Estuaries and Coasts (2016) 39:992–1005
DOI 10.1007/s12237-015-0053-1

Effects of Oxygen Loss on Carbon Processing and Heterotrophic Prokaryotes from an Estuarine Ecosystem: Results from Stable Isotope Probing and Cytometry Analyses

Rémy D. Tadonléké[1,4] · Thomas Pollet[1] · Pieter Van Rijswijk[2] · Brigitte Leberre[1] · Jack J. Middelburg[2,3]

Received: 3 April 2015 / Revised: 6 November 2015 / Accepted: 19 November 2015 / Published online: 3 December 2015
© Coastal and Estuarine Research Federation 2015

Abstract Many aquatic ecosystems are experiencing a decline in their oxygen (O_2) content and this is predicted to continue. Implications of this change on several properties of bacterioplankton (heterotrophic prokaryotes) remain however are poorly known. In this study, oxic samples (~170 μM O_2= controls) from an oligohaline region of the Scheldt Estuary were purged with N_2 to yield low-O_2 samples (~69 μM O_2= treatments); all were amended with ^{13}C-glucose and incubated in dark to examine carbon incorporation and cell size of heterotrophic prokaryotes, and relationships between organic matter (OM) degradation and phosphate (P) availability in waters following O_2 loss. Stable isotope (^{13}C) probing of phospholipid fatty acids (PLFA) and flow cytometry were used. In samples that have experienced O_2 loss, PLFA biomass became higher, prokaryotic cells had significantly larger size and higher nucleic acid content, but P concentrations was lower, compared to controls. P concentration and OM degradation were positively related in controls, but uncoupled in low-O_2 samples. Moreover, the dominant PLFA 16:1ω7c (likely mainly from Gram-negative bacteria) and the nucleic acid content of heterotrophic prokaryotic cells in low-O_2 samples explained (62–72 %) differences between controls and low-O_2 samples in P amounts. Shortly after incubations began, low-O_2 samples had consistently lower bacterial PLFA ^{13}C-enrichments, suggesting involvement of facultatively anaerobic metabolism in carbon incorporation, and supporting the view that this metabolic pathway is widespread among pelagic bacteria in coastal nutrient-rich ecosystems. Estimates based on ^{13}C-enrichment of PLFAs indicated that grazing by protozoa on some bacteria was stronger in low-O_2 samples than in controls, suggesting that the grazing pressure on some heterotrophic prokaryotes may increase at the onset of O_2 deficiency in nutrient-rich aquatic systems. These findings also suggest that physiological responses of heterotrophic prokaryotes to O_2 loss in such ecosystems include increases in cell activity, high carbon incorporation, and possibly phosphorus retention by cells that may contribute to reduce phosphate availability in waters.

Keywords Oxygen decline · Carbon processing · Heterotrophic prokaryotes · Phosphorus availability · Phospholipid fatty acids · Stable isotope probing · Flow cytometry

Communicated by Zhanfei Liu

Electronic supplementary material The online version of this article (doi:10.1007/s12237-015-0053-1) contains supplementary material, which is available to authorized users.

✉ Rémy D. Tadonléké
rtadonleke@hotmail.com

[1] INRA, UMR CARRTEL 042, F-74203, Thonon-Les-Bains, France

[2] Department of Ecosystem Studies, Netherlands Institute for Sea Research (NIOZ), Yerseke, The Netherlands

[3] Faculty of Geosciences, Utrecht University, Utrecht, The Netherlands

[4] Département des Sciences Biologiques, Université du Québec à Montréal, Montréal, Canada

Introduction

Many aquatic ecosystems experience seasonal hypoxia (Oxygen<60–89 μM) and even anoxia in the hypolimnion during thermal stratification. Beside this seasonal depletion, many of these ecosystems are experiencing a long-term decline in their dissolved oxygen (O_2) content due to eutrophication (Diaz and Rosenberg 2008). and/or climate warming (Deutsch et al. 2011). as surface heating reduces gas solubility and enhances thermal stratification. When O_2 declines and anoxia

develops in aquatic ecosystems, most of the energy is processed by microbial communities (Diaz and Rosenberg 2008).

Among microbial communities, bacteria play a key role in the functioning of aquatic ecosystems through the mineralization of organic matter and uptake and transfer of dissolved organic carbon to consumers in the higher trophic levels of the food web via protists. The partitioning of carbon demand between respiration (mineralization) and biomass formation plays a key role in carbon flow in bacterial cells and in the ecosystem. Carbon (C) uptake by bacteria may lead to increases in their cell sizes (Simon and Azam 1989) and, if grazing is low, as shown by some studies in O_2-deficient waters (e.g. Bettarel et al. 2004). only a small amount of carbon from living bacteria will reach higher trophic levels. Given that O_2 is one of the most important electron acceptors for microbial processes, its decline may strongly impact C uptake and cell size of bacteria, and therefore aquatic system functioning. Although studies on pelagic bacteria in O_2-deficient waters have increased in number during the last decade, they did not explore C incorporation by cells and changes in cell size; they deal mainly with microbial interactions and energetics, nitrogen, sulphur and C cycling, and/or phylogenetic composition (e.g. Crump et al. 2007; Lam and Kuypers 2011; Wright et al. 2012).

Larger bacteria have repeatedly been reported in anoxic hypolimnetic waters compared to overlying oxic waters (e.g. Gast and Gocke 1988; Cole et al. 1993). and this has been attributed to lower grazing on bacteria under O_2-deficiency, or to the higher amounts of organic carbon and inorganic nutrients generally found in O_2-deficient waters compared to overlying oxic waters (e.g. Gast and Gocke 1988; Cole et al. 1993). However, it is unclear if grazing really explains differences in bacterial cell size between these conditions, because the few studies that have examined grazing in O_2-deficient waters versus overlying oxic waters did not perform concomitant analyses of changes in cell size or in proxies of cell size of bacteria. Moreover, these studies have revealed contrasting results; some have reported higher grazing rates on these prokaryotes in oxic waters than in O_2-deficient waters, while other have found the reverse (e.g. Detmer et al. 1993; Weinbauer and Höfle 1998; Bettarel et al. 2004; Cuevas and Morales 2006). It is also possible that shifts towards large-cell-dominated communities occur within bacterioplankton following O_2 loss, because of the death of grazers such as metazoan that are able to consume large bacteria. On the other hand, attribution of these differences in cell size to nutrient amounts is, at least for lakes, based only on a cross-system positive correlation between bacterial biomass and total phosphorus (Cole et al. 1993). Remarkably, effects of O_2 loss on cell size of bacterioplankton have seldom been examined.

Likewise, while it is known that O_2 loss often results in suppression of strict aerobic bacteria (Boström et al. 1988) and decrease in bacterial abundance (e.g. Ricciardi-Rigault et al. 2000), whether and how this environmental change affects the capacity of pelagic bacteria that remain or have survived to incorporate organic carbon is poorly known. Some studies have reported bacterial production rates higher in anoxic hypolimnions than in overlying oxic waters (e.g. Bettarel et al. 2004; Cole and Pace 1995). However, it is often difficult to attribute such differences to O_2 loss per se, given that O_2-deficient deep waters are often richer in organic carbon and inorganic nutrients (Gast and Gocke 1988; Wu 2002; Naqvi et al. 2010). An approach that can help overcome these problems and explore this issue is to submit pelagic communities that live in oxic waters to O_2 depletion and then examine their responses. However, few studies have performed such experiments (see Alonso and Pernthaler 2005).

Mineralization of organic matter (the other key bacterial function of interest in this study) fuels ambient waters with inorganic nutrients such as ammonium (NH_4^+) and phosphates (P) which can therefore be used by other organisms, including primary producers. This process occurs in both oxic and O_2-deficient water columns. In oxic water columns this process has well been studied and it is known that bacteria respire organic matter (OM) using oxygen as electron acceptor (aerobic respiration) and release P. In O_2-deficient water columns in contrast, denitrification, and in some cases sulphate reduction, are reported to be the dominant metabolic pathways in OM decomposition (Jorgensen 1982; Lam and Kuypers 2011). However, how OM mineralization by pelagic microorganisms is related to P availability when O_2 declines has seldom been examined (Benitez-Nelson et al. 2004). Such information is of interest for at least two reasons. First, the O_2 content of many aquatic ecosystems is declining, and OM mineralization by pelagic microorganisms may be a major source of the P sustaining primary production during thermal stratification or when sediments have no or weak influence on upper water columns in terms of nutrient supply. Second, a study from engineered systems has shown that some denitrifying bacteria can remove P from the water and accumulate it in cells under anaerobic conditions (Barak and van Rijn 2000). Occurrence of such a process in water columns, when O_2 declines, may affect P availability.

The aim of this study was to examine changes in carbon incorporation and cell size of heterotrophic bacteria within mixed plankton communities (see methods), as well as linkages between carbon mineralization by pelagic microorganisms and phosphorus availability in waters, following O_2 loss. We used a combination of methods including flow cytometry, fatty acid analyses and fatty acid stable isotope probing.

