ORIGINAL ARTICLE

Magnitude and regulation of bacterioplankton respiratory quotient across freshwater environmental gradients

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Bacterioplankton respiration (BR) may represent the largest single sink of organic carbon in the biosphere and constitutes an important driver of atmospheric carbon dioxide (CO_2) emissions from freshwaters. Complete understanding of BR is precluded by the fact that most studies need to assume a respiratory quotient (RQ; mole of CO_2 produced per mole of O_2 consumed) to calculate rates of BR. Many studies have, without clear support, assumed a fixed RQ around 1. Here we present 72 direct measurements of bacterioplankton RQ that we carried out in epilimnetic samples of 52 freshwater sites in Québec (Canada), using O_2 and CO_2 optic sensors. The RQs tended to converge around 1.2, but showed large variability (s.d. $= 0.45$) and significant correlations with major gradients of ecosystem-level, substrate-level and bacterial community-level characteristics. Experiments with natural bacterioplankton using different single substrates suggested that RQ is intimately linked to the elemental composition of the respired compounds. RQs were on average low in net autotrophic systems, where bacteria likely were utilizing mainly reduced substrates, whereas we found evidence that the dominance of highly oxidized substrates, for example, organic acids formed by photo-chemical processes, led to high RQ in the more heterotrophic systems. Further, we suggest that BR contributes to a substantially larger share of freshwater CO_2 emissions than presently believed based on the assumption that RQ is ~ 1. Our study demonstrates that bacterioplankton RQ is not only a practical aspect of BR determination, but also a major ecosystem state variable that provides unique information about aquatic ecosystem functioning.
The ISME Journal (2012) **6,** 984–993; doi:10.1038/ismej.2011.157; published online 17 November 2011
Subject Category: geomicrobiology and microbial contributions to geochemical cycles
Keywords: respiratory quotient; bacterioplankton; freshwater

Introduction

Bacterioplankton respiration (BR) in oceanic and inland water systems may represent the largest single sink of organic carbon (C) in the biosphere (del Giorgio and Williams, 2005a). Inland waters are often supersaturated in carbon dioxide (CO_2) (del Giorgio *et al.*, 1997; Prairie *et al.*, 2002) and, although covering only 1% of the earth's surface, they seemingly emit CO_2 to the atmosphere at a rate that is of a similar order of magnitude as that of fossil fuel combustion or C uptake by oceans (Battin *et al.*, 2009; Tranvik *et al.*, 2009). Some of these CO_2 emissions originate from BR fueled by terrestrially derived organic C (Karlsson *et al.*, 2007; McCallister and del Giorgio, 2008). However, uncertainties

in the quantitative importance of BR, by itself and relative to other processes that also generate aquatic CO_2 (Granéli *et al.*, 1996; Humborg *et al.*, 2010), still precludes a complete understanding of BR and overall aquatic carbon cycling.

One seldom addressed uncertainty that is central to the process of BR is the bacterial respiratory quotient (RQ). Most studies of aquatic bacterial metabolism have inferred BR from O_2 consumption rates, measured using traditional chemical approaches (Winkler, 1888) or modern spectrometric (del Giorgio *et al.*, 2011), electrode and optode (Marchand *et al.*, 2009) techniques. At a resolution several-fold higher than that for CO_2, the O_2 techniques have expanded the possibilities to measure BR in many types of aquatic systems, however, at the cost of needing to apply a RQ (moles produced CO_2 per mole consumed O_2) to derive the rates of CO_2 production.

Although the bacterioplankton RQ has profound conceptual importance and significant practical implications, few empirical determinations exist. There have been a handful of studies of marine RQ (Oviatt *et al.*, 1986; Robinson *et al.*, 2002), but to our knowledge, only one study targeting the RQ of

natural freshwater bacterioplankton (Cimbleris and Kalff, 1998). Because of the lack of data on either the magnitude or the patterns of variability in ambient bacterial RQs, studies to date have applied fixed RQs in the range of 0.7–1.2 based on more or less arbitrary assumptions.

A common theoretical way to assess RQ is to analyze the stoichiometry of complete oxidation of different substrates (Table 1). In glucose respiration, $6O_2$ is required for transformation of $C_6H_{12}O_6$ into $6H_2O$ and $6CO_2$ at the RQ of exactly 1 CO_2/O_2. Oxidation of average phytoplanktonic material (*sensu* Takahashi *et al.*, 1985; Anderson, 1995; Hedges *et al.*, 2002) implies a low theoretical RQ of 0.7, as plankton are constituted by reduced and oxygen-poor components such as lipids and proteins. Humic-rich colored dissolved organic carbon (DOC) of terrestrial origin, which globally dominates freshwater bacterial substrates (del Giorgio *et al.*, 1997; Jansson *et al.*, 2007; Karlsson *et al.*, 2009), is completely oxidized at an RQ of ca 0.9 (Dilly, 2001). However, recent studies show that bacteria selectively utilize sub-pools of the bulk DOC (Berggren *et al.*, 2007; Guillemette and del Giorgio, 2011). Small organic acids that contribute to high RQ are preferentially metabolized (Berggren *et al.*, 2010) and through photo-transformation of humic substances, organic acids with theoretical RQ above 1 are systematically formed and may have a key role for bacterial metabolism (Kieber *et al.*, 1989; Bertilsson and Tranvik, 1998; Pullin *et al.*, 2004).

