environmental microbiology
society for applied microbiology sfam
Environmental Microbiology (2012) doi:10.1111/1462-2920.12042

Does microbial stoichiometry modulate eutrophication of aquatic ecosystems?

A. K. Steenbergh,[1]* P. L. E. Bodelier,[1] M. Heldal,[2] C. P. Slomp[3] and H. J. Laanbroek[1,4]
[1]*Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 50, 6700 AB Wageningen, The Netherlands.*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, University of Bergen, P.O. Box 7803, N-5020 Bergen, Norway.*
[3]*Department of Earth Sciences – Geochemistry, Faculty of Geosciences, Utrecht University, P.O. Box 80.021, 3508 TA, Utrecht, The Netherlands.*
[4]*Institute of Environmental Biology, Faculty of Sciences, Utrecht University, P.O. Box 80.084, 3508 TB, Utrecht, The Netherlands.*

Summary

The stoichiometry of prokaryotes (*Bacteria* and *Archaea*) can control benthic phosphorus (P) fluxes relative to carbon (C) and nitrogen (N) during organic matter remineralization. This paper presents the first experimental data on benthic microbial stoichiometry. We used X-ray microanalysis to determine C : N : P ratios of individual prokaryotes from C-limited Baltic Sea sediments incubated under oxic or anoxic conditions. At approximately 400:1, C : P ratios of prokaryotes from both oxic and anoxic incubations were higher than the Redfield ratio for marine organic matter (106:1), whereas prokaryotic C : N ratios (6.4:1) were close to the Redfield ratio. We conclude that high microbial C : P ratios contribute to the enhanced remineralization of P from organic matter relative to C and N observed in many low oxygen marine settings.

Introduction

Phosphorus (P) is a key nutrient and may limit primary production in marine systems both on short and long time scales (Tyrrell, 1999). Higher phosphorus fluxes from sediments overlain by anoxic bottom waters can intensify eutrophication leading to more primary production and anoxic conditions in the water column, affecting marine ecosystems on both regional and global scales (Ingall and Jahnke, 1994; Conley *et al*., 2002). At the onset of anoxia, liberation of phosphate from iron oxyhydroxides and microbial polyphosphates (Goldhammer *et al*., 2010) can be responsible for a higher efflux of P from sediments. The ongoing higher release of P after this initial phase is frequently attributed to preferential remineralization of P from organic matter (Ingall *et al*., 1993; Ingall and Jahnke, 1994; Slomp *et al*., 2002). It is not known whether the preferential remineralization is solely caused by preferential degradation of P-rich organic matter, or whether microbial stoichiometry contributes to this process (Hall *et al*., 2011) through a low P-demand of benthic prokaryotes.

The cellular carbon : nitrogen : phosphorous (C : N : P) stoichiometry of benthic prokaryotes is not readily determined, as cells and sediment particles form structural aggregates. Based on studies of various cultured bacteria, benthic bacteria are considered to be rich in nutrients (Sterner and Elser, 2002), i.e. show a C : N : P ratio lower than the Redfield ratio for marine organic matter of 106:16:1 (Redfield, 1958). However, no experimental data on the stoichiometry of benthic microorganisms are available to verify this assumption. As a consequence, the effect of microbial stoichiometry on remineralization of C, N and P under oxic or anoxic conditions is unknown.

In this study, we determined the C : N : P stoichiometry of individual prokaryotes from Baltic Sea sediments. Sediment top layers from two stations in the vicinity of the Fårö Deep (LF1 and LF1.5; see *Experimental procedures* section) were incubated as slurries under either oxic or anoxic conditions. To promote microbial growth under the imposed redox conditions, we amended half of the slurries with a combination of C, N and P ('control' and 'CNP'). The elemental content of the microorganisms at the end of the incubations was determined by X-ray microanalysis. This technique makes use of fluorescence emission of samples during Transmission Electron Microscopy (TEM). The wavelength of the X-ray fluorescence is specific for each chemical element, and the quantity of each element essentially correlates with the fluorescence intensity. X-ray microanalysis has been used since the 1960s for the elemental characterization of a wide range of biological samples (Sigee *et al*., 1993). To be able to analyse the elemental content of microorganisms from sediments, we

Received 25 January, 2012; revised 31 October, 2012; accepted 1 November, 2012. *For correspondence. E-mail a.steenbergh@nioo.knaw.nl; Tel. (+31) 317 473400; Fax (+31) 317 473675.

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd

Table 1. Specification of cell numbers analysed by X-ray microanalysis.

	Total : 154	Oxic: 65	Anoxic: 89
LF1.5	101	37	64
LF 1.5 CNP	60	18	42
LF1.5 control	41	19	22
LF1	53	28	25
LF1 CNP	48	23	25
LF1 control	5	5	0

first separated microorganisms from sediment particles by blending and subsequent density centrifugation with Nycodenz density medium. Using X-ray microanalysis, we subsequently determined the stoichiometry of individual benthic microorganisms from these samples to gain insight into the role of benthic microorganisms in enhanced benthic P release.