Material and Methods

Sample Collection and Analyses

Experimental samples were collected on October 29, 2007, in surface waters of the sampling station Temse (Belgium) in the Scheldt Estuary, using 20 L carboys, and then transferred in a 200 L carboy. This estuary receives large amounts of nutrients and has high turbidity. The sampled station (Temse) is characterized by high bacterial production, with dissolved organic carbon and particulate organic carbon up to 400–500 μM, and chlorophyll *a* concentrations>20 μg L^{-1} (Boschker et al. 2005). This station has been reported to frequently experience O_2 depletion. O_2 concentration is often below 1 mg L^{-1} (Billen et al. 2005) and O_2 saturation can be below 10 % (Boschker et al. 2005). The salinity at this station varies seasonally between 0.5 and 3 PSU (Van den Meersche et al. 2009). Collected samples were transported immediately to the laboratory at the Yerseke branch of the Netherlands Institute for Sea Research. At the laboratory, sub-samples were taken prior to experiments for analyses of initial conditions (day 0). The remaining water was split into two sub sets. One was kept without any manipulation, whereas the other was purged to decrease O_2 concentration, using pure gaseous nitrogen (N_2). The initial O_2 saturation, measured using a Presens optode, was 61 % (~170 μM). The purging was performed gently to avoid strong manipulation of the communities. It was stopped when O_2 saturation reached 18 % (~ 69 μM) in order to avoid total elimination of O_2 and to allow gradual shift, if any, in microbial processes during the study. The whole plankton community was used for two main reasons. First, the study aimed to trace carbon incorporation into bacteria and carbon degradation. Second, in ecosystems that experience O_2 deficiency, the whole plankton communities are present prior to the onset of O_2-deficient conditions and not all potential grazers of bacteria are killed following oxygen O_2 loss. Separation of bacteria from the other communities would have removed grazers, therefore eliminating interactions that may occur in nature following O_2 loss. Moreover, as such a separation is known to remove large-size bacteria (a situation more marked with samples from nutrient-rich ecosystems), it may eliminate a significant number of bacteria that might actually be important players in carbon processing. Experiments were carried out in four 25 L carboys, two (replicates) for samples without manipulation (oxic samples=controls) and the two others for the purged samples (treatments, thereafter called "low-O_2 samples"). ^{13}C-glucose was added (at~90 μM carbon) to each of the four carboys in order to examine changes in carbon incorporation (and potential flux within microbial communities), as well as mineralisation, following O_2 loss. ^{13}C-glucose has previously been used in this ecosystem to study carbon flows in the pelagic microbial food web (e.g. Van den Meersche et al. 2011). Other studies have used approaches similar to ours (i.e. oxic samples as controls versus samples flushed with gaseous nitrogen to remove O_2 as treatments, all with additions of labelled glucose, and no separation of bacteria) to examine carbon incorporation by bacterial communities when O_2 declined (e.g. Alonso and Pernthaler 2005; Schellenberger et al. 2010). Glucose is a labile sugar that is taken up by most of the known heterotrophic bacteria. The added glucose in our study corresponded to ~19 % of in situ carbon amount at the sampled station (Boschker et al. 2005; DOC concentration in our initial sample was 463 μM). High amounts of DOC can be consumed rapidly during incubations, following glucose amendments [e.g. 217 μM consumed within 48 h from the initial day, after addition of 100 μM glucose in the study of Karl et al. (2008). they added 25 μM to 1 mM glucose]. After amendments, carboys were put in black plastic bags (for darkness) to avoid photosynthesis and minimize influence of phytoplankton exudates on incorporation of the labelled carbon by bacteria. Darkness also helped avoid O_2 that may be produced by photosynthesis. It should be noted that for ecosystems experiencing O_2-deficiency, the latter generally occurs out of the euphotic zone. The black bags containing the carboys were then placed in an anaerobic chamber (for low-O_2 samples) or outside and below the anaerobic chamber (for controls). In the chamber, hydrogen (gas) was between 2 and 4 % to ensure anaerobic conditions. Samples in the chamber were opened only for few minutes for sub-sampling, which minimized exposure to hydrogen and the potential impacts that it might have on microbial communities. Samples were incubated for 16 days, and were frequently gently mixed to avoid settlement of particles. Sub-samples were taken on days 1, 2, 4, 8 and 16 for analyses, in the anaerobic chamber for low-O_2 samples (to avoid contamination by O_2) and in any other place in the laboratory for controls (to ensure their continuous aeration). There was no difference in temperature between inside and outside the anaerobic chamber.

Nutrients (ammonium, nitrite, nitrate and dissolved inorganic phosphorus=DIP) were analyzed by standard methods on a SKALAR segmented flow autoanalyzer. Carbon cycling was examined by analyzing changes in the concentration and isotopic signature of dissolved inorganic carbon (DIC) and $\delta^{13}C_{DIC}$. Samples for DIC and $\delta^{13}C_{DIC}$ were preserved with mercury chloride. DIC and $\delta^{13}C_{DIC}$ were determined by headspace analysis after acidification of samples, using an elemental analyzer-isotopic ratio mass spectrometer (Moodley et al. 2000). The labelled DIC expressed as $\Delta\delta^{13}C_{DIC}$ ($\delta^{13}C_{sample}-\delta^{13}C_{initial}$) was used to evaluate carbon mineralization.

The microbial variables analyzed include microbial lipid concentrations, carbon isotopic composition of specific microbial lipids, abundance, relative cell size and nucleic acid content of heterotrophic prokaryotic cells (see next paragraph).

Microbial lipids were collected by filtration of samples (1 L) through pre-combusted GF/F filters, and extracted with a modified Bligh and Dyer extraction (Boschker et al. 1999). Details on the analytical procedure can be found in Middelburg et al. (2000) and Boschker et al. (2005). Briefly, the lipid extract was fractionated on silicic acid (60, Merck) into different polarity classes by sequential elution with chloroform, acetone and methanol. The methanol fraction containing the phospholipid ester-linked fatty acids (PLFA) was derivatized using mild alkaline methanolysis to yield fatty acid methyl ester (FAME). Concentrations were determined using gas chromatography-flame ionization detection. Identification of FAME was based on comparison of retention time with reference material on two analytical columns with different polarities and by gas chromatography-mass spectrometry. Internal standards (12:0 and19:0) were used. Carbon isotopic composition of individual FAME was determined using a gas chromatography combustion-interface isotope ratio mass spectrometer. Stable carbon isotope ratio for individual PLFA was calculated from FAME data by correcting for the one atom of carbon in the methyl group that was added during derivatization. Stable carbon isotope data are expressed in the delta notation relative to Vienna Pee Dee Belemnite.

Heterotrophic prokaryote abundance was determined from formaldehyde-preserved samples (2 %*v*/*v*) using the nucleic acid dye SYBR green II, a flow cytometer (FacsCalibur, Becton-Dickinson) equipped with a laser emitting at 488 nm and 0.92 μm fluorescent beads as an internal standard (Tadonléké et al. 2005). The term “heterotrophic prokaryotes” was used and will be used thereafter (where applicable) because flow cytometer likely analyzed both bacteria and archaea. Effects of O_2 decline on these prokaryotes, at a cell level, were investigated by examining changes in side scatter (SSC) and green fluorescence (FL1) recorded for individual cells during cytometry analyses, and normalized to beads. SSC and FL1 are known to be related to cellular characteristics of individual cells (e.g. Steen et al. 1981; Troussellier et al. 1999). they were used as indexes of relative size and nucleic acid content of cells, respectively (Gasol and del Giorgio 2000; Troussellier et al. 1999; Tadonléké et al. 2005). SSC of heterotrophic prokaryotes obtained using the dye SYBR green II has been found to be strongly related, positively, to their cell volume (Tadonléké et al. 2005).

Statistical Analyses

The Wilcoxon two-sample test was used to test for difference between the two sets of data (i.e. control samples and low-O_2 samples) for each of the studied variables (Sokal and Rohlf 1995). Relationships between variables were examined using simple regressions. Comparison of regression lines from controls samples with those from low-O_2 samples (e. g., for bacterial growth rates) was done using an analysis of covariance (ANCOVA) (Sokal and Rohlf 1995). All the statistics were performed using the software JMP 7.0 (SAS).

Results

Chemical Variables

Concentrations of nitrate, nitrite and ammonium (in terms of nitrogen) in the initial sample were 364, 5.6 and ~17 μM, respectively. Nitrite and nitrate amounts changed much less in controls than in low-O_2 samples, ranging from 0.28 to 5.67 μmol L^{-1}, and from 341 and 385 μM, respectively (Fig. 1a, b). Overall, there was an increase of ~18 μM in nitrate and a decrease of 4.6 μM in nitrite in controls. In low-O_2 samples in contrast, these nutrients varied strongly and showed opposite temporal patterns. Nitrite increased from ~5.6 μM at day 0 to ~313 μM at the end of the study, while nitrate decreased from 364 μM to less than 11 μM (Fig. 1a, b). Overall, nitrite concentration was significantly higher in low-O_2 samples than in controls, while the reverse was found for nitrate concentration (Table 1).

NH_4^+ concentration decreased by more than 50 % (from ~17 to ~7 μM) in both controls and low-O_2 samples after only 2 days of incubation (Fig. 1c). From day 2, concentrations varied little in low-O_2 samples (~ 7 to 4.7 μM), while in controls, they further decreased strongly (−74 %), reaching 1.8 μM at the end of the study. However, low-O_2 samples and controls were not significantly different in NH_4^+ amounts (Table 1).

Initial DIP concentrations were high (2.8 μM), characteristic of nutrient-rich aquatic systems. Concentrations strongly decreased only after one day of incubation, and remarkably, the decline was more than two times higher in low-O_2 samples (−70 %) than in controls (−32 %) (Fig. 1d). After day 1, DIP amounts continuously rose in controls, exceeding the initial value at the end of incubation, whereas in low-O_2 samples, they remained constant at ~1.5 μM (Fig. 1d). On average, DIP concentration was significantly lower in low-O_2 samples than in controls (Table 1).

Initial DIC concentration was ~3035 μM CO_2. Concentrations decreased in all samples during the experiments, and were significantly lower in low-O_2 samples (Fig. 2a, Table 1).