The RQ is also theoretically regulated by biochemical pathways of metabolism. For example, instead of being completely oxidized, glucose may be used for growth or enzyme production according to chemical equations (Herbert, 1976) that correspond to an RQ of ~ 1.2. Hypothetically, the bacterial RQ could be particularly affected by enzymatic production in humic systems, where exoenzymes have a major role in the depolymerization of large humic molecules and in the release of their contents of phosphorus (Münster *et al.*, 1992; Middelboe and Søndergaard, 1993).

Thus, natural bacterioplankton utilize complex substrate mixtures, and the cells themselves have a wide range of physiological states. It could be expected that the resulting community-level RQs is not constant, but vary across freshwater gradients to reflect the dominant pools of organic C (algal, terrestrial, photochemically derived) respired by bacterioplankton and the factors that influence bacterial activity. Quantifying this variability in RQ is not only important to improve freshwaters BR estimation: the patterns in RQ will, in turn, inform on fundamental shifts in bacterial physiology and C consumption that may occur along the environmental gradients, and that are not evident from any other measurement.

We here present the first systematic, large-scale study of bacterioplankton community RQ carried out in freshwaters. We explore the variation in RQ in 52 different lakes, ponds and tarns in Québec (Canada), spanning wide ranges in water chemical characteristics, productivity and degree of terrestrial influence. We measured the RQ non-invasively during relatively short incubations at dark *in situ* conditions. It was hypothesized that the RQ is <1 in net autotrophic systems, where phytoplanktonic carbon sources are likely to dominate the bacterial metabolism, but >1 in other systems where photochemically produced organic acids may have a larger role.

Materials and methods

Sites
A total of 52 sites in Québec, Canada, were sampled from mid July to mid August, 2010 (Supplementary Table S1). A subset of 17 sites were sampled again in early October and additional sampling was carried out occasionally at Lakes Croche and Hébécourt. The sites ($0.01–511\,km^2$) were situated in the clay belt of western Québec ($n = 29$), in the wetland-dominated area south of James Bay ($n = 13$), and in the Laurentian shield in south–eastern Québec ($n = 10$). Many of the waters were shallow, sometimes dammed by beavers, and often brown, turbid and macrophyte rich. In this paper, we refer to all small water bodies ($<1\,km^2$) with a depth at the center of $<2\,m$ as 'shallow ponds and tarns' ($n = 17$; one $2.2\,km^2$ site of $0.9\,m$ depth was also included). The remaining sites are defined as 'lakes' ($n = 35$). See Supplementary Table S1 for site characteristics and coordinates.

Table 1 Stoichiometry of aerobic complete oxidation of selected compounds

Compound	Chemical equation	CO_2 produced	O_2 consumed	RQ
Methane	$CH_4 + 2\,O_2 \rightarrow CO_2 + 2\,H_2O$	1	2	0.50
Palmitic acid	$C_{16}H_{32}O_2 + 23\,O_2 \rightarrow 16\,CO_2 + 16\,H_2O$	16	24	0.70
Glucose	$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,H_2O + 6\,CO_2$	6	6	1.00
Pyruvic acid	$C_3H_4O_3 + 2.5\,O_2 \rightarrow 2\,H_2O + 3\,CO_2$	3	2.5	1.20
Citric acid	$C_6H_8O_7 + 4.5\,O_2 \rightarrow 4\,H_2O + 6\,CO_2$	6	4.5	1.33
Tartaric acid	$C_4H_6O_6 + 2.5\,O_2 \rightarrow 3\,H_2O + 4\,CO_2$	4	2.5	1.60
Oxalic acid	$C_2H_2O_4 + 0.5\,O_2 \rightarrow 1\,H_2O + 2\,CO_2$	2	0.5	4.00

Abbreviation: RQ, respiratory quotient.

Sampling and in situ analyses

Lakes with road access were sampled by boat, but over 1/3 of the lakes had to be sampled from a hydroplane. Water was collected at the center of each lake, at 0.5 m depth, in 18 l cubitainers using a peristaltic pump. Shallow ponds and tarns with areas $> 0.10 \, km^2$ were sampled as lakes, whereas those with $< 0.10 \, km^2$ were sampled a few meter from the shore using a pole sampler.

Ambient dissolved O_2, water temperature, pH and depth were measured in situ with a combination probe (YSI Inc., Yellow Springs, OH, USA). In situ pCO_2 was measured using an EGM-4 infrared gas analyzer (PP Systems, Amesbury, MA, USA) receiving air, equilibrated with the water by pumping $(100 \, l \, h^{-1})$ through a Liqui-Cel MiniModule (Membrana, Charlotte, NC, USA). In some smaller ponds, pCO_2 was measured using a headspace equilibration method. A 60-ml syringe was filled with sample water and ambient air (in equal proportions), sealed and shaken, following direct injection of the headspace gas into the EGM-4. Sample pCO_2 was calculated to take into account for CO_2 movements between the water and gas phase during equilibration. CO_2 fluxes at the water–air interface were measured daytime using a floating chamber (diameter 38 cm, above water volume 17 l) connected to an EGM-4. Readings of CO_2 concentration in the chamber headspace were taken each minute for duplicate periods of 10 min. Day-time flux measurements from these floating chambers are overestimating daily fluxes because of induced water turbulence (Vachon et al., 2010), implying that a calculated contribution (%) to the measured CO_2 efflux by BR becomes conservative.