Results

The C : N : P stoichiometry of in total 154 cells was analysed by X-ray microanalysis, as specified in Table 1. Not all cells could be analysed in full, as they were visibly overlapping with other cells or sediment particles. When it was not possible to analyse a cell in full, care was taken to analyse a representative area of these cells (for example, one half of the cell). The size of the cell area that is scanned per microorganism can be seen directly from the TEM image. Scanned cell areas were on average 0.96 μm^2, ranging from 0.16 to 3.08 μm^2.

Although TEM images are two dimensional, the volume of analysed complete and partial cells can be approximated by assuming that all microbial cells are essentially rod shaped (i.e. cylindrical with hemispherical ends; Fagerbakke *et al.*, 1996). However, it must be noted that this method leads to an underestimation of the analysed volume of cells that are not scanned in full. The average calculated cell volume was 0.54 μm (ranging 0.04–2.53 μm). The relation between cell volume and C content is shown in Fig. 1. This relation between two measured variables is best described using standardized major axis estimation (SMA, see *Experimental procedures* section; Warton *et al.*, 2006). The volumes and C contents of the analysed cell areas are linearly correlated, as the slopes of the SMA fits were not different from 1. From the elevation of the SMA fit (i.e. the *y*-intercept of the fit, where $\log(x) = 0$ and therefore $x = 1$; see Fig. 1) of the total dataset follows a C content of 131 fg C μm^{-3}. The C : volume ratios are different for the oxic and anoxic incubations (149 and 115 fg μm^{-3}; 95% confidence intervals = 127–182 and 89–138 fg μm^{-3}, respectively, $P = 0.01$), and also for the control and CNP amended slurries (195 and 111 fg μm^{-3}, confidence intervals = 138–220 and 99–141, respectively, $P < 0.001$).

The C content of prokaryotes can be overestimated if the density-gradient medium Nycodenz ($C_{19}H_{26}I_3N_3O_9$) that was used during the separation of cells from sediment particles enters microbial cells (see *Experimental procedures* section). As Nycodenz contains iodine, the microbial iodine contents determined by X-ray microanalysis can be used to correct microbial C contents, assuming all iodine to be originating from Nycodenz. The resulting C contents were on average less than 0.05% lower than the uncorrected C contents (data not shown). Because of this minor potential contribution of Nycodenz to microbial C contents and the possibility that the measured iodine contents are of biological origin, the uncorrected C contents are used in all calculations.

The relation between microbial C contents on the one hand, and N and P contents on the other was also determined with SMA estimation (Fig. 2). The slope of the C : N SMA fit was not significantly different from 1 (0.985; 95% confidence interval = 0.928–1.046), suggesting a linear relation between untransformed C and N contents over the whole range of measured data. Because of this linear relationship the elevation of the SMA fit is an estimation of the C : N ratio (see Fig. 2). The C : N of ratio 6.42:1 of the analysed cell areas was not significantly different across treatments (oxic and anoxic incubations of control and CNP amended sediment slurries) and sampling stations (not shown), and is close to the Redfield ratio (i.e. 6.63:1).

The relation between C and P contents of the analysed cell areas is non-linear, as the slope of the (log-transformed) SMA fit is significantly lower than 1 (0.896; 95% confidence interval = 0.808–0.994; see Fig. 2). This indicates that analysed cell areas on the high end of the measured P values contain less C relative to P than those with a low P content. Because of this non-linear relation between C and P contents the elevation of the SMA fit at

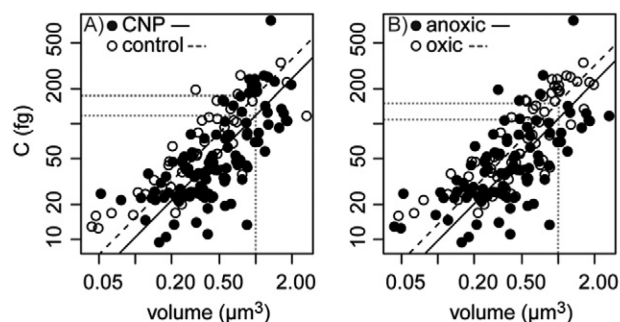

Fig. 1. C contents and cell volumes of microbial cells (both complete cells as well as representative parts of cells) as determined by X-ray microanalysis. Note that the values on the (logarithmic) axes have been substituted by their antilogs. Panel A displays the data separately for CNP-amended and control incubations, and panel B for oxic and anoxic incubations. Black solid lines represent the SMA fits, whereas dashed grey lines mark the C : volume ratio determined by the elevation of the fit (see text).

