **limitation. We have therefore reformulated all sentences in this regard, clarifying that C was the least bioavailable**
element out of the three key macronutrients that we work with. We also have now made clear that our bioavailability
estimates are informative of maximum potential bioavailability under specific conditions, i.e. when all other macro-
and micronutrients of relevance are in excess. Thus, while we can state that access to bioavailable C in our samples
tended to be in scarcity relative to the microbial need and access to N and P, the apparent C limitation is not directly
35 **transferrable to natural systems, especially not when considering the dynamic nature of natural ecosystems and the**
potential presence of top-down controls and/or micronutrient limitation.

40 Referee comment 2: Page 3 Line 8. While the long incubations have their shortcomings, it is overstated and confusing to say
that these are not 'ecologically relevant timescales'. Certainly the majority of the consumption and respiration in fresh DOM
happen in a matter of hours to days. However, longer-term degradation rates of more recalcitrant forms are of key
importance. Specific to this study, the rapid rates of consumption observed are due to

the high concentrations of CNP added and thus, the timescale of the experiment is not ecologically relevant. I suggest that the authors focus this section and justification on the multi-element aspect of their design, which is the important and novel part.

5 **Authors' comment: We agree with the Referee on the ecological importance of long-term degradation of more recalcitrant DOM, particularly in systems with long water residence times. However, resource bioavailability measured with long-term incubations does not reflect readily bioavailable pool sizes that control bacterial metabolism at a given moment. Moreover, during long incubation periods various factors can interfere with the uptake of bioavailable resources. For example, the dynamics of viruses and the development of toxic conditions that can appear**
10 **from repeated bacterial regeneration of resources can interfere in long-term measurements (Cho et al., 1996). By using our seven-day approach and by maximizing bacterial metabolism, we reduce the incubation length to a minimum and sufficient time period during which bacteria take up most of the readily bioavailable pool (Fig. 1). Our estimates can be used to understand the potential C, N and P bioavailability, as they are performed during "ecologically relevant timescales". In our revised manuscript we will clarify that the relevance refers to how**
15 **meaningful the measurements are for understanding the direct controls of bioavailable nutrient pools on the metabolism – not the controls the nutrient pools may have months ahead in time.**

20 Referee comment 3: Page 3 Line 30. The third question seems certain to be true, and thus not informative as a question or hypothesis. Yet, quantifying this mismatch is important, so I suggest that the authors rewrite these questions.

Authors' comment: The third question was changed to "By how much do total C:N, C:P and N:P ratios exceed bioavailable C:N, C:P and N:P ratios".

25

Referee comment 4: Page 4 Line 10. By sampling the rivers at their outlet, much of the bioavailable forms have presumably been consumed in transit. What is the rationale for sampling far downstream from the sources of DOM?
30

Authors' comment: Our main goal was to capture bioavailability patterns across a landscape gradient with different boreal freshwater properties (please see revised manuscript version page 4 lines 7-9) and not to determine the amount of bioavailable element coming from terrestrial soils.

35

Referee comment 5: Page 5 Line 2. This standardized inoculum has important implications for interpreting the results. Elaborate on why this single community was used as opposed to the communities present in the source water.

Authors' comment: **We wanted to ensure that differences in bacterial community composition did not influence our estimates of resource bioavailability (Martinez et al., 1996). This was achieved by using a standard bacterial community in all our assays. We have now explicitly motivated the use of a singular bacterial inoculum in the manuscript. By using a pooled inoculum representing both headwater inlet and lake water from four different lakes with different properties, we ensured a high diversity of the microbial assemblage that was used to inoculate.**

Referee comment 6: "Page 5 Line 15-30. This experimental approach is rather involved. If space allows, the authors should include a schematic diagram that shows how they forced limitation by CNP and measured the response to addition of the limiting resource. Presumably this method is based on the Wright-Hobbie technique and thus it is important to show how the estimates of ambient concentrations were derived."