In vitro analyses

Water from each site was gently filtered through 'A/E Glass' filters (PALL Life Sciences, Port Washington, NY, USA; 142-mm diameter, 1.0-μm pore size). This filtration separated bacterioplankton from most other planktonic components (del Giorgio and Pace, 2008). Bacterial O_2 consumption was measured on the filtrate with a 'Fibox 3′' system (PreSens, Regensburg, Germany) as described by Marchand et al. (2009). Within 2–6 h from sampling, duplicate 500 ml Erlenmeyer flasks, equipped with O_2 optodes, were filled for each sample, sealed with silicone stoppers and incubated at ambient epilimnetic temperatures using dark circulating water baths. The incubations were allowed to stabilize for ca 1 h before the first sensor reading was performed in a series of daily measurements (1–3 times per day). The protocol allowed for repeated non-invasive readings of each optode from the outside of the closed flasks (Marchand et al., 2009). The CO_2 production was measured in the same incubation flasks using a 'pCO_2 mini' optode system (PreSens). Every time an O_2 sensor was read with the Fibox 3, a parallel CO_2 sensor (attached 3 cm from the O_2 optode) was read with the pCO_2

mini. Each incubation was stopped when all sensors (duplicates of O_2 and CO_2 optodes) had recorded significant rates of O_2 consumption and CO_2 production (one-tail test of significance of the O_2-time and CO_2-time correlations, $\alpha = 0.05$). The O_2 concentration during the incubations never dropped to levels that are known to slow down bacterioplankton process rates $(< 2 \, mg \, l^{-1})$.

We used O_2 optodes that had recently been calibrated by the manufacturer. The CO_2 optodes were calibrated at our laboratory. Ten out of 42 sensors were selected for a 9-point calibration. A series of CO_2 dilutions was prepared, with pCO_2 (measured with EGM-4 using the headspace equilibration method) from 200 to 20 000 p.p.m.v. The CO_2 optodes were equilibrated with each dilution for 2 h before measurement. Using the pCO_2 mini software, a four-parameter logistic calibration curve was generated (Supplementary Figure S1).

Single-substrate experiments

Single-carbon substrate $(4 \, mg \, l^{-1}$ of DOC) solutions of tryptophan, glucose, citric acid, tartaric and oxalic acid were prepared in Nanopure water. Inorganic nitrogen and phosphorus (0.5 mg of nitrogen per litre in the form of NH_4NO_3 and 50 μg of phosphorus per litre in the form of KH_2PO_4) were added to all substrate solutions, which were then inoculated (10% inoculum of final volume) with freshly sampled water from one of the study sites (oligotrophic lake Croche; see Supplementary Table S1). The inoculum, which had been pre-filtered through PALL A/E Glass filters (see above), was assumed to contain sufficient concentrations of essential micronutrients. Incubations in 500 ml Erlenmeyer flasks equipped with O_2 and CO_2 optodes were then carried out in the same way as described above, but at a fixed temperature of 20 °C and with incubation times proceeding until a stationary phase had been reached. As blank control, a Nanopure water sample was inoculated, enriched with nutrients and treated in the same way as the substrate samples.

Blank-corrected curves describing cumulative O_2 consumption and CO_2 production during the single-substrate incubations were constructed (Supplementary Figure S2). Finally, the rates of O_2 consumption and CO_2 production were calculated from the slopes of the curves during the periods of apparent linear respiratory activity, between the lag phase and stationary phase (see details in Supplementary Figure S2).

Additional analyses of ecosystem properties

Site and catchment characteristics were determined from a digital elevation model in ArcGIS 10 (Esri, Redlands, CA, USA). Total phosphorus was measured using the molybdenum-blue method after persulfate digestion, DOC on an OI Analytical TIC/TOC Analyzer and water color at 440 nm (and other

wavelengths) using a UV/Vis UltroSpec 2100 spectrophotometer (Biochrom Ltd, Cambridge, UK). Phytoplankton primary production and the ecosystem P:R ratio (primary production divided by total respiration) were calculated from O_2 saturation, $\delta^{18}O$ of dissolved O_2, $\delta^{18}O$ of water and community respiration, according to Quiñones-Rivera et al. (2009). Oxygen isotopes were determined using routine methods at the G.G. Hatch Stable Isotope Laboratory (Ottawa, ON, Canada). For the purpose of these calculations, we assumed that the total respiration equaled the bacterial total CO_2 (TCO_2) production multiplied with the ratio between bacterial O_2 consumption rates in unfiltered (P del Giorgio, unpublished) and filtered water (this study) measured using identical methodology. We further assumed 0 exchange of O_2 between the mixed layer and the hypolimnion. All data needed to calculate primary production and P:R were available for 56 out of the 72 samples.