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd,

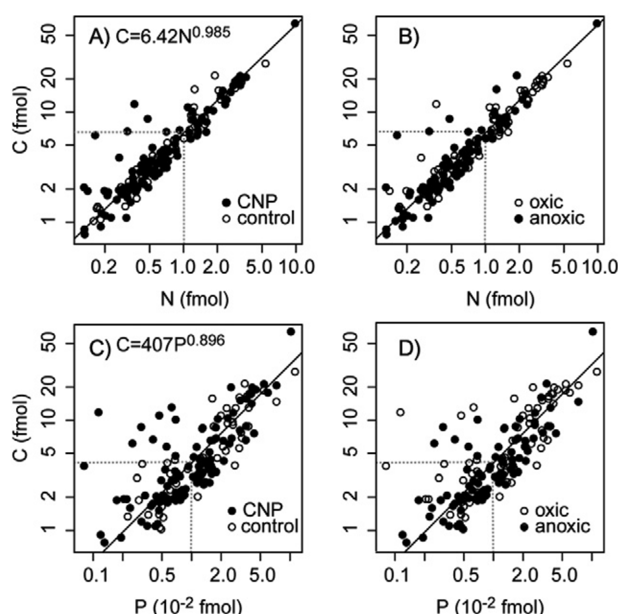

Fig. 2. C, N and P contents of microbial cells as determined by X-ray microanalysis. Note that while the C : nutrient ratios are accurately determined for each cell, the absolute C, N and P contents are not the actual contents per cell, as both partial and complete cells were analysed (see *Experimental procedures*). Panels A and C display the values separately for each incubation amendment (CNP-amended and control incubations), and panels B and D for redox condition during incubation (oxic and anoxic incubations). Fitted lines (solid black) and equations were obtained by SMA estimation. Note that all values on the (logarithmic) axes have been substituted by their antilogs, and the unit of the P values is 10^{-2} fg in order to determine the elevation of the SMA fit (dashed grey lines; see text).

log(P) = 0 only is an estimation of the C : P ratio for values that are close to this P value. The elevation at 1×10^{-2} fmol P, which is close to the median P value of the total dataset of 1.1×10^{-2} fmol, is approximately 4 fmol C (see dashed grey lines in Fig. 2). This C : P ratio of approximately 400:1 for cell areas at median P content therefore is considerably higher than the Redfield C : P ratio (which is 106:1). To determine the C : P ratio of the full range of measured P contents, the highest and lowest measured P contents were normalized to 1 and the elevation of the SMA fits through the normalized datasets were calculated. This results in a C : P ratio range of 318:1 to 531:1 for cell areas at the high and low end of measured P contents respectively. Because both complete and partial cells were included in the analysis, it is not known whether there is a relation between C : P ratio and microbial cell size. As for C : N values, no significant difference was found for C : P values of cells across treatments and sampling stations.

Discussion

The average analysed cell volume of 0.54 μm^3 calculated from the TEM images in this study is at the upper range of volumes determined for marine pelagic bacteria (Malfatti *et al*., 2010), even though not only complete, but also partial cells were analysed. The relatively large volumes found by us can in part be explained by the method of sample preparation; volumes determined by X-ray microanalysis have been found to be 40% larger than those determined for fixed cells by fluorescence microscopy, as formaldehyde can cause shrinkage of bacterial cells (Fagerbakke *et al*., 1996). In addition, the low *in situ* and incubation temperatures of the sediments used in this study may have led to larger than average cell volumes, as bacterial cell volume and temperature have been found to be negatively correlated (Sjöstedt *et al*., 2012).

The C : volume ratios determined in this study lie within the range of values observed for bacteria (both cultured and native aquatic) by Fagerbakke and colleagues (1996). The higher C : volume ratios of cells in oxic incubations may be explained by a higher membrane content of aerobic cells to host membrane-bound electron transport systems (Frerman and White, 1967). We do not know what causes the higher C : volume ratios of the cells from CNP-amended incubations.

The C : N of ratio 6.42:1 of the analysed cell areas is close to the Redfield ratio of 6.63:1. Therefore, accumulation of microbial biomass during organic matter degradation will have little effect on sediment organic matter C : N ratio. The microbial C : N ratios displayed less variation than the C : P ratios, which has been observed previously for bacteria in the water column of lakes (Elser *et al*., 1995; Cotner *et al*., 2010).

The C : P values of benthic prokaryotes determined in this study are 3–5 times higher than the Redfield ratio. Prokaryotes are often considered to be P rich, for example because of the high RNA contents needed to support rapid growth rates (Sterner and Elser, 2002), and several studies have shown that bacterial communities in the water column of lakes and oceans indeed have low C : P ratios (Vadstein *et al*., 1988; Jürgens and Güde, 1990; Chrzanowski *et al*., 1996; Fagerbakke *et al*., 1996). Despite this general concept of high P content, high bacterial C : P ratios have been observed in water column studies (Tezuka, 1990; Elser *et al*., 1995; Makino and Cotner, 2004; Cotner *et al*., 2010). Generally, these high bacterial C : P ratios occur at high resource C : P ratios, and are explained in light of phosphorus limitation (Tezuka, 1990; Elser *et al*., 1995; Makino and Cotner, 2004). However, the high C : P ratios of carbon-limited prokaryotes determined in this study show that P limitation is not a prerequisite for high microbial C : P ratios. Similarly, Cotner and colleagues (2010) determined the community C : P ratio of bacteria from the water column of a freshwater lake to be 259:1, and these values did not differ for bacteria that had received a phosphate amendment 2 days prior to the measurements.