The $\delta^{13}C_{DIC}$ of initial samples (−10.2‰) was within the range of values generally reported for natural abundance isotope ratios for heterotrophic rivers. Shortly after addition of ^{13}C-glucose, DIC became enriched in ^{13}C reflecting degradation of glucose. The labelled DIC expressed as $\Delta\delta^{13}C_{DIC}$ ($\delta^{13}C_{sample}-\delta^{13}C_{initial}$) however showed opposite patterns in controls versus low-O_2 samples (Fig. 2b). In controls, $\Delta\delta^{13}C_{DIC}$ reached ~540 ‰ on day 1, and continued to increase until the end of the study. In low-O_2 samples in contrast, $\Delta\delta^{13}C_{DIC}$ increases were steeper than in controls during

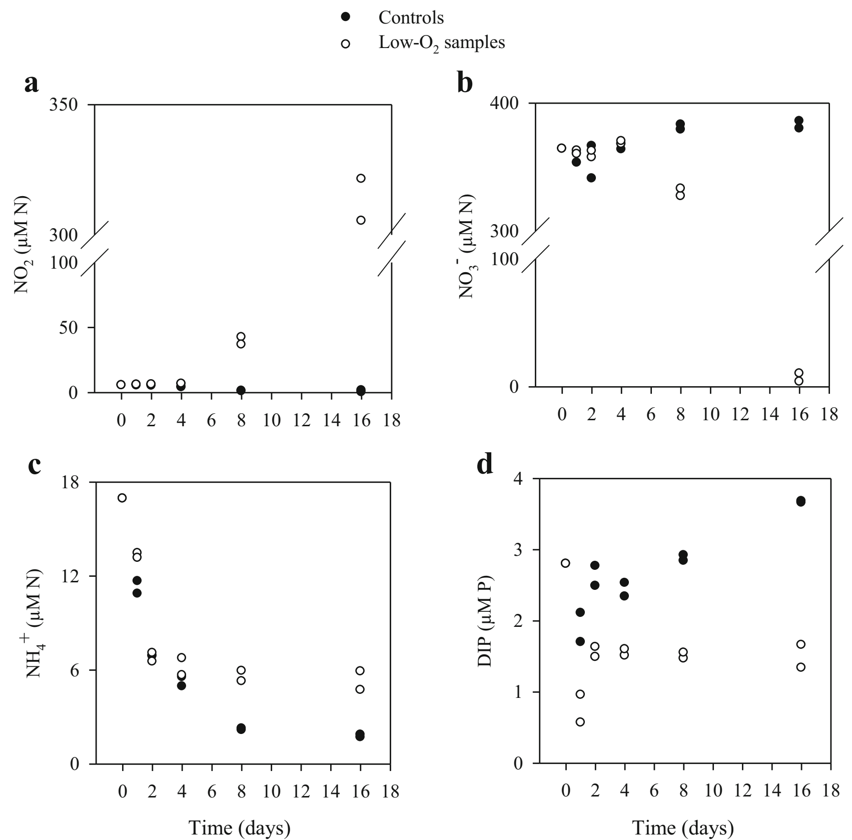

Fig. 1 Temporal changes in nitrite (**a**), nitrate (**b**), ammonium (**c**) and dissolved inorganic phosphorus (**d**) in controls (*filled circles*) and low-O_2 samples (*opened circles*) during the experiments

the first four days, and values strongly decreased thereafter. The mean value for these first 4 days of incubation (+629.1‰ ±7.7‰) was significantly higher than that in controls (+553.8 ‰±10.4‰) (p=0.009), a pattern that persisted when comparison dealt with all the five sampling time points (Table 1).

Remarkably, $\Delta\delta^{13}C_{DIC}$ was positively related to DIP concentration in controls, explaining 87 % of its variability, whereas in low-O_2 samples these two variables were not related at all (Fig. 2c).

Microbial Variables

The abundances of heterotrophic prokaryotes increased from day 0 to day 2 in both controls and low-O_2 samples (Fig. 3a). The estimated net growth rates during this period (i.e. using data from day 0, day1 and day 2), assuming exponential growth, showed no significant differences between low-O_2 samples (slope±standard error=0.22±0.01 day^{-1}, N=5) and controls (0.18±0.05 day^{-1}, N=5) (ANCOVA, p>0.4 for both

Table 1 Wilcoxon test comparing controls (N=10) with low-O_2 samples (N=10) for abiotic and microbial variables

Variables	Mean±SD (control)	Mean±SD (low-O_2 samples)	Probability
NO_2^- (µM N)	3.5±2.1	74±126	< 0.001
NO_3^- (µM N)	368±14.3	285±147	0.03
NH_4^+ (µM N)	5.5±3.7	7.5±3.2	0.19
DIP (µM P)	2.7±0.6	1.4±0.4	< 0.001
DIC (µM CO_2)	2774±97	2522±159	< 0.01
$\Delta\delta^{13}C_{DIC}$ (o/oo)	579±29	621±26	< 0.01
Green fluorescence of prokaryotes (FL1)	0.13±0.02	0.16±0.02	< 0.01
Side scatter of prokaryotes (SSC)	0.037±0.002	0.042±0.003	< 0.01
PLFA 16:1ω 7c (µg L^{-1})	4.6±3	8.8±4.1	0.03

$\Delta\delta^{13}C_{DIC}$ is the index of mineralization; *FL1* and SSC are variables without units. *FL1* is the indicator of heterotrophic procaryotes nucleic acid content, *SSC* is the index of heterotrophic procaryotes cell size. *SD* stands for standard deviation

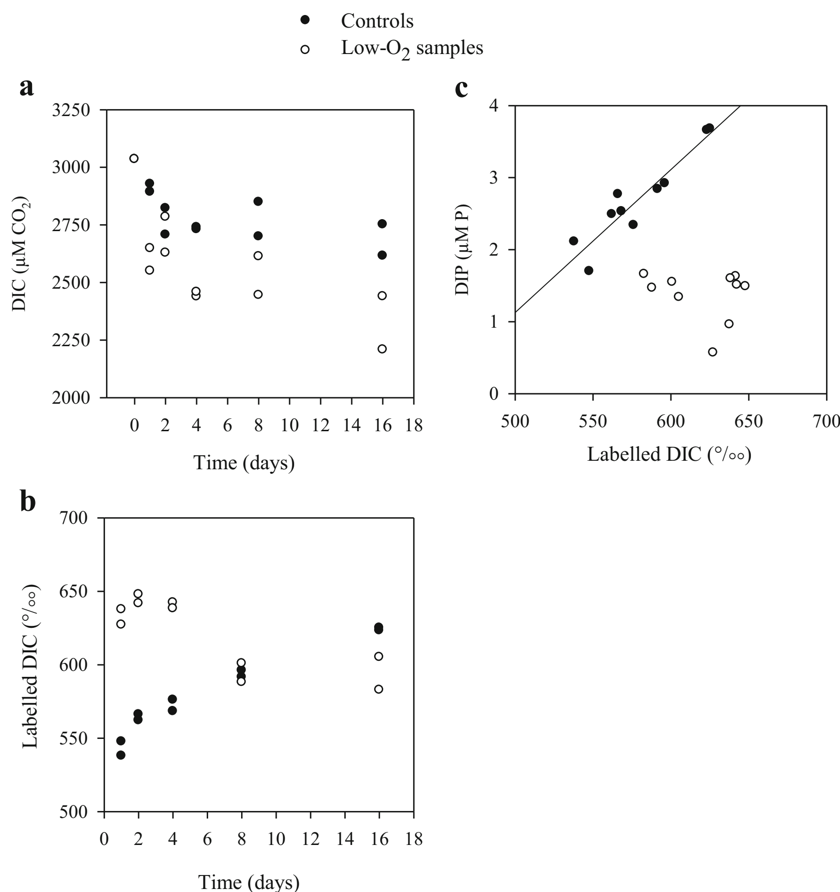

Fig. 2 Temporal changes in dissolved inorganic carbon (DIC) (**a**) and the labelled DIC ($\Delta\delta^{13}C_{DIC}$ [$\delta^{13}C_{sample}-\delta^{13}C_{initial}$]) used as indicator of carbon mineralization (**b**) in controls (*filled circles*) and low-O_2 samples (*opened circles*). (**c**) Relationships between dissolved inorganic phosphorus (DIP) concentration and the labelled DIC (indicator of carbon mineralization). The symbols are the same as for panels "A" and "B". For controls samples, i.e. the significant relationship, the equation is DIP= $0.02\times[\Delta\delta^{13}C_{DIC}]-8.8$, $r^2=0.87$, $p<0.001$, $N=10$

slopes and intercepts). Although abundances strongly decreased in all samples between day 2 and day 4, the decrease was more marked in low-O_2 samples (−67 versus −48 % in controls), which thus had lower abundances. After day 4, abundances increased in low-O_2 samples until the end of the study, whereas in controls they increased until day 8, and declined thereafter (Fig. 3a).

FL1 values, i.e. the index of nucleic acid content of heterotrophic prokaryote cells, were significantly higher for low-O_2 samples than for controls (Fig 3b, Table 1), indicating that these cells had significantly higher nucleic acid content in low-O_2 samples. Heterotrophic prokaryotes also had significantly higher side scatter (SSC, index of relative cell size) in low-O_2 samples than in controls (Fig. 3c, Table 1). Overall SSC and FL1 of these cells were positively related coupled ($r^2=0.89$ for controls and 0.73 for low-O_2 samples, $p<0.0001$), which indicated that heterotrophic prokaryotes with higher nucleic acid content also had larger cell size.