Substrate characterization
The ratio between absorbance at 254 and 365 nm (a_{254}/a_{365}), which tends to be negatively correlated to the average molecular weight of DOC (Dehaan, 1993; Dahlén et al., 1996), was calculated along with the specific ultraviolet absorbance (SUVA; a_{254} divided by DOC) that indicates contents of aromatic carbon (Weishaar et al., 2003). Fluorescence intensity was measured on a Shimadzu RF5301 PC (Shimadzu, Kyoto, Japan), across excitation wavelengths of 250–450 nm and emission wavelengths of 280–600 nm. Modeling of fluorescence components (parallel factors) was conducted, on inner-filter effect-corrected samples (McKnight et al., 2001), with MATLAB (MathWorks, Natick, MA, USA) using the DOMfluor 1.7 toolbox (Stedmon and Bro, 2008) (see description of components in Supplementary Figure S4). Biological lability (Bio-lability) and photoreactivity (UV-lability) expressed as DOC loss, calculated as the slopes of linear regression lines for DOC vs time, were obtained from 2-week 20 °C dark incubations with ambient microbial communities, and 48 h incubations under the equivalent of midday tropical sunlight in a solar simulator (Qsun Xe-1-BC, Q-Lab, Cleveland, OH, USA), respectively. DOC was determined every second day in the bio-lability incubations and every 6–12 h in the UV-lability incubations. To obtain indices of substrate quality (rather than quantity), the lability measures were expressed as fractions of the DOC pool and the fluorescence component scores were expressed as fractions of the sum of all components. The cross-sample mean UV-sensitivity of each fluorescence component (% loss after 24 h solar simulation) was used to calculate an index of the total share (%) of UV-sensitive fluorescence in each sample.

Bacterial community characterization
The capacity of the communities to degrade 31 different substrates (each replicated three times) was measured using the Biolog Ecoplate (Biolog, Carlsbad, CA, USA). The respiration by the community reduces a tetrazolium dye that is included with each substrate, thus inducing a color development that is measured in an optical assay (Garland and Mills, 1991). We calculated the mean color development values for each class of compounds, measured at the overall 'average well color development' of 0.5.

Calculations
Measured CO_2 production was recalculated to TCO_2 ($CO_2 + HCO_3^- + CO_3^{2-}$) production according to equations in Stumm and Morgan (1996). Thus, we accounted for the share of respired carbon that was transformed into carbonates, thus not being recorded by the pCO_2 mini. Results from these calculations (Supplementary Figure S3) show that net transformation of respired carbon into carbonates was only significant in rare cases with extremely high pH combined with high ratios between alkalinity and CO_2 production. In short, the equilibration constants that regulate the ionization fractions were calculated from incubation temperature. Alkalinity was calculated from in situ pH, dissolved inorganic carbon (measured using the IO Analytical TIC/TOC Analyzer) and temperature. The equilibration constants, in combination with alkalinity and incubation pCO_2, was used to calculate the pH at each incubation time point, assuming constant alkalinity and that no other process other than CO_2 production caused changes in pH (Oviatt et al., 1986). As the respiratory CO_2 affects pH though a feedback loop involving changed ionization fractions, the incubation pH was not algebraically calculated, but solved iteratively using the 'Solver' module in Excel 2007. Finally, the share of produced TCO_2 that did not contribute to measured CO_2 (but to carbonates) was calculated as function of initial pH, temperature, and the ratio between alkalinity and cumulative TCO_2 production (Supplementary Figure S3).

The standard error (s.e.) of each duplicate O_2 consumption and TCO_2 production estimate was calculated as the 'standard error of slope' using the LINEST function in Excel 2007. The s.e. of RQ was calculated by combining the s.e. of TCO_2 production (s.e.[Y]) and O_2 consumption (s.e.[X]) according to the equation:

$$s.e.\left(\frac{Y}{X}\right) = \frac{Y}{X}\sqrt{\frac{s.e.(Y)^2}{Y^2} + \frac{s.e.(X)^2}{X^2}}.$$

Statistics
The regulation of RQ was analyzed exploratively by correlating RQ to principal components (PCs) that described the major patterns of variation in potential explanatory variables. PCs with eigenvalues greater than one were extracted using Varimax rotation (missing values replaced by mean). The extractions

resulted in three models with two significant components each describing the variation in: (a) ecosystem properties, (b) substrate characters and (c) bacterial community functional traits (Biolog data).

All statistical analyses were performed in PASW Statistics 18 (SPSS, Chicago, IL, USA). Tests for comparing different means and to assess correlations were performed using two-tailed P-values except the tests for significant rates of O_2 consumption and CO_2 production during incubations (see above).