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

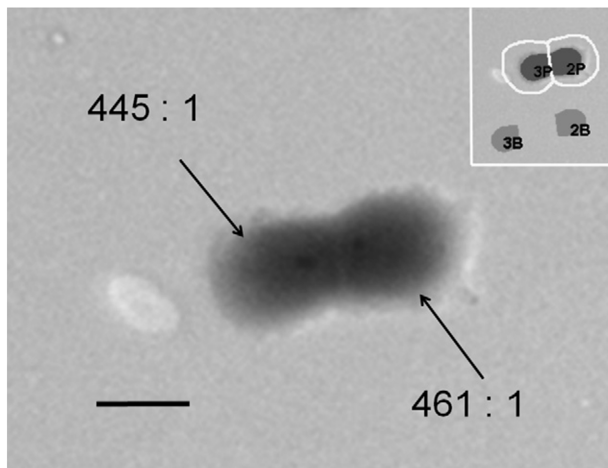

Fig. 3. Example of dividing microbial cells as seen during X-ray microanalysis. The values denote the C : P ratios determined for the two cells, scale bar = 1 μm. The resized inset in the upper right hand corner shows the scanned area of the two cells (2P and 3P) and their accompanying background areas (2B and 3B) used to subtract the carbon content of the formvar support film.

In addition to resource stoichiometry, the growth state of microorganisms can influence microbial C : P ratios (Chrzanowski *et al.*, 1996). Non-growing microorganisms potentially contain less RNA than growing microorganisms, resulting in higher C : P ratios. If during the incubation time substrates in both control and CNP-amended slurries were depleted, this would have resulted in a mostly resting microbial community. However, during X-ray microanalysis dividing cells could be seen, as shown for example in Fig. 3. The high C : P ratios measured in these cells (> 400:1) demonstrate that growing prokaryotes also can have high C : P ratios.

Apart from high RNA contents, microorganisms can become P rich as a result of microbial polyphosphate storage (Ingall *et al.*, 1993, and references therein). Polyphosphates are energy-rich compounds that can be accumulated under oxidizing conditions by many bacteria when energy and phosphate are available in surplus, but growth is limited, for example by N-availability (Gächter *et al.*, 1988). Polyphosphates have been detected in oxic top layers of marine sediments, but not in anoxic sediments (Sannigrahi and Ingall, 2005). If polyphosphates had accumulated in the oxic slurry incubations, this would have led to lower C : P values in the oxic than in the anoxic incubations. However, as the microbial activity was limited by C availability (Steenbergh *et al.*, 2011), the energy surplus necessary for polyphosphate accumulation was lacking. In natural C-limited sediments with oxic top layers, reduced inorganic compounds from deeper, anoxic sediment layers can diffuse to the oxic zone. Chemolithotrophic prokaryotes can use this energy source in combination with pore water phosphate to store polyphosphates (Schulz and Schulz, 2005; Goldhammer *et al.*, 2010). Whether C : P ratios of prokaryotes from oxic top layers in natural sediments are lower than the values in this study needs to be determined. However, polyphosphates are rapidly broken down under anoxic conditions, shifting the C : P value of the autotrophic microorganisms towards their higher 'structural' value.

Benthic prokaryotes are not solely catalysts of the mineralization of organic matter, but also alter the composition of the organic matter remaining in the sediment through the production of microbial biomass (Gächter and Meyer, 1993). The microbial C : N ratios found in this study were close to the Redfield ratio. Thus, accumulation of microbial biomass will not lead to a change in sediment C : N ratios in this system. In contrast, the high microbial C : P ratios can increase sediment organic matter C : P ratios, and can contribute to a decreased P burial efficiency. The influence that microbial biomass exerts on sediment C : P ratios not only depends on the microbial stoichiometry, but also on the extent of accumulation of microbial biomass. There is no consensus on the amount of microbial biomass that accumulates in sediments during diagenesis. Whereas some studies suggest that microbial C is just a minor component of sediment organic C (e.g. Van Oevelen *et al.*, 2009), other studies suggest a larger contribution of up to 40% of sediment organic matter (Gong and Hollander, 1997). It has been proposed that increased microbial biomass accumulation resulting from a lower microbial mortality due to reduced grazing pressure and lack of redox fluctuations (Aller, 1994) can be responsible for enhanced preservation of organic matter under anoxic conditions (Lee, 1992). This hypothesis is supported by a lower turnover of microbial biomass under anoxic conditions than under oxic or fluctuating redox conditions (Sun *et al.*, 2002), and a higher bacterial biomass in anoxic sediments (Gong and Hollander, 1997). When assuming a maximal microbial fraction of sediment organic matter of 40% and a microbial C : P ratio of 400:1 as observed in this study, burial of P in sediments for a given C burial rate can be up to approximately 30% lower than when microbial C : P ratios are conform Redfield stoichiometry. Such a decreased burial efficiency of organic P can be accompanied by an increase in the benthic P flux to the water column. The microbial C : P ratios determined in this study for sites in the vicinity of the Fårö Deep (Baltic Sea) can explain up to 70% of the preferential remineralization of P observed in the anoxic centre of the Fårö Deep (Jilbert *et al.*, 2011). In addition to the accumulation of intact microbial cells, microbially derived organic matter, such as dead biomass or exudates, can contribute to sediment organic matter (Parkes *et al.*, 1993). As the stoichiometry of this organic matter can be different from the stoichiometry of

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

intact microbial cells, the effect of high microbial C : P ratios on this organic matter pool is not known.