Although the nucleic acid content of heterotrophic prokaryotes and DIP concentration were not statistically related in low-O_2 samples or in controls ($p>0.05$), pooled data (low-O_2 samples and controls) showed that DIP concentration significantly decreased as cell nucleic acid content increased (DIP$=-14.7\times$FL1$+4.1$; $r^2=0.25$; $p=0.024$, $N=20$). Remarkably, the nucleic acid content of heterotrophic prokaryote cells in low-O_2 samples was also positively related to the difference in DIP concentration between controls and low-O_2 samples, explaining 62 % of its variance (Fig. 3d).

Thirteen PLFAs were detected in our samples, but one of them (22:4ω6) could not be interpreted or linked to an organism because of lack of published information on it. Therefore, although presented in the supplementary figure (Fig. S1), this PLFA will not be emphasized. The interesting feature in the dynamics of the concentrations of the detected PLFAs known to be produced by bacteria (all but 20:5ω3, 20:4ω6 and 22:4ω6) in low-O_2 samples compared to controls was that a temporal shift occurred around day 2 (Fig. 4). Concentrations of these PLFAs were slightly higher in the controls on day 1 (Fig. 4a), but after day 2, and especially on days 4 and 8, they tended to be higher in low-O_2 samples (Fig. 4c, d). This shift was more marked and obvious for some of these PLFAs (e.g. 16:0 and 16:1ω7c). For 16:1ω7c, for instance, concentrations increased in low-O_2 samples from day 4, whereas in the controls they decreased (Fig. 5a). This PLFA was dominant in low-O_2 samples representing, after day 2, 40 to 61 % (mean ±standard deviation=48±11 %) of the biomass of the ten PLFAs known to be produced by bacteria (see Fig. 4). The biomass of this PLFA (16:1ω7c) in low-O_2 samples was significantly higher than in controls (Table 1), and also explained a high proportion (72 %, positive relationship) of the variance

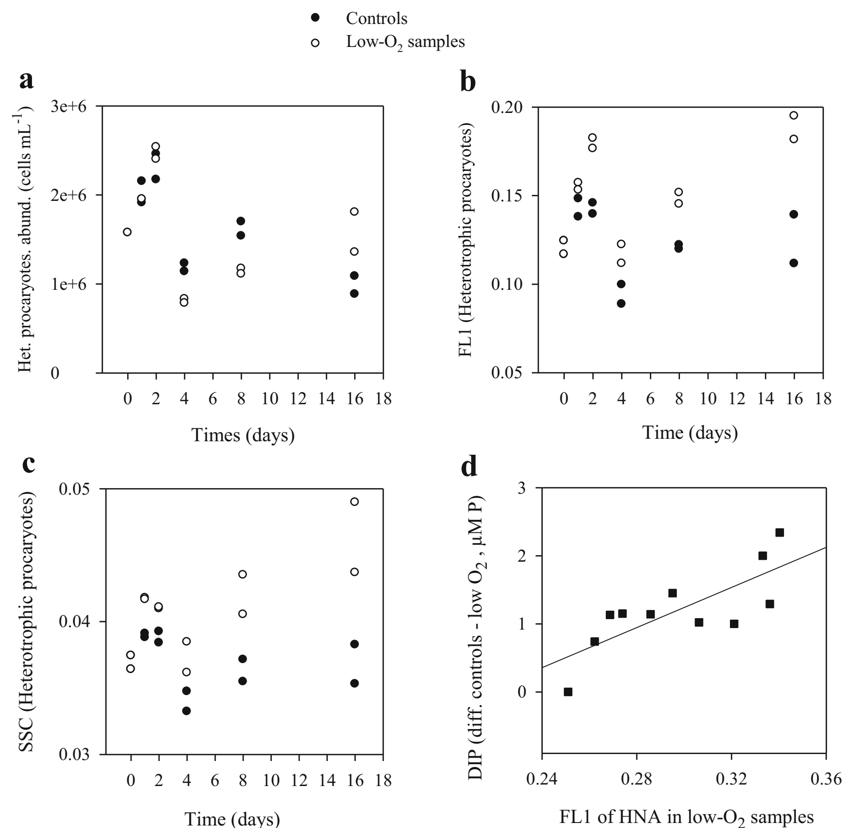

Fig. 3 Temporal changes in the abundance (**a**), the green fluorescence (FL1), i.e. the nucleic acid content (**b**), and side scatter (SSC) (**c**) of heterotrophic prokaryotes obtained from cytometry analyses in controls (*filled circles*) and low-O_2 samples (*opened circles*) during the experiments. Relationships of the difference in dissolved inorganic phosphorus (DIP) concentration between controls and low-O_2 samples with the nucleic acid content of heterotrophic prokaryotes in low-O_2 samples (**d**). For panel "E", HNA stands for heterotrophic prokaryotes with high nucleic acid content, and the equation is DIP (controls minus low-O_2 samples)=14.5×[FL1 of HNA]−3.1; r^2=0.62, p=0.004, N=10. The analyses were also performed with the nucleic acid content of the whole heterotrophic prokaryotic community (whole HP FL1). The relationship was also significant (r^2=0.51, p=0.01, N=10), and the equation is DIP (controls–low-O_2 samples)=15.7×[FL1 whole HP]−1.2. Note that FL1 and SSC are unitless variables

of the difference in phosphate concentration between controls and low-O_2 samples (difference DIP concentration= 0.1×[biomass16:1ω7c]+0.45, p=0.0018, N=10).

Temporal patterns in the ^{13}C-enrichment of PLFAs (i.e. incorporation of the labelled compound by PLFAs) were similar between control and low-O_2 samples (Fig. 5b–f and Fig. S1). These patterns showed that PLFAs could be divided into three groups depending on the magnitude of ^{13}C-enrichment on day 1 and on the date when the enrichment peaked. Nine of the PLFAs (group 1), were strongly ^{13}C-enriched one day after the addition of ^{13}C-glucose. These included i15:0, ai15:0, 16:1ω7c, 18:1ω7c, 16:1ω5c+t, i14:0, 14:0, 15:0 and 16:0. δ^{13}C values of these PLFAs strongly increased, peaked on day 2, and thereafter decreased over time (Fig. 5b–d and Fig. S1). PLFAs of group 2 (20:4ω6, 20:5ω3 and 22:4ω6) showed time lags in the early ^{13}C-enrichment, and in the date of enrichment peaks, compared to PLFAs of group 1. Indeed δ^{13}C values for PLFAs of groups 2 were comparatively very low on day 1 and their peaks were recorded rather on day 4, i.e. 2 days after enrichment peaks of group 1 PLFAs (Fig. 5b–f and Fig. S1). Average δ^{13}C values for replicates for these PLFAs on day 1 were +314‰ for controls and +40‰ for low-O_2 samples for the PLFA 20:5ω3, +1220‰ for controls and +409‰ for low-O_2 samples for the PLFA 20:4ω6 and + 1243‰ for controls and +203‰ for low-O_2 samples for the PLFA 22:4ω6. Note that, as stated earlier, the PLFA 22:4ω6 will not be further discussed. The only PLFA of group 3 (18:0) showed characteristics of both group 1 PLFAs (strong ^{13}C-enrichment on day 1) and group 2 PLFAs (peaks on day 4), as well as the lowest magnitude of ^{13}C-enrichment among all PLFAs (Fig. S1 D). Regarding the level of enrichment, the common feature of all these curves was that on day 1, PLFAs were consistently less ^{13}C-enriched in low-O_2 samples than in controls (Figs 5b–f and Fig. S1). For the other sampling days, patterns in ^{13}C-enrichment in controls versus low-O_2 samples were not consistent for a given PLFA, and varied between PLFAs. The fact that δ^{13}C of PLFAs exceeded $\delta^{13}C_{DIC}$ (Figs. 2b and 5 b–f) indicated that incorporation of the labelled carbon by PLFAs was due to non-photosynthetic processes, as it is expected in darkness.