Results

The time required for observing significant linear changes in both TCO_2 and O_2 (86 ± 45 h; mean $\pm$ s.d.) was negatively correlated with water temperature ($r^2 = 0.50$, $n = 72$, $P < 0.001$; data not shown). At higher temperatures ($> 18\,^\circ C$), most incubations could be stopped after ~ 48 h. On average, the TCO_2-time and O_2-time regressions comprised 5.0 time points and showed coefficients of determination (r^2) of 0.88 and 0.92, respectively. The mean calculated standard error (s.e.; based on individual regression slopes) amounted to 18% of TCO_2 production, 12% of O_2 consumption and 24% of RQ. The between-replicate standard deviation of TCO_2 production, O_2 consumption and RQ was on average 23%, 10% and 24%, respectively. Measured RQs during bacterial assays with single substrates never deviated from theoretical RQ values by $> 20\%$ (Figure 1). Control incubations showed non-significant changes in TCO_2 and O_2 (Supplementary Figure S2).

Bacterial TCO_2 production and O_2 consumption rates spanned two orders of magnitude, from $0.65\,\mu M$ per day to $52\,\mu M$ per day, generating RQ values in the range of 0.25–2.26. All respiration data from this study, compiled with unpublished and previously published data on freshwater bacterioplankton TCO_2 production and O_2 consumption are shown in Figure 2. Overall, the TCO_2 production could be roughly calculated from O_2 consumption according to the equation $y = 1.23x^{0.99}$ ($r^2 = 0.75$, $n = 110$, $P < 0.001$; Figure 2), implying a general tendency of freshwater RQs to converge at values around 1.2 across all sites and respiration rates. The variation around the central tendency of RQ, however, was not random. The RQ showed distinct patterns of distribution along gradients of classical limnological variables, particularly in the lakes. Excluding the shallow ponds and tarns, there were highly significant negative correlations between RQ and *in situ* O_2 saturation ($r^2 = 0.17$, $n = 50$, $P < 0.01$; Figure 3a) and pH ($r^2 = 0.29$, $n = 50$, $P < 0.001$; Figure 3b), and a relatively strong positive log–log relationship between RQ and *in situ* pCO_2 ($r^2 = 0.42$, $n = 50$, $P < 0.001$; Figure 3c). Overall, systems with very small areas ($< 0.10\,km^2$, $n = 15$) had significantly higher RQ than larger ($> 0.10\,km^2$, $n = 57$) systems ($t = 2.735$, df $= 71$, $P < 0.01$, independent t-test; Figure 3d).

We further explored the distribution of RQs across all systems (lakes, shallow ponds and tarns) relative to ecosystem-level, substrate-level and bacterial community-level PCs of variation. At the ecosystem level, RQ was not correlated to the first PC (PC1), describing increasing tendencies in DOC, color, total phosphorus, shallowness and catchment wetland coverage (Figure 4a). However, there was a relatively strong negative correlation between RQ and ecosystem PC2, characterized by positive loadings
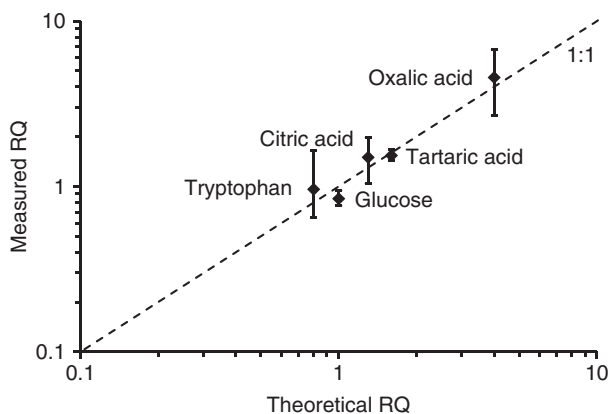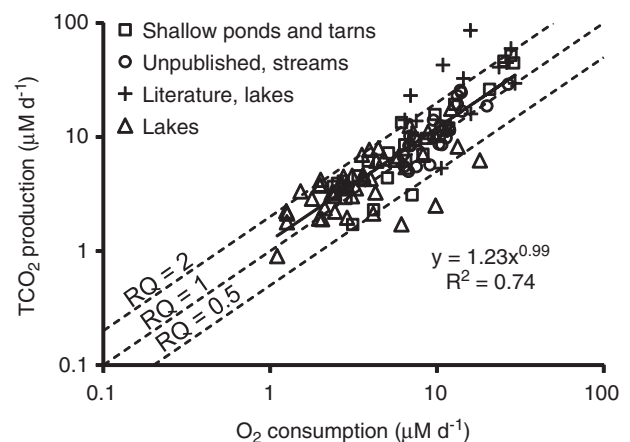

Figure 1 Measured RQs during single-substrate bacterial bioassays plotted against theoretical RQ values for complete aerobic oxidation of the different substrates. Error bars show the range of values obtained by duplicate measurements. The theoretical RQs of the substrates are derived from Table 1, except tryptophan that is attributed the typical text book value of 0.8 for protein and amino-acid degradation. For complete incubation data, see Supplementary Figure S3.

Figure 2 Relationship between the rate of bacterial production of TCO_2 and the rate of bacterial consumption of dissolved oxygen (O_2) shown for different freshwater systems ($n = 110$). Diagonally dashed lines represent different bacterial RQs. The figure compiles present ('Lakes' and 'Shallow ponds and tarns'), unpublished ('Unpublished, rivers'; M. Berggren unpublished*) and previously published data ('Literature, lakes'; Cimbleris and Kalff (1998)**). *Measured as in this study, but at a fixed temperature ($20\,^\circ C$) and controlled inorganic nutrient concentrations. **Measured during 2-day dark incubations at a controlled temperature of $21\,^\circ C$ using Winkler titration (O_2 consumption) and gas chromatography (TCO_2 production).