The preferential release of P during organic matter mineralization can be attenuated in sediments with oxic top layers by, e.g. polyphosphate accumulation and adsorption to iron oxyhydroxides. These processes facilitate burial of P through precipitation of authigenic P minerals (i.e. *in situ* formed; Ruttenberg and Berner, 1993). Polyphosphate concentrations of up to 8% (Sannigrahi and Ingall, 2005) of total P have been found in oxic top layers of marine sediments. Although polyphosphates are inorganic compounds, their location inside bacterial cells can cause them to be classified as organic phosphorus during P speciation (Ingall *et al.*, 1993). Polyphosphate accumulation in combination with a proposed lower abundance of microbial biomass in oxic sediments due to high microbial mortality can explain the low organic C : P ratios observed in some sediments (Ingall *et al.*, 1993; Slomp *et al.*, 2002).

The release of P from sediments is an important factor in sustaining eutrophication and reduced oxygen availability in both freshwater and marine systems (Conley *et al.*, 2009) and contributes to the worldwide expansion of coastal 'dead zones' (Diaz and Rosenberg, 2008). A full understanding of the processes affecting the intensity of benthic P fluxes is therefore vital. Our data show that marine benthic prokaryotes are poor in P compared with the Redfield ratio, resulting in lower C : P ratios of remineralization products than when nutrient-rich or equal to Redfield ratio stoichiometry is assumed for prokaryotes. The generality of high microbial C : P values and the extent of microbial biomass accumulation needs to be ascertained for diverse types of marine sediment experiencing oxic and anoxic conditions.

Experimental procedures

Site characteristics and sampling

Sediment samples of recently deposited, organic C-rich top layers (0–1 cm) were collected using a multicorer in May–June 2009 at stations LF1 (57°35′22″N, 21°07′28″E) and LF1.5 (57°35′23″N, 21°07′28″E) on the margins of the Fårö Deep in the Baltic Sea onboard R/V Aranda as described previously (Steenbergh *et al.*, 2011). At the time of sampling these stations were positioned in oxic water above the Baltic Sea halocline, with a bottom water salinity of 7.9 and 8.2 respectively, and a temperature of approximately 4°C. The oxygen penetration depth into the sediment was less than 1.5 mm. The C : P ratio of organic matter was above Redfield at both stations (340:1 and 437:1 respectively). In contrast, the ratio of organic C : total P was below Redfield at station LF1 (57:1) and approximately at Redfield stoichiometry at station LF1.5 (116:1), likely due to the presence of iron-bound P (Jilbert *et al.*, 2011). The microbial activity of the sediments prior to incubation has been shown to be limited by the availability of utilizable carbon (Steenbergh *et al.*, 2011). For a more extensive description of the sediment and site characteristics, see Jilbert and colleagues (2011).

Directly upon sampling the top 1 cm layer of several cores from a single multicore cast were collected as bulk sample in an airtight container and transported at 4°C in the dark. In addition, bottom water samples were taken from each station. The water was filtered (0.22 μm) and stored at 4°C in the dark.

Sediment incubation

To minimize contact with oxygen, all sediment manipulations were conducted in a glove bag (Glas-Col, Terre Haute, USA) under continuous nitrogen-flushing. Sediment from each station was mixed and approximately 5 g of sediment and 12.5 ml of bottom water were weighed into 50 ml serum bottles. To ensure microbial growth in the slurry incubations, half of the slurries were amended with a combination of C, N and P. To these CNP-amended slurries glucose, NH_4Cl, and Sørensen's phosphate buffer were added (final concentrations of 10, 1, and 1 mM respectively) resulting in a molar C : N : P stoichiometry of the amendment of 60:1:1. To both CNP-amended and control slurries Hepes buffer was added (pH 7.4, final concentration 25 mM). The serum bottles were closed with butyl rubber stoppers. The anoxic slurries were flushed for approximately 1 h with nitrogen, and the oxic slurries with compressed air. The resulting sediment slurries were incubated for approximately 80 days at 5.2°C in the dark on a gyratory shaker. When assuming a specific growth rate of 0.05 per day for benthic bacteria at this temperature (Sander and Kalff, 1993), the time period would on average allow for four division cycles during incubation. In the first week of incubation, the CO_2 production of the slurries was monitored as part of a previous study (Steenbergh *et al.*, 2011). After this period the slurries were flushed weekly with either compressed air or nitrogen for the oxic and anoxic incubations respectively.