Authors' comment: **We agree that it is important to include a schematic diagram to help to better visualize our approach. We will add a schematic diagram of the method to the supplementary material in our next manuscript version.**

Referee comment 7: "Page 5 Line 30. "The total amount of bioavailable nutrient taken up" is not precise. Especially for C, the nutrient need not be assimilated in order for the bacteria to exhibit a growth response."

Authors' comment: **We think that our sentence is well formulated. We used leucine incorporation as an experimental response variable of all bioavailable element uptake, which in the case of C can be used either for growth or respiration.**

Referee comment 8: "Page 6 Line 15. The use of complementary validation methods is an important strength of this paper. Well done."

Author's comment: **Thank you for pointing this out.**

Referee comment 9: "Page 6 Line 32. This method of calculating cellular N content is strange. What are the assumptions of this method? At the least it assumes that all of the added N is assimilated and that no other N is used."

Authors' comment: This method encompasses several assumptions: 1) bacterial growth in the bioassays was effectively limited by N, 2) different N compounds yield similar bacterial biomass increases, 3) all bioavailable N was assimilated when bacterial growth ceased and 4) N bioavailability was independent from the bacterial inocula. The paper from Stepanauskas et al. (1999) describes in detail the experimental setup and the method's assumptions.

5

Referee comment 10: "Page 7 Line 5. The validation method used for P availability is more straightforward than for N. Why not use this method for N also? Additionally, were these fitler-P measurements corrected/checked for phosphate binding to
10 the filter?"

Authors' comment: We lacked the equipment necessary to measure bioavailable N (and C) with the same method as the one used to determine P bioavailability.

**Estimates of P bioavailability were corrected for potential P filter content, binding of dissolved P species and
15 abiotic formation of particles (Jansson et al., 2012).**

Referee comment 11: "Page 7 Line 30. Needs clarification. No difference between slopes for C, N, and P or among lakes?
20 Also, it is unclear why the regressions were performed individually for each analytical replicate instead of using all of the analytical replicates for a given site/date. From what I can tell, the standard curves were computed individually for each of five analytical replicates and then the standard deviation of their estimates is presented in table 2?"

Authors' comment: We have changed lines 24-25 on page 8 of the revised manuscript.
25 **We first performed the regressions individually (Figure 2), precisely because we wanted to test whether there were differences on the bacterial response to nutrient additions between the different lakes. Since we found no statistically significant differences between lake slopes (this is mentioned on page 8 line 24 and page 13 lines 30-31 of the revised manuscript), we combined all datapoints and performed a new regression for each element based the entire dataset. This rendered the "mean slope" given on Figure 2 (C slope=784 nmol L^{-1} per µg C L^{-1}, N=slope 2667 µg
30 N L^{-1}, P slope=67575 µg P L^{-1}).**

No, in table 2, the mean slope of the standard curves was used to translate amounts five replicate measurements of leucine uptake. The standard deviation of the estimates is given within brackets.

35

Referee comment 12: "Page 9 Line 20. Were the total and bioavailable concentrations (or elemental ratios) positively correlated?"

Authors' comment: No, there was no correlation between the total and bioavailable concentrations.

Referee comment 13: "Page 9 Line 23. Again, what is the evidence that C was most limiting, or even limiting at all? The traditional lines of evidence for this (single nutrient bioassays) are not presented, so this is either inferred from the stoichiometry estimated for resources or

from the low proportional bioavailability of C compared to N and P. Neither of these

shows that C was the strongest limiting factor. Pease elaborate on this and explain 1)

the assumptions used for this claim and 2) the specific evidence from this study"

Authors' comment: We agree with the Referee that we do not have the evidence needed to claim that C is limiting in boreal waters (see answer to Referee comment 1). We have changed the sentence in question (see revised manuscript page 10, lines 17-18).

"Surprisingly, in these systems where absolute surface water DOC concentrations are large, C bioavailability was low and was the strongest limiting factor for heterotrophic aquatic production." to "In these systems where absolute surface water DOC concentrations are large, C bioavailability was lowest, relative to N and P."