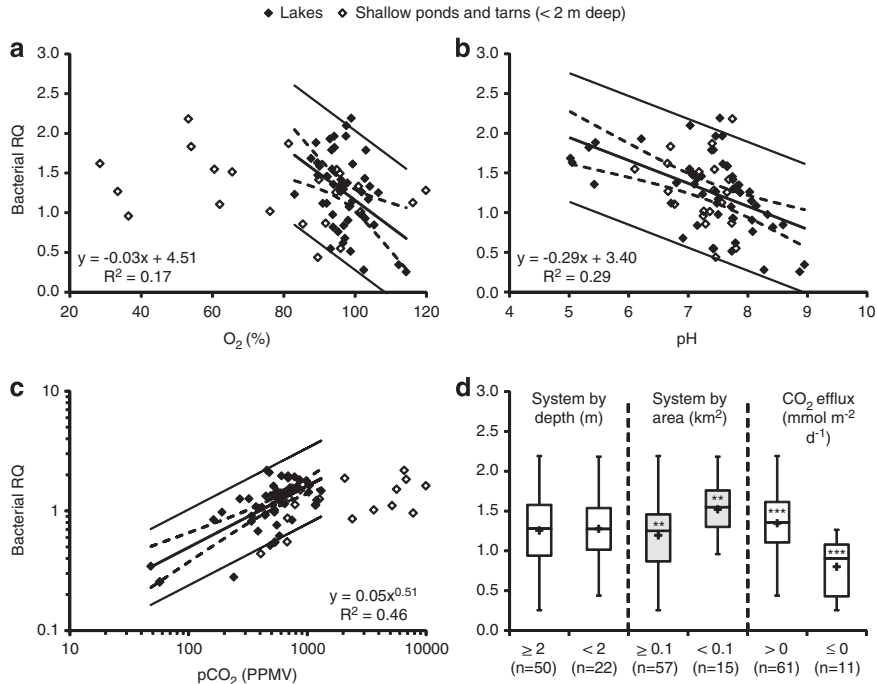Lakes ◆ Shallow ponds and tarns (< 2 m deep) ◇

Figure 3 Relationships between the RQ of lake bacterioplankton (filled diamonds, $n = 51$) and *in situ* (**a**) oxygen saturation, (**b**) pH and (**c**) partial pressure of CO_2. The RQ of bacterioplankton in shallow ponds and tarns (open diamonds, $n = 21$) is shown is shown for comparison, although not being significantly correlated to any of the explanatory variables. (**d**) Box and whisker plots of all RQ observations grouped by system depth, area and CO_2 flux to air. Plus signs denote means and asterisks denote the significance of difference between means: **0.01, ***0.001 (independent samples *t*-test). Note the logarithmized axes of (**c**).
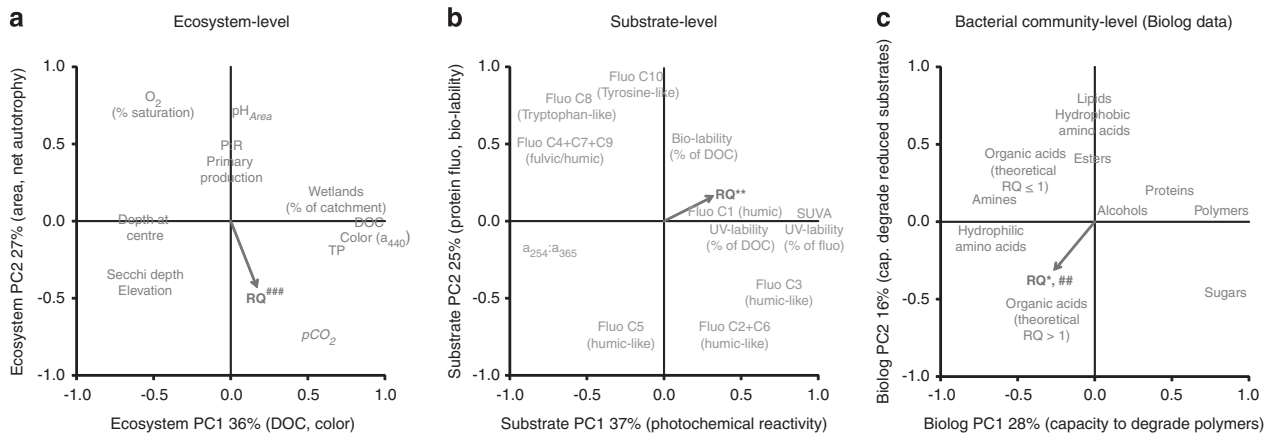