Sample preparation

Directly after incubation, sediment microorganisms were detached from sediment particles by blending. This method was chosen over methods that are based on phosphate to disperse bacteria from sediment particles, as this might have increased microbial P contents. To 5 ml of sediment slurry approximately 10 ml of particle-free water (9.34‰ NaCl in Milli-Q water, filtered over 0.22 μm) was added in a 50 ml centrifuge tube. The samples were blended for 3 × 1 min with an Ultra-Turrax blender (IKA-werke, Stauten, Germany), and cooled in-between and afterwards on ice. The slurries were then brought to a volume of approximately 40 ml with particle-free water and shaken thoroughly. Larger sediment particles were allowed to settle for approximately 15 min, after which 29 ml of the suspension was transferred to a 43 ml polycarbonate centrifuge tube (Nalgene, Rochester, NY).

Intact microbial cells in these samples were concentrated through density centrifugation with Nycodenz density medium (Axis-Shield PoC, Rodelokka, Norway; Lindahl, 1996), as described in Steenbergh and colleagues (2011). Nycodenz is a carbon-based compound ($C_{19}H_{26}I_3N_3O_9$) and can therefore potentially increase microbial C contents.

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

However, as it also contains iodine, the iodine contents per cell that are determined during X-ray microanalysis can be used to calculate the theoretical maximal amount of Nycodenz present per cell. A 6 ml Nycodenz cushion was pipetted underneath each sample (80% w/v in particle-free water), and samples were centrifuged for 1 h at 10.000 *g* at 4°C in a Sorvall RC-6 Plus (Thermo Scientific) with a Sorvall HB-6 swing-out rotor (Thermo Scientific). Approximately 27 ml of the top layer was pipetted off and discarded. Three millilitres of sample at the interface between Nycodenz cushion and top layer, which is the expected location of intact cells, were transferred to a clean centrifuge tube. The samples were again brought to a volume of 29 ml with particle-free water, and a Nycodenz cushion was pipetted underneath. Samples were centrifuged under the same conditions for 2 h. Three millilitres at the Nycodenz-water interface was transferred to a clean centrifuge tube and brought to a volume of 35 ml with 9.34‰ NaCl solution and vortexed. The cells were pelleted by another hour of centrifugation under the same conditions. Thirty millilitres of the supernatant was pipetted off and discarded. The cells were resuspended in the remaining 5 ml.

A subsample of the separated microorganisms was used to prepare the X-ray microanalysis grids, as described by Norland and colleagues (1995), with minor modifications. Approximately 100 μl of resuspended microorganisms and 5 μl of 10× diluted polystyrene latex particles used for calibration during X-ray microanalysis (0.520 μm, Van Loenen Instruments, Zaandam, the Netherlands) were added to 4 ml of particle-free water and vortexed to reduce clumping. Microorganisms and latex beads were centrifuged onto formvar-coated X-ray microanalysis grids (aluminum, 100 mesh, 3.05 mm, Van Loenen Instruments, Zaandam, the Netherlands) for 20 min at 12.000 *g* (Sorvall Discovery M120 SE with swinging bucket rotor S52-ST, Thermo Scientific).

X-ray microanalysis

X-ray microanalysis was performed with a Philips CM200 scanning transmission electron microscope in scanning mode with a data accumulation time of 30 s [80 keV accelerating voltage, spot size 3 (14 nm probe size), 7000× magnification], with an ADDA slowscan imaging system (Soft Imaging System Corp.) and DX-4 EDS detector (EDAX). The image analysis program analySIS (Soft Imaging System Corp.) was used to acquire images and for low-pass filtering and intensity adjustment. To correct for the carbon contribution of the supporting film of the X-ray microanalysis grids, an area identical in size and shape adjacent to each analysed cell was scanned and its elemental contents subtracted from the contents of each particle. Polystyrene latex beads with known elemental contents ($(CH_2)_n$) were included as standards on every X-ray microanalysis sample grid to calibrate the relation between detector counts and carbon contents. The calibration constants for other elements relative to carbon have been previously determined for the used set-up according to Norland and colleagues (1995). With this method, samples with a thickness of up to 20 μm can be analysed without the loss of accuracy due to absorption of the X-ray fluorescence by the sample itself (Norland *et al*., 1995).

Not only complete, but also partial cells were scanned because of overlapping microbial cells, particles or calibration beads. As the main objective of this study was to determine C : N : P ratios and not absolute contents of C, N and P, it is not important whether whole microbial cells were analysed, as long as a representative part of each microorganism was scanned.

Statistical analysis

All data were log-transformed to achieve a normal distribution. The relation between sets of variables (i.e. volume and C content, and C and nutrient content) was assessed through standard major axis estimation (SMA) on log-transformed data (Warton *et al*., 2006; Cotner *et al*., 2010), using the R-package ‘smatr’ (Standardised Major Axis Estimation and Testing Routines, version 2.1, author: David Warton, translated to R by John Ormerod). The SMA line-fitting method is better suited to estimate the relation between two variables than linear regression. SMA takes into account that both variables contain measurement error, instead of only one of the variables in the case of linear regression (where the other variable is the predictor variable; Warton *et al*., 2006). Using this method, the slope of the line fitted through log-transformed data expresses the power of the relationship between the two variables (e.g. linear or exponential); a slope of 1 indicates a linear relationship between – untransformed – data. For fitted lines with a slope not significantly different to 1, the ratio between the two variables is described by the elevation of the fit. The elevation is the *y*-value of the SMA fit at the *y*-intercept (where $\log(x) = 0$; in Figs 1 and 2 this is at $x = 1$, as the antilog values are displayed on the axes). If the slope is different to 1, the elevation only is a measure for the ratio between the variables close to this log-transformed *y*-intercept as the relation between the variables before log-transformation is non-linear. To position the range of P-content data around this *y*-intercept, P-content data were multiplied by 100 before log-transformation (see Fig. 2; as a result the antilogs of the P contents on the *x*-axes are given in 10^{-2} fg). All given ratios are molar ratios. P values lower than 0.05 were considered to be statistically significant.