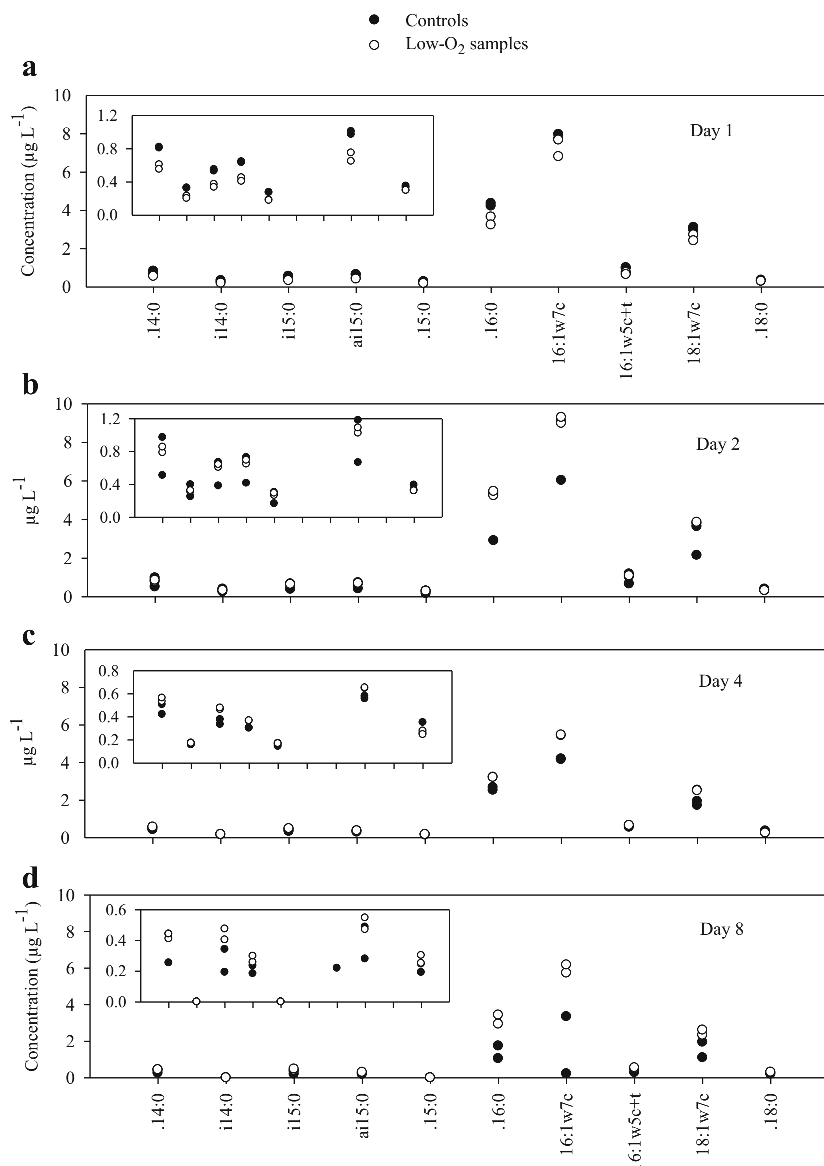

Fig. 4 Concentrations of the 10 PLFAs known to be related to bacteria on day 1 (**a**), day 2 (**b**), day 4 (**c**) and day 8(**d**) in controls (*filled circles*) and low-O_2 samples (*opened circles*) during the experiments. The insets allow a good visibility for concentrations below 1.2 µg L^{-1}. For 16:7w5c+t, "c" stands for "cis" and "t" stands for "trans". These compounds are presented as one compound because they could not be separated under the chromatographic conditions used. The isomere "cis" of this PLFA (16:1ω5c) is produced by both fungi and bacteria (Zak et al. 1996; Madan et al. 2002). The PLFA 16:1ω7c is produced by both Gram-negative bacteria and algae (Ratledge and Wilkinson 1988). The PLFAs i14:0, i15:0, ai15:0 are considered markers of Gram-positive bacteria and the PLFA 18:1w7c is considered marker of Gram-negative bacteria (Ratledge and Wilkinson 1988; Tunlid and White 1992; McKindley et al. 2005). The PLFAs 20:4ω6 is produced by protozoa (Erwin 1973; McKindley et al. 2005). The PLFA 20:5ω3 is reported to be produced by dinoflagellates (Parrish et al. 1994; Volkman et al. 1998). The PLFAs 14:0, 16:0, 18:0 are general PLFAs produced by many organisms, including bacteria. The PLFA 15:0 is reported to belong to many bacterial groups, i.e. it is not specific to a bacterial group

Discussion

O_2 decline is a source of stress for many living organisms. It is known to negatively affect fitness and reduce activities of large eukaryotic aquatic organisms (Wu 2002). Our results showed for the first time that the nucleic acid content, the size of heterotrophic prokaryotic cells, and the biomass of these communities became significantly higher, while the P amount was significantly lower in samples that had experienced O_2 loss than in controls, suggesting that O_2 loss in nutrient-rich aquatic systems may have profound impacts on heterotrophic prokaryotes at both community (biomass formation) and cell levels, as well as on P availability. Our analysis suggests that these changes were due to a combination of factors, including grazing, enhanced microbial activity and, possibly, retention of P by cells.

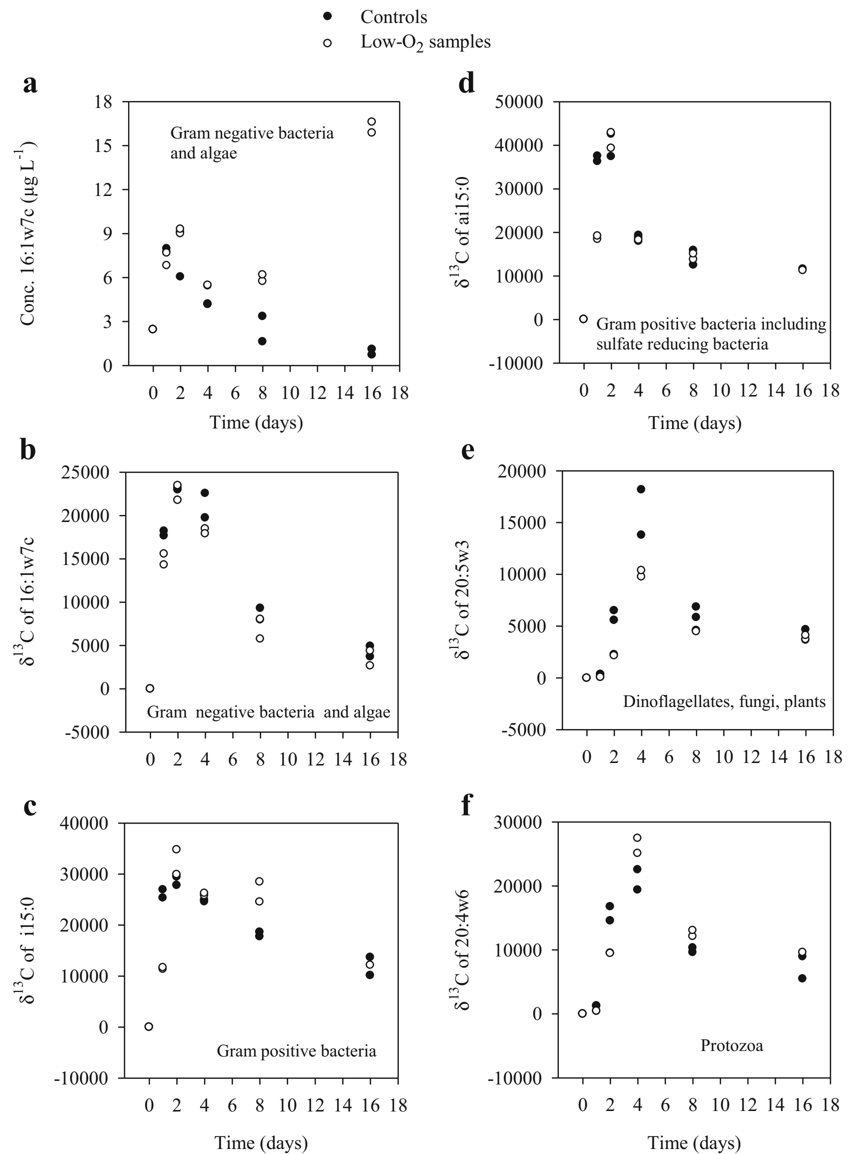

Fig. 5 Temporal changes in the concentration of the dominant PLFA 16:1ω7c (**a**), the ^{13}C isotopic signature of the PLFAs 16:1w7c (**b**), i15:0 (**c**), ai15:0 (**d**), 20:5ω3 (**e**) and 20:4ω6 (F) in controls (*filled circles*) and low-O_2 samples (*opened circles*) during the experiments. The origin of each PLFA is given in the corresponding panel. The PLFA 16:1ω7c is produced by both Gram-negative bacteria and algae (Ratledge and Wilkinson 1988). but since the experiments were conducted in the dark, incorporation of carbon by this PLFA and changes in its concentration were considered to be due mainly to Gram-negative bacteria and non-photosynthetic processes (see the "Results" section of the text)

It is generally assumed that lower grazing in O_2-deficient conditions results in increase in heterotrophic prokaryotes cell size (Cole et al. 1993). However, grazing studies in such conditions have not examined the cell size of prokaryotes, and some authors have reported grazing rates on them similar to or higher than those in oxic conditions (e.g. Detmer et al. 1993). In both controls and low-O_2 samples in the present study, two PLFAs known to be produced by protists (20:4ω6 and 20:5ω3 produced by protozoa and dinoflagellates, respectively [Erwin 1973; McKindley et al. 2005; Parrish et al. 1994; Volkman et al. 1998]), showed time lags in their ^{13}C-enrichments, compared to those of PLFAs considered markers of bacteria (i14:0, i15:0, ai15:0 and 18:1w7c, Ratledge and Wilkinson 1988; Tunlid and White 1992; McKindley et al. 2005) (Fig. 5c–f). Time lags are commonly found in prey-predator relationships (Wangersky and Cunningham 1957). Because the time lags between peaks of ^{13}C-enrichments (protists peaked 2 days after prokaryotes) coincided with the strong decline in prokaryotes abundance from day 2 to day 4 (Fig 3a), these patterns were considered indicative of grazing. These data were therefore used to estimate the fraction of carbon in the grazer that is derived from these prokaryotes, in order to examine if grazing intensity could help explain the higher index of cell size of prokaryotes in low-O_2 samples. A significantly higher fraction would indicate stronger grazing pressure.

This fraction, which was expressed as the ratio $\Delta\delta^{13}C_{grazer}/\Delta\delta^{13}C_{bacteria}$, where $\Delta\delta^{13}C$ (‰)=$\delta^{13}C_{sample}$–$\delta^{13}C_{background}$ (van Oevelen et al. 2006). at least, doubled from day 2 to day 4, supporting the idea that the strong decline in prokaryotic abundance between these sampling time points was, at least in part, due to grazing (Fig. 3a, Table 2). Interestingly, this ratio (called "grazing index" below) was also significantly higher in low-O_2 samples than in controls, but only for the PLFA 20:4ω6 (grazer), and when the PLFA 16:1ω7c (prey)

Table 2 Mean values of the fraction (%) of carbon of grazer that is derived from bacteria in the two replicates (R1 and R2) during the experiments.