Referee comment 14: Page 10 Line 33. There are many other factors related to seasonality that could explain this (light, plant production, hydrology, etc), so how can you conclude that soil microbial activity is the predominant driver? Overall, I found this discussion of seasonality too speculative

Authors' comment: We acknowledge the important role of other seasonal factors for the amount of bioavailable dissolved organic carbon measured in our study. We have now removed the sentences from the revised manuscript page 11 lines 26-31.

Referee comment 15: Page 11 Line 27. These calculations seem to be the core of the argument that C is limiting and thus require elaboration. Even then, this only shows that C is more likely to be limiting than N or P, but does not show that C was in fact limiting at ambient concentrations.

Moreover, the ranges here are so large that they are not really meaningful. Why not use the ratio of slopes presented in figure 2 to estimate the relative consumption rates

of CNP? In your calculations, you already assume that the ratio of leucine:cell is invariant, so the ratio of 1/C-slope to 1/P-slope (=86) is the ratio of C consumption to P consumption when those elements are limiting. No?

5   In both the lakes and the rivers, the DOM pools have already undergone much degradation by bacteria, light, and reactive oxygen. This needs to be acknowledged, or better yet, discussed in some detail.

Authors' comment: We agree with the Referee. We have thus, reformulated our conclusion and all statements related

10 **to the topic (please see also answers to Referee comment 1 and 13).**

We decided to exclude the calculations from the manuscript, as we acknowledge that the use of natural ranges of BGE may not be truly representative of BGE values in our bioassays in which element limitation was strongly induced. We appreciate the reviewer's suggestion of assuming a ratio for the C consumption in relation to P but we now think it is better to remove the discussion of C limitation of BP.

15 **We agree with the Referee regarding the loss of most of the riverine bioavailable pool. We added a discussion paragraph on the subject to the revised manuscript version (page 13 lines 9-19). This however does not apply to the lakes DOM as we targeted the short-term bioavailable resource pool (see revised manuscript version page 13 lines 9-19).**

20

Referee comment 16: "Page 13, line 1. Avoiding these uncertainties is important, but those are typically on the order of a few percent and can be constrained by experimental validation. Without a robust analysis of the resulting uncertainties from the present approach, it is not

25 possible to discern which method is advantageous. Form Table 2 and Figure 1/2, it appears that the uncertainty in concentration estimated for a single date/site is large. Without such an analysis of the uncertainty in the final estimates, I suggest that the authors focus on the multi-element aspects of their study"

30 **Authors' comment: As suggested we will focus our discussion on the multi-element aspect of our study. Thus, we have removed from the revised manuscript lines 21 to 25 on page 14.**

35 Referee comment 17: "Figure 4. What do the diamonds represent in this figure?"

Authors' comment: The diamonds represent average resource ratio values for the lakes for all dates (n=26). We have added to Figure's 4 caption the following sentence: "Data shown as boxplots and includes mean as diamonds.

Referee comment 18: "Figure 5. The vertical axis scale should be fitted to the range of data presented."

Authors' comment: Vertical axis scale has been changed from 1 to 100000 to 100 to 100000.

References**:**

Cho, B. C., Park, M. G., Shim, J. H., and Azam, F.: Significance of bacteria in urea dynamics in coastal surface waters, Mar Ecol Prog Ser, 142, 19-26, 10.3354/meps142019, 1996.

Jansson, M., Berggren, M., Laudon, H., and Jonsson, A.: Bioavailable phosphorus in humic headwater streams in boreal Sweden, Limnol. Oceanogr., 57, 1161-1170, 2012.

Martinez, J., Smith, D. C., Steward, G. F., and Azam, F.: Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea, Aquatic Microbial Ecology, 10, 223-230, 10.3354/ame010223, 1996.

Stepanauskas, R., Leonardson, L., and Tranvik, L. J.: Bioavailability of wetland-derived DON to freshwater and marine bacterioplankton, Limnol. Oceanogr., 44, 1477-1485, 1999.