Equipment and settings for Fig. 3

The image was taken during X-ray microanalysis as described above. Microsoft Office PowerPoint 2007 was used to insert arrows, C : P ratios, scale bar, and inset, to crop the image, and to convert the inset to greyscale.

Acknowledgements

We thank the captain and crew of the R/V Aranda for excellent assistance during the May/June cruise in 2009, Egil Severin Erichsen for his expertise and ceaseless support during XRMA, and Svein Norland for his help with data analysis. C. P. S. acknowledges funding from the EU-Bonus project HYPoxia Mitigation for Baltic Sea Ecosystem Restoration (HYPER) and the Netherlands Organisation for Scientific Research (Vidi grant), and A. K. S. from the Darwin Center for Biogeosciences, the Schure-Beijerinck-Popping

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

fund of the Royal Netherlands Academy of Arts and Sciences, and BE-Basic. This publication is publication number 5374 of the Netherlands Institute of Ecology (NIOO-KNAW), and publication number DW-2012-1008 of the Darwin Center for Biogeosciences.

References

Aller, R. (1994) Bioturbation and remineralization of sedimentary organic matter: effects of redox oscillation. *Chem Geol* **114:** 331–345.

Chrzanowski, T.H., Kyle, M., Elser, J.J., and Sterner, R.W. (1996) Element ratios and growth dynamics of bacteria in an oligotrophic Canadian shield lake. *Aquat Microb Ecol* **11:** 119–125.

Conley, D.J., Humborg, C., Rahm, L., Savchuk, O.P., and Wulff, F. (2002) Hypoxia in the Baltic Sea and basin-scale changes in phosphorus biogeochemistry. *Environ Sci Technol* **36:** 5315–5320.

Conley, D.J., Pearl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., *et al*. (2009) Controlling eutrophication: nitrogen and phosphorus. *Science* **323:** 1014–1015.

Cotner, J.B., Hall, E.K., Scott, T., and Heldal, M. (2010) Freshwater bacteria are stoichiometrically flexible with a nutrient composition similar to seston. *Front Microbiol* **1:** Article 132.

Diaz, R.J., and Rosenberg, R. (2008) Spreading dead zones and consequences for marine ecosystems. *Science* **321:** 926–929.

Elser, J.J., Chrzanowski, T.H., Sterner, R.W., Schampel, J.H., and Foster, D.K. (1995) Elemental ratios and the uptake and release of nutrients by phytoplankton and bacteria in three lakes of the Canadian shield. *Microb Ecol* **29:** 145–162.

Fagerbakke, K.M., Heldal, M., and Norland, S. (1996) Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. *Aquat Microb Ecol* **10:** 15–27.

Frerman, F.E., and White, D.C. (1967) Membrane lipid changes during formation of a functional electron transport system in *Staphylococcus aureus*. *J Bacteriol* **94:** 1868–1874.

Gächter, R., and Meyer, J.S. (1993) The role of microorganisms in mobilization and fixation of phosphorus in sediments. *Hydrobiologia* **253:** 103–121.

Gächter, R., Meyer, J.S., and Mares, A. (1988) Contribution of bacteria to release and fixation of phosphorus in lake sediments. *Llimnol Oceanogr* **33:** 1542–1558.

Goldhammer, T., Brüchert, V., Ferdelman, T., and Zabel, M. (2010) Microbial sequestration of phosphorus in anoxic upwelling sediments. *Nature Geoscience* **3:** 557–561.

Gong, C.R., and Hollander, D.J. (1997) Differential contribution of bacteria to sedimentary organic matter in oxic and anoxic environments, Santa Monica Basin, California. *Org Geochem* **26:** 545–563.

Hall, E.K., Maixner, F., Franklin, O., Daims, H., Richter, A., and Battin, T. (2011) Linking microbial and ecosystem ecology using ecological stoichiometry: a synthesis of conceptual and empirical approaches. *Ecosystems* **14:** 261–273.

Ingall, E.D., and Jahnke, R. (1994) Evidence for enhanced phosphorus regeneration from marine sediments overlain by oxygen depleted waters. *Geochim Cosmochim Acta* **58:** 2571–2575.

Ingall, E.D., Bustin, R.M., and Van Cappellen, P. (1993) Influence of water column anoxia on the burial and preservation of carbon and phosphorus in marine shales. *Geochim Cosmochim Acta* **57:** 303–316.