Figure 4 PC loading plots, showing major axes (PCs) of variation in (**a**) ecosystem properties, (**b**) substrate quality and (**c**) bacterial community capacity to degrade different compounds on the Biolog Ecoplate. Percent explained variance by each PC is indicated in the axis title. Vectors show Pearson correlations between RQ and PC scores. Variables in italics have been log-transformed to improve normality. Asterisks and squares denote significant correlations with horizontal and vertical axis, respectively (single symbol $P < 0.05$; double symbol $P < 0.01$; triple symbol $P < 0.001$).

for water surface area and variables related to ecosystem metabolism, indicating net autotrophy (O_2 saturation, P:R and primary production). At the substrate level, samples with high RQ tended to score high on PC1, which was related to substrates of high photochemical reactivity, high SUVA (aromaticity) and high molecular weight, as indicated by low values on the absorbance ratio a_{254}/a_{365} (Figure 4b). There was no significant correlation between RQ and substrate PC2, characterized by

positive loadings for bio-lability and protein-like fluorescence, and negative loadings for most humic-like components. Also the PC analysis of Biolog data extracted two significant components, the first describing the capacity of the bacterial community to degrade polymers and second describing the capacity to degrade reduced compounds (Figure 4c). RQ showed negative correlations to the scores of both axes and particularly PC2. The highest RQ was found for the bacterial commu-
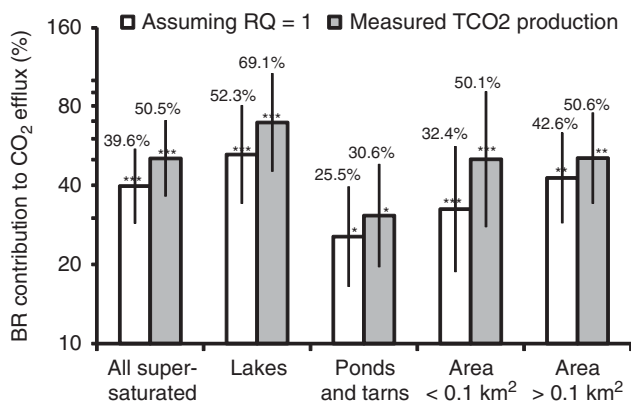
Figure 5 Contribution of epilimnetic bacterial respiration (BR) to CO_2 efflux from different types of CO_2 supersaturated freshwaters. White bars show BR calculated from O_2 consumption assuming a fixed RQ of 1 and grey bars show BR measured directly as TCO_2 production. Bars show mean values and error bars denote 95% confidence intervals of the means (based on log values). Asterisks denote the significance of difference between paired log means: *0.05, **0.01, ***0.001 (paired samples *t*-test).

nities with the highest capacity to degrade highly oxidized organic acids.

Our results suggest a link between the degree of CO_2 supersaturation (and O_2 undersaturation) and the RQ in these systems. Most systems were supersaturated in CO_2, and a total of 61 observations of CO_2 efflux to the atmosphere were obtained with floating chambers. In the remaining 11 cases, typically representing situations of supersaturated O_2 and undersaturated pCO_2, the mean RQ was 0.81, which is significantly lower than the mean RQ of 1.35 in CO_2 effluxing systems ($t = 3.358$, df $= 71$, $P < 0.001$, independent *t*-test; Figure 3d).

The influence of variable RQ on the perceived contribution of BR to the measured CO_2 fluxes in supersaturated systems is explored in Figure 5. We found that this contribution was systematically higher if BR was measured as TCO_2 production rates compared with when BR was inferred from O_2 consumption assuming a fixed RQ of 1 (Figure 5). The effect was particularly pronounced in lakes and in systems with small areas. In the lakes, the mean of BR contribution to CO_2 efflux was 69% based on TCO_2 production, but only 52% assuming an RQ of 1. In systems smaller than $< 0.10 \, km^2$, the contribution was 50% using the measured TCO_2 production, and 32% assuming an RQ of 1 (Figure 5).

Discussion

Our results clearly show that the common assumption of the bacterioplankton RQ fixed at a specific value, often 0.8 or 1.0, may not be justified. The estimates of RQ across 52 freshwaters sites tended to exceed 1 (Figure 2), and there were patterns of RQ along environmental gradients (Figures 3 and 4). We argue that our RQ estimates are robust and representative of ambient values, as the sensors

were carefully calibrated and could effectively detect meaningful differences in RQ when bacterioplankton were respiring different single-carbon substrates (Figure 1). The mean replicate-level error of our RQ estimates, assessed either from calculated standard error (s.e.), or from standard deviation (s.d.) of replicates, was 24%, which is much less than the variability of the observed natural RQs (Figures 2 and 3). Moreover, by using the average of duplicate measurements and by compensating the error of individual RQs by a large sample number ($n = 72$), we were able to capture consistent patterns of RQ across major gradients of ecosystem-level, substrate-level and bacterial community-level variations (Figure 4).