Potential grazer	Bacteria	Sample	Day 1	Day 2	Day 4	Day 8	Day 16*	*P* (2–4)	*P* (4–16)
20:4ω 6 (Protozoa)	i14, i15, ai15, 18:1ω7c	Controls	1.1 (0.1)	11.7 (3)	27.5 (4.6)	24.7 (0.6)	23		
		Low-O_2	2.6 (0.2)	12.27 (3)	29 (4.5)	19.7 (4.4)	25	0.67	0.83
	i14, i15, ai15, 18:1ω7c, 16:1ω7c	Controls	0.9 (0)	10.0 (2.4)	21.5 (2.9)	20.3 (0.4)	21		
		Low-O_2 **	0.7 (0)	6.3 (0.7)	29.1 (2.6)	21.9 (2.2)	24	0.99	0.037**
20:5ω 3 (Dinoflagellates)	i14, i15, ai15, 18:1ω7c	Controls	0.3 (0)	4.6 (1.2)	21 (6.1)	15.7 (1)	9		
		Low-O_2	0.16 (0)	1.7 (0.3)	13.9 (0.8)	9 (1)	10	0.31	0.31
	i14, i15, ai15, 18:1ω7c, 16:1ω7c	Controls	0.2 (0.1)	3.9 (1)	16.4 (4.2)	13 (1.4)	8		
		Low-O_2	0.12 (0)	1.5 (0.2)	11.2 (0.7)	7.9 (1.6)	9	0.31	0.094

P (2–4) and *P* (4–16) are the probabilities for the comparison (Wilcoxon test) of controls and Low-O_2 samples using data from days 2 and 4, and from day 4 to day 16, respectively, assuming that the steady-state between grazers and bacteria occurred between day 2 and day 4. Statistics were performed using data from individual replicates. Values in the parantheses are absolute values of differences between the two replicates. The determination of the steady-state time/period (point of equilibrium between grazers and prey) required for this calculation (van oevelen et al. 2006) was based on time lags of ^{13}C-enrichements of grazers versus prey (day 4 versus day 2) and the graphical superimposition of their curves as recommended by Noy-Meir (1975) (see references in the text). The PLFA i14, i15, ai15 and 18:1w7c are markers of bacteria. The PLFA 16:1ω7c was included in a different scenario because it is produced by both Gram-bacteria and algae. The compound 16:1ω5c+t was not included in this calculation because the two isomers "cis" and "trans" could not be separated, and the isomere "cis" is produced by both fungi and bacteria. *δ13C data for some bacteria (i14, i15, ai15) were available only for one replicate (R1). ** Low-O_2 samples had significantly higher mean than controls ($P<0.05$)

was included in the calculation (Table 2). These findings supported the view that the grazing pressure from protists on some prokaryotes was stronger in our low-O_2 samples than in controls. O_2 loss might have resulted in the death of metazoan grazers and thus in release of grazing pressure on prey that they are known to consume, i.e. larger-sized heterotrophic prokaryotes and protists, therefore leading to enhanced grazing pressure from some protists on some heterotrophic prokaryotes, with shifts in cell size within these communities. Peaks of protist abundance or clearance rates have been reported for example at the oxic-anoxic interfaces or in hypoxic zones of the water column in other studies (Park and Cho 2002; Edgcomb et al. 2011). Protist grazers are known to preferentially crop on medium-sized (0.4–2.4 μm) heterotrophic prokaryotic cells, and grazing-induced shifts in cell size directed towards larger cells have been observed within these communities, especially when the grazing pressure is strong (Hahn and Höfle 2001). Ciliates can be significant grazers of these prokaryotes. A study on changes in protist communities during O_2 loss in Saanich inlet (Canada) has shown a shift towards strong dominance of ciliates (66 % of sequences) at the onset of seasonal anoxia (Orsi et al. 2012). To our knowledge our study is the first to explore protist grazing within natural communities in low-O_2 waters using stable isotope probing, and results suggest that grazing by protists on some heterotrophic prokaryotes may increase at the onset of O_2 deficiency in nutrient-rich aquatic systems. If this is true, this would mean that, besides changes in the electron acceptors type (e.g. Crump et al. 2007). changes in protist grazing pressure may play a significant role in shifts observed in the composition of prokaryotes (and the resulting nutrient cycling) during O_2 loss in aquatic ecosystems.

Our data showed that internal and morphological characteristics of cells were affected following O_2 loss, pointing to enhanced microbial activity, and suggesting that this has contributed to increase the cell size of prokaryotes in low-O_2 samples, compared to controls. Selective grazing cannot fully explain this difference because the nucleic acid content of prokaryotic cells (FL1) and the index of cell size (SSC) became significantly higher in low-O_2 samples, and increased over time, while initially they were low and similar in controls and low-O_2 samples (Fig. 3b). Flow cytometry measurements (e.g. SSC and FL1) capture changes that occur at individual cell level. Increases in FL1 and SSC in low-O_2 samples were already visible at early incubation (day 1–day 2), although controls and low-O_2 samples did not differ in abundance-based prokaryotic net growth rates (see results), or in the grazing index, which was still relatively low (Fig. 3a-c, Table 2). It is known that, in addition to size per se, cellular characteristic such as internal granulosity and cell membrane thickness may be related to SSC (Steen et al. 1981; Troussellier et al. 1999). It has also been shown that heterotrophic prokaryotes cells with high nucleic acid content tend to have larger size (Gasol et al. 1999; Tadonléké et al. 2005). a trend that was supported in this study by the strong positive relationship between FL1 and SSC. Besides this, the biomass of the dominant PLFA 16:1ω7c increased over time and became significantly in low-O_2 samples (Fig. 5a). PLFAs are essential components of microbial cell membrane, known to indicate viable, active recently-formed biomass (Harvey et al. 1986).

Except for day 1 of the experiment, controls and low-O_2 samples were generally similar in ^{13}C-enrichments of PLFA (i.e. incorporation of labelled carbon), supporting occurrence of high microbial activity in low-O_2 samples and providing

evidence that heterotrophic prokaryotes cells continued to highly incorporate carbon despite O_2 loss. Such results are consistent with field studies that have found similar heterotrophic prokaryote production in O_2-deficient waters and overlying oxic waters at the onset of anoxia (Crump et al. 2007). A remarkable result of this stable isotope probing was, however, that ^{13}C-enrichments of all PLFAs were consistently lower in low-O_2 samples than in controls on day 1, a pattern more marked for bacterial PLFAs (Fig. 5b–d and Fig. S1). Similar results have been found in studies of a facultative bacterial strain comparing aerobic and anaerobic conditions, and attributed to changes in the pathways of fatty acid synthesis (changes in the primary route of carbon assimilation from aerobic to anaerobic conditions), and increase in the isotopic fractionation in the absence of O_2 (Scott and Nelson 1994; Teece et al. 1999). This similarity suggests that changes in the route of carbon incorporation into cells occurred in our low-O_2 samples shortly after incubations, and supported the view that these samples (which initially could be considered hypoxic) became O_2-depleted (NO_3^- was depleted and NO_2^- produced), in contrast to controls where NH_4^+ and NO_2^- decreased while NO_3^- increased indicating that they had significant amount of O_2 throughout the study (Fig. 1a–c). Due to technical problems with the O_2 optode, we could not know however whether these low-O_2 samples had reached anoxia, but it is important to note that the boundaries between hypoxic versus suboxic and suboxic versus anoxic conditions based on O_2 concentrations vary and are considered arbitrary (Yakushev and Newton 2013). NO_3^--based respiration of organic matter generally occurs when O_2 is lacking (Fenchel and Blackburn 1979). Glucose, used here, undergoes fermentation in such conditions. It is therefore conceivable that in our low-O_2 samples, these processes provided at least part of the energy required for incorporation of glucose by cells.

With an approach similar to ours, but using ^{14}C-labelled glucose and the micro-autoradiography-FISH technique with, in some cases, genera-specific probes, Alonso and Pernthaler (2005) found that several bacteria living in oxic coastal waters of the North Sea were able to take up glucose when incubated in anoxic conditions in short-term (4 and 24 h) experiments. These authors concluded that facultatively anaerobic metabolism is widespread in costal marine waters with anaerobic microzones. Similar to their results, several bacterial groups were actively incorporating carbon after O_2 loss in the present study. It should be noted however that identification of phylotypes involved in this incorporation or analyses of shift in bacterial phylogenetic composition were not our aims. If all these bacteria were strict anaerobic bacteria (exploiting anaerobic microzones) and/or strict aerobic or microaerophilic bacteria, we likely would not have found consistently lower ^{13}C-enrichments of bacterial PLFAs in low-O_2 samples as on day 1 of the experiments. The similarity between the latter result and those indicating changes in pathways of fatty acid synthesis for a facultative bacterial strain in anaerobic conditions (Teece et al. 1999) therefore suggests that facultatively anaerobic bacteria (known to be able to switch their metabolism) were among bacteria actively incorporating glucose in our low-O_2 samples, at least on day 1. Our findings therefore seem to support the view that facultatively anaerobic metabolism is widespread among pelagic bacteria in coastal waters (Alonso and Pernthaler 2005).