Interactive comment on "New insights on resource stoichiometry: assessing availability of carbon, nitrogen and phosphorus to bacterioplankton" by Ana Soares et al.

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We thank referee 2 for constructive and relevant comments and suggestions of technical corrections, which helped us to improve the manuscript. Please find our response below.

General Comments

This manuscript presents the results from a test of a new method of determining the relative bioavailability of carbon, nitrogen and phosphorus for lake and riverine bacterioplankton. The technique, which combines radiolabeled leucine incubations with reciprocal nutrient amendments, is a novel approach to backing out the proportion of total dissolved C, N and P that bacteria can rapidly take up if other factors are not limiting. The authors test the approach with seasonal samples from four Swedish lakes and single-date samples from seven rivers. Overall, the authors provide a very interesting analysis and the paper is in good shape. Please see below for my specific and technical comments. The only general comment that I would make is that the approach explicitly considers bioavailability in the absence of any co-limitation. In other words, the method cannot incorporate any interactions between limiting factors. While this may be a necessary shortfall of the approach, its significance perhaps deserves some thought and maybe some treatment in the discussion.

Authors' comment to the general comment: The Referee is correct. Our method determines the maximum pool sizes of readily bioavailable macronutrient fractions that can be used given that all other nutrients are provided in access. In the revised manuscript version we clarify that our bioavailability estimates are defined under these specific operational conditions. We also explain that, in order to translate the implications of the results to natural systems, factors like nutrient co-limitation and potential limitation by micronutrients or even top-down controls (e.g., grazing as pointed out by Reviewer 1) need to be taken into account (see revised manuscript version page 13 lines 21-26).

Specific Comments

Referee comment: 1) "Page 1, line 17-18: Make sure the readers know that these percentages are based on the initial concentrations. I know that this might sound obvious, but I was initially confused about whether these were percentages of final (post-incubation) or initial
(pre-incubation) amounts."

Authors' comment: We thank the Reviewer for pointing this out. This has been clarified in the new manuscript version (page 1, lines 16-18).

Referee comment: 2) "Page 5, line 2: Where exactly was the inoculum sampled? And how could it have been sampled only once, given that the lake and river samples were collected over a lengthy period and the incubations run soon after each sample collection? Was it maintained in the laboratory?"

Authors' comment: The inoculum consisted of a mixture of water from both the epilimnion and inlet of the lakes. By including communities from several different sampling sites, we ensured a large microbial diversity on the inoculum. The inoculum was maintained in the fridge at approximately 4 ˚C. Because our experiment strongly maximized bacterial metabolism (selecting for fast-growing opportunistic bacteria), we do not think that the inoculum played a large role on the outcome of our experiment. Previous studies have further demonstrated bacterial growth to be independent of bacteria inocula (Tranvik and Hofle, 1987).

Referee comment: 3) Page 5, line 8: Could there be an effect of incubating bacterioplankton in such a small volume of water? Could biofilms on the walls of these small vials start to have a disproportionate impact on the results?

Authors' comment: We did not test or control for the potential development of biofilms in the tubes walls. However, based on the results for phosphorus presented in Figure 5, we can compare our measurement of the amount of leucine incorporation (normalized per unit of bioavailable P; filled square) with corresponding data extracted from Jansson et al. (2012; the box plot). In the latter case, Jansson et al. did not involve incubations in Eppendorf tubes but in much larger (700+ ml) volumes. There was an overlap in magnitude of leucine incorporation when comparing these two data sources, but it can be noted that our measurements are in the upper range compared to those from Jansson et al. (2012). Biofilm accumulation could have potentially contributed to this difference in our incubation tubes. However, when looking at the time series of our incubations (Figure 1), it is clear that most of the leucine incorporation in our case happened already within 3 days, which should be a time-frame too short for substantial biofilm formation. Thus, we do not consider that biofilms strongly influenced our results.