The overall trend for high RQ (Figure 2) is consistent with previous observations from temperate lakes (Cimbleris and Kalff, 1998), but in contrast to theoretical studies of bacterial metabolism, which have questioned large departures of RQs from 1 (del Giorgio and Williams, 2005b). In line with the often assumed bacterial use of relatively reduced phytoplanktonic compounds (Jansson *et al.*, 2007), marine studies on plankton RQs have generally reported values slightly below, or around, 1 (Hopkinson, 1985; Oviatt *et al.*, 1986; Berdalet *et al.*, 1995; Robinson *et al.*, 2002) (P del Giorgio, unpublished). Although high RQ might be physiologically explained by biogeochemical pathways of DOC degradation, it is not obvious how the community-level RQ could rise to values as high as 2 in response to cell biochemistry. For example, if bacteria used glucose for maximum possible enzyme production (see equation for complete glucose anabolism in Herbert, 1976), the RQ would only be *ca* 0.2 units higher than if the glucose was exclusively respired.

Instead, our study points to a substrate control of RQs. The RQ in the substrate experiments was close to 1 when bacteria were provided amino acid or carbohydrate DOC, but in the range of 1.5–4.6 for different organic acids (Figure 1). All these observations agreed well with theoretical RQs for complete aerobic oxidation of the different substrates (Table 1). Thus, the high RQ values that we observed in many natural epilimnetic samples are strongly indicative of bacterial use of compounds of high degree of oxidation, for example, low molecular weight organic acids. This type of compounds can be exported directly from terrestrial systems to streams (Berggren *et al.*, 2010), but in lakes these compounds also originate from photochemical degradation of DOC by ultraviolet (UV) light (Kieber *et al.*, 1989; Bertilsson and Tranvik, 1998), and the fact that RQ was strongly related to DOC UV-reactivity in our lakes (Figure 4) suggests that the preferential consumption of photochemically produced substrates may cause the high RQs observed.

We also observed some surprisingly low RQ values, outside of the range of theoretical RQs for different substrates (Table 1), especially at extremely

high pH (Figure 3b). It is possible that the high pH and temperature in hard-water systems, in combination with microbial processes, caused some of the TCO_2 to be transformed into calcite (Dittrich and Obst, 2004; Homa and Chapra, 2011), thus lowering the apparent RQ. Another possibility is that water column methane oxidation (Bastviken et al., 2008), for which the theoretical RQ is 0.5 (Table 1), partially explained the low RQs. However, pCH_4 determinations performed on most samples using a headspace technique combined with gas chromatography (P del Giorgio, unpublished), revealed no significant relationships with RQ. Thus, methane was probably of small relative importance.

Systems that were net autotrophic (CO_2 influx from atmosphere and high calculated P:R ratios) had a mean RQ of 0.81 (Figure 3d), suggestive of a dominant phytoplanktonic source of respired substrates (Anderson, 1995; Hedges et al., 2002). The remaining systems appeared to be net heterotrophic to varying degrees and had significantly higher mean RQs (1.35; Figure 3d). These patterns suggest that the net metabolic balance of freshwater ecosystems is not only related to their nutrient and organic matter inputs, but also to the nature of dominant pools of organic carbon that are consumed and respired by pelagic bacteria. Knowledge of the character of these pools may, in turn, contribute to understanding of why lakes in different regions differ in their relationships between DOC and pCO_2 (Larsen et al., 2011). The fundamental role of substrate control of bacterial RQ and community metabolism was further highlighted by the Biolog results, showing that bacterial communities in high RQ systems were characterized by their capacity to degrade oxidized organic acids, whereas communities in low RQ systems were optimized for degrading reduced compounds, such as lipids (Figure 4c).

Many studies have characterized lake metabolic balances (as a P/R ratio) by comparing planktonic primary production (measured, for example, by ^{14}C-bicarbonate incorporation) and planktonic respiration from O_2 consumption, often assuming an RQ of 1 (del Giorgio et al., 1997; Koch et al., 2007). Our results suggest that assuming a fixed RQ is dampening the apparent variations in the net metabolic balance: In lakes that are net heterotrophic, the use of fixed RQ of around 1 will result in an underestimation of the actual degree of net heterotrophy, whereas the same fixed RQ will result in an underestimation of the degree of net autotrophy in more productive lakes. Moreover, high RQ values in net heterotrophic systems imply that bacteria have a significantly larger role in generating the excess pCO_2, and thus the CO_2 efflux, than what would be estimated based on the assumption of RQ = 1 (Figure 5).

The above conclusions apply mostly to lakes, while shallow ponds and tarns showed less clear patterns in RQ (Figure 3). Overall, the contribution of BR to CO_2 efflux was also smaller from ponds and tarns, in comparison to that of the deeper lakes (Figure 5). In shallow systems, the water column CO_2 and O_2 dynamics are probably dominated by benthic processes (Pace and Prairie, 2005; Gudasz et al., 2010) that take control over the regulation of pCO_2 and O_2 dynamics (Figure 3). However, regardless of the systems considered, there were no significant relationships between RQ and DOC, suggesting that it is not the amount of DOC, but rather its origin and composition that have a major role for the pathways of ecosystem metabolism.

Our results clearly show that variations in RQ can have profound implications on the perceived magnitude of BR and of the metabolic balance of the ecosystem (Figure 5). Although this study focuses on bacterial RQ along freshwater environmental gradients, there is no reason to think that other aquatic ecosystems, such as coastal and open oceans, will behave differently, thus, prompting the urgent need to assess bacterial RQ broadly.

Acknowledgements

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)