Support for the contention that heterotrophic prokaryotes played a significant role in reducing phosphate availability in our low-O_2 samples, likely owing to their enhanced activity and retention of P by cells came from analyses of relationships between carbon degradation and DIP amounts in waters. Studies at the water-sediment interfaces (similar studies in water columns are not available for comparison) indicate that phosphate release from the sediments to the overlying waters is generally enhanced due to microbial OM mineralization, death of cells and dissolution of metal oxides (normally in particulate forms) when conditions shift from oxic to anoxic (Boström et al. 1988; Hupper and Lewandowski 2008; Jilbert et al. 2011). The lower P concentrations in our low-O_2 samples therefore contrasted with this knowledge, especially since O_2 loss likely killed some organisms. Moreover, DIP was uncoupled from $\delta^{13}C_{DIC}$ in these samples, in contrast to controls where these two variables were positively related (Fig. 2c), consistent with the commonly observed phosphate release in nutrient-rich waters due to mineralization in oxic conditions. Yet, the higher ^{13}C-signature of DIC during the first four days of incubation, and its strong decrease thereafter (dilution of ^{13}C) in low-O_2 samples, in contrast to controls (Fig. 2b) suggested higher degradation of the labelled glucose and of non-labelled OM in these samples. O_2 removal by purging with N_2 can remove dissolved CO_2 and possibly alter DIC and $\delta^{13}C_{DIC}$. Moreover, fermentation and CO_2 reduction can occur in O_2-deficient waters and affect $\delta^{13}C_{DIC}$ (Whiticar 1999). Dark DIC fixation is also known within phytoplankton and chemoautotrophic prokaryotic communities. However links between natural phytoplankton per se and the magnitude of this process remain unclear, since the latter has been found to increase with bacterial production (Li et al. 1993). A study under axenic bubbling has shown that dark DIC fixation by phytoplankton species is low when phosphorus is abundant (Theodorou et al. 1991). Our samples were rich in DIP. On the other hand, similarities or significant differences have been found between oxic and O_2-deficient waters regarding dark DIC fixation by chemoautotrophic prokaryotes (see e.g. Varela et al. 2011). While these processes might have contributed to uncouple DIP from $\delta^{13}C_{DIC}$ in our low-O_2 samples (and help explain some of the initial difference observed in DIC and labelled DIC between controls and treatments), they cannot explain the observed lower phosphate concentration, as they do not consume P to our knowledge. For water-sediment interfaces, it has been argued that the presence of

redox-insensitive P-binding systems such as Al $(OH)_3$ and unreducible Fe (III) minerals can prevent phosphate release, or that the C:P ratio of substrate can decrease mineralization if P is poorly available in the environment (Hupper and Lewandowski 2008). It is unlikely that these factors played a major role in our case, given that our samples were rich in P and glucose-amended. Moreover, redox-insensitive P-binding systems, if present, would have been the same in all our samples because they originated from the same initial sample.

A few studies, none of which concerned pelagic bacteria however, have shown that bacteria in O_2-deficient conditions can reduce phosphate availability in the milieu by transforming it nearly instantaneously into apatite, i.e. crystal phosphate minerals (Goldhammer et al. 2010). or by accumulating it into cells as polyphosphates (Barak and van Rijn 2000). This study by Barak and van Rijn (2000) showed, in an engineered system, that this accumulation was done by denitrifying bacteria using nitrate as electron acceptor when an external source of carbon was present. These conditions (presence and decline of nitrate and presence of an external carbon source) were similar to those in our low-O_2 samples. For prokaryotes that come from the same initial samples to become significantly richer in P-rich compounds (nucleic acids at the cell level) or to have significantly higher biomass of P-rich compounds (PLFAs at the community level) than their counterpart in controls, as observed for our low-O_2 samples, P uptake from the waters must be higher and its release weak. Interestingly, we found that DIP decrease in our low-O_2 samples was, indeed steeper (−70 %) than in controls (−32 %) on day 1. Moreover, DIP varied inversely with the index of cell activity (nucleic acid content) for the whole study, and change in this index in low-O_2 samples explained 62 % of the variance of difference in DIP concentration between controls and low-O_2 samples (Figs. 1c and 3d). Since they had a higher bacterial PLFA biomass than controls, but similar or lower abundance of heterotrophic prokaryotes (Fig. 3a, 4 and 5a) low-O_2 samples likely also had, on average, a higher PLFA biomass-derived P per cell. If one assumes that the difference in DIP concentration between controls and low-O_2 samples (average ~1.3 μM for day1 to day 16), was taken up by heterotrophic prokaryotes in low-O_2 samples (average ~1.6×10^6 cells mL^{-1}) there would be, on average, an extra 25 fgP per cell. This value is certainly overestimated because the calculation does not take into account other processes (e.g. transformation of phosphate into apatite, which cannot be ruled out regarding our low-O_2 samples), nor heterotrophic prokaryotic cells that were grazed. However, it compares well with values that have been found during growth phase for bacteria such as *Vibrio natriegens* (17 fgP $cell^{-1}$) and *E. coli* (31 fgP $cell^{-1}$) (Fagerbakke et al. 1996). indicating that our calculation is realistic, even though P content may vary from a cell to another. It is thus conceivable that the reduced availability of phosphate in our low-O_2 samples was due, at least in part, to retention of P by heterotrophic prokaryotic cells. Perhaps these observations were signs of cell accumulation of phosphate using nitrate as electron acceptor, as observed for denitrifying bacteria by Barak and van Rijn (2000). Denitrification is common in the water column anaerobic zones in the river system of the Scheldt Estuary, and has been estimated at 60 to 75 % of total denitrification in the Scheldt basin (Billen et al. 2005).

In conclusion, our results provide insights on how heterotrophic prokaryotes may respond, including at cell level, to O_2 loss in nutrient-rich aquatic systems. These results suggest that, at least at short-term scales, O_2 loss may not have negative impacts on carbon incorporation into cells and on the overall activity of heterotrophic prokaryotes, nor result in weak grazing on these communities as it is often stated. We have indeed shown that the size and the activity of heterotrophic prokaryotes cells increased significantly, while phosphate availability in waters was significantly reduced, following O_2 loss, likely as a result of both increase in grazing pressure and physiological responses involving facultatively anaerobic prokaryotes, significant carbon incorporation into cells, and possibly phosphorus retention by heterotrophic prokaryotes cells. Further studies on links that may exist between OM decomposition, heterotrophic prokaryote cells P content and both P and nitrate availability, as well as studies on the partitioning of cellular carbon demand into biomass (incorporation) and respiration when O_2 declines, will likely be of interest for a better understanding of how future O_2 decline might affect aquatic ecosystem functioning.

Acknowledgments R.D.T. was supported during his 3-month scientific visit at the Centre for Estuarine and Marine Ecology (Yerseke, The Netherlands) by funding from the Mission des Relations Internationales of INRA (Institut National de la Recherche Agronomique, France). He thanks Paul del Giorgio for hosting him at UQAM and providing him with a research facility. We also thank journal reviewers for their constructive comments which have helped improve the manuscript.

References

Alonso, C., and J. Pernthaler. 2005. Incorporation of glucose under anoxic conditions by bacterioplankton from coastal North Sea surface waters. *Applied and Environmental Microbiology* 71: 1709–1716.

Barak, Y., and J. van Rijn. 2000. Atypical polyphosphate accumulation by the denitrifying bacterium *Paracoccus denitrificans*. *Applied and Environmental Microbiology* 66: 1209–1212.

Benitez-Nelson, C.R., L.C. Kolowitch, P. Pellichia, and R. Thunell. 2004. Phosphonates and particulate organic phosphorus cycling in an anoxic marine basin. *Limnology and Oceanography* 49: 1593–1604.

Bettarel, Y., T. Simè-Ngando, C. Amblard, and J. Dolan. 2004. Viral activity in two contrasting lake ecosystems. *Applied and Environmental Microbiology* 70: 2941–2951.

Billen, G., J. Garnier, and V. Rousseau. 2005. Nutrient fluxes and water quality in the drainage network of the Scheldt basin over the last 50 years. *Hydrobiologia* 540: 47–67.

Boschker, H.T.S., J.F.C. de Brouwer, and T.E. Cappenberg. 1999. The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: stable carbon isotope analysis of microbial biomarkers. *Limnology and Oceanography* 44: 309–319.

Boschker, H.T.S., J. Krompkamp, and J.J. Middelburg. 2005. Biomarkers and carbon isotopic constraints on bacterial and algal community structure and functioning in a turbid, tidal estuary. *Limnology and Oceanography* 50: 70–80.

Boström, B., J.M. Andersen, S. Fleischer, and M. Jansson. 1988. Exchange of phosphorus across the sediment–water interface. *Hydrobiologia* 170: 229–244.

Cole, J.J., and M.L. Pace. 1995. Bacterial secondary production in oxic and anoxic freshwaters. *Limnology and Oceanography* 40: 1019–1027.

Cole, J.J., M.L. Pace, N.F. Caraco, and G.S. Steinhart. 1993. Bacterial biomass and cell size distribution in lakes: More and larger cells in anoxic waters. *Limnology and Oceanography* 38: 1627–1632.

Crump, B.C., C. Parenteau, B. Beckingham, and J.C. Cornwell. 2007. Respiratory succession and community succession of bacterioplankton in seasonally anoxic waters. *Applied and Environmental Microbiology* 73: 6802–6810.

Cuevas, L.A., and C.E. Morales. 2006. Nanoheterotroph grazing on bacteria and cyanobacteria in oxic and suboxic waters in coastal upwelling areas of northern Chile. *Journal of Plankton Research* 28: 385–397.

Detmer, A.E., H.C. Giesenhagen, V.M. Trenkel, H. Auf dem Venne, and F.J. Jochem. 1993. Phototrophic and heterotrophic pico- and nanoplankton in anoxic depths of the central Baltic Sea. *Marine Ecology Progress Series* 99: 197–203.

Deutsch, C., H. Brix, T. Ito, H. Frenzel, and L. Thompson. 2011. Climate-forced variability of ocean hypoxia. *Science* 333: 336–338.

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

Edgcomb, V., W. Orsi, J. Bunge, S. Jeon, S. Christen, C. Leslin, M. Holder, G.T. Taylor, P. Suarez, M. Varela, and S. Epstein. 2011. Protistan microbial observatory in the Cariaco Basin, Carribean. I. Pyrosequencing vs Sanger insights into species richness. *ISME Journal* 5: 1344–1356.