Referee comment: 4) Page 5, line 16: Maybe I'm missing something, but why didn't the controls consist of lake water without any added C, N or P?

Authors' comment: Since our design is based on the idea of inducing strong limitation of the nutrient to be evaluated for maximum potential bioavailability, we did not consider relevant to incubate lake water without any nutrient additions. On the lines that the Reviewer refers to, we tested whether the inoculum or L16 added any bioavailable C, N and P to our assays. By using Mili-Q water instead of lake water, we made sure the inoculum and L16 were the only possible sources of limiting resource in our bioassays. At the same time, this also tested that leucine incorporation (or bacterial growth) was in fact controlled by the induced limiting resource and that no bacterial growth occurred in the absence of the bioavailable limiting resource (see revised manuscript version page 14 lines 3-5).

Referee comment: 5) Page 5, line 24: Presumably these standard curves would be system-specific? Or at least limited to similar environments within a region? Some discussion of should perhaps be added to the discussion.

Authors' comment: We did not find significant differences among standard curves for the different lakes (page 13, line 6 revised manuscript version), which is interesting since the lakes represent gradients in DOC and catchment features representative to a range of boreal conditions. Possibly, corresponding standard curves from the rivers could have been different from those in the lakes, but it would have been a major time-consuming effort to determine those curves for all of the rivers. Therefore, results from the rivers should be interpreted with caution, even if the rivers do not represent fundamentally different chemical conditions. We added a short section on the subject to the discussion part in the new manuscript (pages 13-14 lines 33-3).

Referee comment: 6) Page 6, line 24 to page 7, line 8: It sounds like these methods assume no changes in cellular stoichiometry with nutrient availability (i.e. elemental homeostasis).

Authors' comment: Yes the Reviewer is correct; an invariant cellular stoichiometry was assumed in the validation method used to calculate N bioavailability. This, as well as other method assumptions, has been scrutinized in Stepanauskas et al. (1999).

However, there were no assumptions regarding cellular stoichiometry for the method used to calculate P bioavailability since this was based on direct measurements of the content of P in bacterial growth cultures harvested from filters.

Referee comment: "7) Page 11, line 5: Is this consistent with turnover rates of these elements in these ecosystems?"

Authors' comment: We do not know of studies looking at the turnover rates of these elements in the soils in the study area. We have deleted the sentence in question and replaced it with a clearer sentence that brings into attention the main mechanism suggested by Jansson et al. (2012), i.e., the apparent temperature-dependence of mobilization of bioavailable P from soils (see revised manuscript page 11-12, lines 33-3).

Referee comment: 8) Page 11, line 23: Perhaps mention threshold elemental ratios here, as well as the work that has focused on them in bacteria (Sinsabaugh, Chrzanowski, etc).

Authors' comment: We have accepted the suggestions from Referee one (see Referee comment 1, 13, 15) and removed the discussion on threshold element ratios and inferences on C limitation. As the section in question has been deleted, we did not include these references.

Referee technical corrections: 9 – 35)

Authors' comment: We thank the Referee for technical corrections. All have been addressed in the new manuscript version, apart from the technical corrections in sentences that have been removed from the manuscript.

36) Fig. 1: Why are the data points from the different treatments not differentiated
here?

Authors' comment: The majority of the data points overlap, differentiating these points would make the figure more complex and not necessarily more informative.

References:

Jansson, M., Berggren, M., Laudon, H., and Jonsson, A.: Bioavailable phosphorus in humic headwater streams in boreal Sweden, Limnology and Oceanography, 57, 1161-1170, 2012.

Stepanauskas, R., Leonardson, L., and Tranvik, L. J.: Bioavailability of wetland-derived DON to freshwater and marine bacterioplankton, Limnology and Oceanography, 44, 1477-1485, 1999.

5   Tranvik, L. J., and Hofle, M. G.: Bacterial growth in mixed cultures on dissolved organic carbon from humic and clear waters, Applied and Environmental Microbiology, 53, 482-488, 1987.