Jilbert, T., Slomp, C.P., Gustafsson, B.G., and Boer, W. (2011) Beyond the Fe-P-redox connection: preferential regeneration of phosphorus from organic matter as a key control on Baltic Sea nutrient cycles. *Biogeosciences* **8:** 1699–1720.

Jürgens, K., and Güde, H. (1990) Incorporation and release of phosphorus by planktonic bacteria and phagotrophic flagellates. *Mar Ecol Prog Ser* **59:** 271–284.

Lee, C. (1992) Controls on organic carbon preservation: the use of stratified water bodies to compare intrinsic rates of decomposition in oxic and anoxic systems. *Geochim Cosmochim Acta* **56:** 3323–3335.

Lindahl, V. (1996) Improved soil dispersion procedures for total bacterial counts, extraction of indigenous bacteria and cell survival. *J Microbiol Methods* **25:** 279–286.

Makino, W., and Cotner, J. (2004) Elemental stoichiometry of a heterotrophic bacterial community in a freshwater lake: implications for growth- and resource-dependent variations. *Aquat Microb Ecol* **34:** 33–41.

Malfatti, F., Samo, T.J., and Azam, F. (2010) High-resolution imaging of pelagic bacteria by Atomic Force Microscopy and implications for carbon cycling. *ISME J* **4:** 427–439.

Norland, S., Fagerbakke, K.M., and Heldal, M. (1995) Light element analysis of individual bacteria by X-Ray microanalysis. *Appl Environ Microbiol* **61:** 1357–1362.

Parkes, R.J., Cragg, B.A., Getliff, J.M., Harvey, S.M., Fry, J.C., Lewis, C.A., and Rowland, S.J. (1993) A quantitative study of microbial decomposition of biopolymers in Recent sediments from the Peru margin. *Mar Geol* **113:** 55–66.

Redfield, A.C. (1958) The biological control of chemical factors in the environment. *Am Sci* **46:** 205–221.

Ruttenberg, K.C., and Berner, R.A. (1993) Authigenic apatite formation and burial in sediments from non-upwelling, continental margin environments. *Geochim Cosmochim Acta* **57:** 991–1007.

Sander, B.C., and Kalff, J. (1993) Factors controlling bacterial production in marine and freshwater sediments. *Microb Ecol* **26:** 79–99.

Sannigrahi, P., and Ingall, E. (2005) Polyphosphates as a source of enhanced P fluxes in marine sediments overlain by anoxic waters: evidence from ^{31}P NMR. *Geochem Trans* **6:** 52–59.

Schulz, H.D., and Schulz, H.N. (2005) Large sulfur bacteria and the formation of phosphorite. *Science* **307:** 416–418.

Sigee, D.C., Morgan, A.J., Sumner, A.T., and Warley, A. (1993) *X-Ray Microanalysis in Biology: Experimental Techniques and Applications*. Cambridge, UK: Cambridge University Press.

Sjöstedt, J., Hagström, Å., and Zweifel, U.L. (2012) Variation in cell volume and community composition of bacteria in response to temperature. *Aquat Microb Ecol* **66:** 237–246.

Slomp, C.P., Thomson, J., and De Lange, G. (2002) Enhanced regeneration of phosphorus during formation of

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

the most recent eastern Mediterranean sapropel (S1). *Geochim Cosmochim Acta* **66:** 1171–1184.

Steenbergh, A.K., Bodelier, P.L.E., Hoogveld, H.L., Slomp, C.P., and Laanbroek, H.J. (2011) Phosphatases relieve carbon limitation of microbial activity in Baltic Sea sediments along a redox-gradient. *Limnol Oceanogr* **56:** 2018–2026.

Sterner, R., and Elser, J. (2002) *Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere*. Princeton, NJ, USA: Princeton University Press.

Sun, M.-Y., Aller, R.C., Lee, C., and Wakeham, S.G. (2002) Effects of oxygen and redox oscillation on degradation of cell-associated lipids in surficial marine sediments. *Geochim Cosmochim Acta* **66:** 2003–2012.

Tezuka, Y. (1990) Bacterial regeneration of ammonium and phosphate as affected by the carbon : nitrogen : phosphorus ratio of organic substrates. *Microb Ecol* **19:** 227–238.

Tyrrell, T. (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature* **400:** 525–531.

Vadstein, O., Jensen, A., Olsen, Y., and Reinertsen, H. (1988) Growth and phosphorus status of limnetic phytoplankton and bacteria. *Limnol Oceanogr* **33:** 489–503.

Van Oevelen, D., Soetaert, K., Franco, M.A., Moodley, L., Van IJzerloo, L., Vincx, M., and Vanaverbeke, J. (2009) Organic matter input and processing in two contrasting North Sea sediments: insights from stable isotope and biomass data. *Mar Ecol Prog Ser* **380:** 19–32.

Warton, D.I., Wright, I.J., Falster, D.S., and Westoby, M. (2006) Bivariate line-fitting methods for allometry. *Biol Rev Camb Philos Soc* **81:** 259–291.

© 2012 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*