Erwin, J.A. 1973. *Fatty acids in eukaryotic microorganism*, 41–143. In: Erwin, JA editor. Lipids and Biomembranes of Eukaryotic Microorganisms. Academic Press New York.

Fagerbakke, K.M., M. Heldal, and S. Norland. 1996. Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. *Aquatic Microbial Ecology* 10: 15–27.

Fenchel, T., and T.H. Blackburn. 1979. *Bacteria and mineral cycling*. London: Academic Press. **225 p**.

Gasol, J.M., and P.A. del Giorgio. 2000. Using flow cytometry for counting natural bacteria and understanding the structure of bacterial communities. *Scienca Marina* 64: 197–224.

Gasol, J.M., U. Zweifel, F. Peters, J.A. Fuhrman, and A. Hagström. 1999. Significance of size and nucleic acid content heterogeneity, as measured by flow cytometry in natural planktonic bacteria. *Applied and Environmental Microbiology* 65: 4475–4483.

Gast, V., and K. Gocke. 1988. Vertical distribution of number, biomass and size-class spectrum of bacteria in oxic/anoxic conditions in the central Baltic Sea. *Marine Ecology Progress Series* 45: 179–186.

Goldhammer, T., V. Brüchert, T.G. Ferdelman, and M. Zabel. 2010. Microbial sequestration of phosphorus in anoxic upwelling sediment. *Nature Geosciences* 3: 557–561.

Hahn, M.W., and M.G. Höfle. 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. *FEMS Microbiology Ecology* 35: 113–121.

Harvey, H.R., R.D. Fallon, and J.S. Patton. 1986. The effect of organic matter and oxygen on the degradation of bacterial membrane lipids in marine sediments. *Geochimica et Cosmochimica Acta* 50: 795–804.

Hupper, M., and J. Lewandowski. 2008. Oxygen controls the phosphorus release from lake sediments—a long-lasting paradigm in limnology. *International Review of Hydrobiology* 93: 415–432.

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

Jorgensen, B.B. 1982. Mineralisation of organic matter in the sea bed-the role of sulphate reduction. *Nature* 296: 643–645.

Karl, D.M., L. Berversdorf, K.N. Bjorkman, M.J. Church, A. Martinez, and E.F. DeLong. 2008. Aerobic production of methane in the sea. *Nature Geosciences* 1: 473–478.

Lam, P., and M.M.M. Kuypers. 2011. Microbial nitrogen cycling processes in oxygen minimum zones. *Annual Review Marine Science* 3: 317–345.

Li, W.K.W., B.D. Irwin, and P.M. Dickie. 1993. Dark fixation of ^{14}C: variations related to biomass and productivity of phytoplankton and bacteria. *Limnology and Oceanography* 38: 483–494.

Madan, R., C. Pankhurst, B. Hawke, and S. Smith. 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. *Soil Biology and Biochemistry* 34: 125–28.

McKindley, V.L., A.D. Peacock, and D.C. White. 2005. Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soils. *Soil Biology and Biochemistry* 37: 1946–1958.

Middelburg, J.J., C. Barranguet, H.T.S. Boschker, P.M.J. Herman, T. Moens, and C.H. Heip. 2000. The fate of intertidal microphybenthos carbon: an in situ ^{13}C-labeling study. *Limnology and Oceanography* 45: 1224–1234.

Moodley, L., H.T.S. Boschker, J.J. Middelburg, R. Pel, P.M.J. Herman, E. de Deckere, and C.H. Heip. 2000. The ecological significance of benthic Foraminifera: ^{13}C labeling experiments. *Marine Ecology Progress Series* 202: 289–295.

Naqvi, S.W.A., H.W. Bange, L. Farias, P.M.S. Monteiro, M.I. Scranton, and J. Zhang. 2010. Marine hypoxia/anoxia as a source of CH_4 and N_2O. *Biogeosciences* 7: 2159–2190.

Noy-Meir, I. 1975. Stability of grazing systems: an application of predator-prey graphs. *Journal of Ecology* 63: 459–481.

Orsi, W., Y.C. Song, S. Hallam, and V. Edgcomb. 2012. Effects of oxygen minimum zone formation on communities of marine protists. *ISME Journal* 6: 1586–1601.

Park, J.S., and B.C. Cho. 2002. Active heterotrophic nanoflagellates in the hypoxic water-column of the eutrophic Masan Bay, Korea. *Marine Ecology Progress Series* 230: 35–45.

Parrish, C.C., G. Bodennec, and P. Gentien. 1994. Time courses of intracellular and extracellular lipid classes in batch cultures of the toxic dinoflagellate. *Gymnodinium cf. nagasakiense Marine Chemistry* 48: 71–82.

Ratledge, C. and Wilkinson, S. G. 1988. Microbial lipids. Academic press.

Ricciardi-Rigault, M., D.F. Bird, and Y.T. Prairie. 2000. Changes in viral and bacterial abundance with hypolimnetic oxygen depletion in a shallow eutrophic Lake Brome (Québec, Canada). *Canadian Journal Fisheries and Aquatic Sciences* 56: 1284–1290.

Schellenberger, S., S. Kolb, and H.L. Drake. 2010. Metabolic responses of novel cellulolytic and saccharolytic agricultural soil bacteria to oxygen. *Environmental Microbiology* 12: 845-–861.

Scott, J.H., and K.H. Nelson. 1994. A biochemical study of the intermediary carbon metabolism of *Shewanella putrefaciens*. *Journal of Bacteriology* 176: 3408–3411.

Simon, M., and F. Azam. 1989. Protein content and protein synthesis rates of planktonic marine bacteria. *Marine Ecology Progress Series* 51: 201–213.

Sokal, R.R., and J.F. Rohlf. 1995. *Biometry: the principles and practice of statistics in biological research*, 3rd ed. New York: WH Freeman and Company. **850 p**.

Steen, H.B., E. Boye, and T. Godal. 1981. Applications of flow-cytometry to bacteria. *Cytometry* 2: 128–129.

Tadonléké, D.R., D. Planas, and M. Lucotte. 2005. Microbial food webs in boreal humic lakes and reservoirs: ciliates as a major factor related to the dynamics of the most active bacteria. *Microbial Ecology* 49: 325–341.

Teece, M.A., M.L. Fogel, M.E. Dollhopf, and K.H. Nelson. 1999. Isotopic fractionation associated with biosynthesis of fatty acids by a marine bacterium under oxic and anoxic conditions. *Organic Geochemistry* 30: 1571–1579.

Theodorou, M.E., I.R. Elrifi, D.H. Turpin, and W.C. Plaxton. 1991. Effects of Phosphorus limitation on respiratory metabolism in the green alga *Selenastrum minutum. Plant Physiology* 95: 1089–1095.

Troussellier, M., C. Courties, P. Lebaron, and P. Servais. 1999. Flow cytometric discrimination of bacterial populations in seawater based on SYTO 13 staining of nucleic acids. *FEMS Microbiology Ecology* 29: 319–330.

Tunlid, A. and White, D.C. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil, In Bollag JM, Stotzky G. editors, *Soil Biochemistry*, Marcel Dekker. p. 229–262.

Van den Meersche, K., P. van Rijswijk, K. Soetaert, and J.J. Middelburg. 2009. Autochthonous and allochthonous contributions to mesozooplankton diet in a tidal river and estuary: integrating carbon isotope and fatty acid constraints. *Limnology and Oceanography* 54: 62–74.

Van den Meersche, K., K. Soetaert, and J.J. Middelburg. 2011. Plankton dynamics in a estuarine plume: a mesocosm ^{13}C and ^{15}N tracer study. *Marine Ecology Progress Series* 429: 29–43.

Van Oevelen, D., L. Moodley, K. Soetaert, and J.J. Middelburg. 2006. The trophic significance of bacterial carbon in a marine intertidal sediment: results of an in situ stable isotope labeling study. *Limnology and Oceanography* 51: 2349–2359.

Varela, M.M., H.M. van Aken, E. Sintes, T. Reinthaler, and G.J. Herndl. 2011. Contribution of *Crenarchaeota* and *Bacteria* to autotrophy in the North Atlantic interior. *Environmental Microbiology* 13: 1524–1533.

Volkman, J.K., S.M. Barrett, S.I. Blackburn, M.P. Mansour, E.L. Sikes, and F. Gelin. 1998. Microalgal biomarkers: a review of recent research developments. *Organic Geochemistry* 29: 1163–1179.

Wangersky, P.J., and W.J. Cunningham. 1957. Time lag in prey-predator population models. *Ecology* 38: 136–139.

Weinbauer, M.G., and M.G. Höfle. 1998. Significance of viral lysis and flagellate grazing as factors controlling bacterioplankton production in a eutrophic lake. *Applied and Environmental Microbiology* 64: 431–438.

Whiticar, M.J. 1999. Carbon and hydrogen isotope systematic of bacterial formation and oxidation of methane. *Chemical Geology* 161: 291–31.

Wright, J.J., K.M. Konwar, and S.J. Hallam. 2012. Microbial ecology of expanding oxygen-minimum zones. *Nature Review Microbiology* 10: 381–394.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. *Marine Pollution Bulletin* 45: 35–45.

Yakushev E.V. and Newton A. 2013. Redox interfaces in marine waters, In Yakushev E.V. (ed) Chemical structure of pelagic redox interfaces. Observations and modelling. Springer, 290 p.

Zak, D.R., D.B. Ringelberg, K.S. Pregitzer, D.L. Randlett, D.C. White, and P.S. Curtis. 1996. Soil microbial communities beneath *Populus grandidentata* grown under elevated atmospheric CO_2. *Ecological Applications* 6: 257–262.