Freeze-lysable inorganic nutrients in intertidal sediments: dependence on microphytobenthos abundance

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ABSTRACT: Freezing is a common treatment for the preservation of sediment samples. To test the role of microphytobenthos (MPB) abundance in the release of intracellular nutrients to the pore water due to cell breakage after freezing, referred to as freeze-lysable inorganic nutrients (FL-IN), parallel extractions were carried out from intertidal sediment cores collected in winter and summer from Cádiz Bay. After the determination of net production and dark respiration rates with O_2 microsensors, sediment cores were subcored and sliced into several layers. The samples were divided into 2 fractions; the first was centrifuged to extract pore water (fresh) and the other was frozen at –80°C, thawed and centrifuged to extract pore water after freezing. NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-} were measured in the pore water extracted by both procedures. Chlorophylls *a* and *c* were extracted from the same sediment fractions. Freezing produced a significant increase in the pore water concentrations of all inorganic nutrients. Therefore, if the variable of interest is the inorganic nutrient concentration, pore water should be extracted from fresh samples. In addition, FL-IN correlated significantly with chlorophylls *a* and *c* (p < 0.01), r^2 ranged from 0.54 for NO_3^- to 0.94 for NH_4^+, indicating that most FL-IN were released from MPB biomass. The relationships between chlorophyll *a* and FL-IN in winter and summer were significantly different, suggesting that nutrient accumulation by MPB changes seasonally and might affect nutrient cycling in intertidal sediments.

KEY WORDS: Microphytobenthos · Microbenthos · Freeze-lysable inorganic nutrients · Pore water nutrients · Intertidal sediments · Benthic microalgae · Sediment preservation

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INTRODUCTION

The analysis of inorganic nutrients in the sediment pore water and the overlying water column allows biogeochemists to calculate diffusive fluxes at the sediment–water interface (Boudreau 1997). In addition, numerical modelling of inorganic nutrient profiles within the sediment has been used to calculate the rates and location of several biogeochemical activities within the sediment (Berg et al. 1998). Therefore, any alteration of the *in situ* pore water concentration during collection, storage or extraction of pore water from

the cores would introduce serious errors in the calculation and interpretation of biogeochemical processes (Sayama 2001).

Freezing is often used to preserve sediment samples in biogeochemical studies when a large number of them must be collected. It is assumed, although generally not tested, that this practise will prevent sediment mixing during transportation and 'stop' any further biogeochemical process occurring within the sediment. Once in the laboratory, sediment cores are thawed and processed for the extraction of pore water nutrients. However, this practice may produce erro-

neous results. It is well established that freezing causes cell breakage and release of cytoplasmic compounds into the external medium. Freezing-thawing cycles have been used to extract intracellular pools of inorganic nitrogen (Thoresen et al. 1982) and nucleic acids from marine phytoplankton (More et al. 1994). Freezing of marine sediments has been shown to increase the concentration of extracted pore water inorganic nutrients (Lomstein et al. 1990, Aller 1994, Sayama 2001, Ferguson et al. 2004) and dissolved organic carbon (Otero et al. 2007). The characteristic exponential decrease with depth in the concentration of pore water freeze-lysable (FL) nitrate and ammonium (defined here as the difference between the concentrations of inorganic nutrients in thawed and fresh aliquots of the same sample) and their increase after the sedimentation of a phytoplankton bloom in subtidal sediments lead Lomstein et al. (1990) to suggest that $FL-NO_3^-$ and $FL-NH_4^+$ might represent the intracellular pool of the sedimented phytoplankton cells. However, other components of the benthic community may also release intracellular nutrients after freezing. $FL-NO_3^-$ was at least 10 times higher than the NO_3^- pore water concentration in the hypertrophic subtidal sediments of Tokyo Bay due to the abundance of nitrate-accumulating sulphur bacteria (Sayama 2001). $FL-NH_4^+$ and $FL-PO_4^{3-}$ have also been interpreted as originating mainly from intracellular pools associated with microbial and meiofaunal biomass (Aller 1994, Ferguson et al. 2004). High concentrations of FL inorganic nutrients (FL-IN) were found in intertidal sediments below macroalgal mats, released most likely from macroalgal detritus (Corzo et al. 2009). However, so far, no specific studies have been carried out to quantify the contribution of the different components of the complex biological community inhabiting the marine sediment to the FL-IN pool.

In intertidal areas, benthic microalgae (microphytobenthos, MPB) inhabit the sediment surface. The MPB community is usually dominated by benthic diatoms and cyanobacteria. MPB cells could release intracellular nutrients when sediment samples are frozen. The aim of the present study was to test the hypothesis that FL-IN represents the intracellular nutrient pool of MPB and to assess the contribution of MPB to the release of FL-IN in sediment samples. To achieve this, we compared the concentrations of inorganic nutrients (NO_3^-, NO_2^-, NH_4^+ and PO_4^{3-}) in parallel fresh and freeze-thawed samples from different sediment depths and related these with the concentration of chlorophyll *a* and *c*, used as an indicator of microalgal abundance. The experiment was carried out in winter and summer to test if the seasonal changes in microphytobenthos abundance and activity could affect the FL-IN content of the sediment and its relationship with chlorophyll

and pore water nutrients. The experimental data were analysed at annual level by pooling the results obtained in the winter and summer experiments to cover as much variability as possible and by seasons to reveal possible changes in the relationships among FL-IN, chlorophyll and pore water nutrients.

MATERIALS AND METHODS

Sampling site and collection of samples. Sediment samples were collected in January and July 2008 from an unvegetated intertidal site in Trocadero Island (Cadiz Bay, Spain). Sediment was silty mud and no macroalgae or seagrasses detritus were observed in the samples. Two sediment cores (internal diameter [i.d.] 5.4 cm) were collected from each of 3 randomly selected 1×1 m sediment areas with a typical separation between areas of 10 to 15 m.

Sample processing. After collection, sediment cores were carried to the laboratory and maintained in an aquarium, at a photon flux density (PFD) of 800 µmol m^{-2} s^{-1} provided by halogen lamps, under a 12 h light: 12 h dark photoperiod cycle for 4 to 5 d until further analysis. The aquarium was under a semidiurnal tidal cycle, simulated with the help of 2 pumps connected to time controllers. O_2 profiles (n = 3 to 4) were measured in the light (800 µmol photons m^{-2} s^{-1}) and in the dark at the sediment–water interface in each of the 6 sediment cores using O_2 microelectrodes (Unisense A/S, Denmark). Net primary production (P_n) and dark respiration (R_d) rates were calculated from the O_2 gradient at the diffusive boundary layer of the light and dark profiles respectively (Kühl et al. 1996, García-Robledo et al. 2008, Corzo et al. 2009).

After the determination of the O_2 profiles, 4 smaller cores (i.d. 1.6 cm) were subcored from each of the 6 cores in order to facilitate subsequent slicing at the mm scale. Every subcore was sliced at the following depth intervals: 0–1, 1–2, 2–3, 3–5 and 5–10 mm. Slices from the same depth from each area were pooled and homogenised to obtain a sufficiently large amount of sample for subsequent analyses. These pooled sediment samples were then split into 2: one subsample was processed immediately (fresh subsample) and the other was frozen at –80°C for a minimum of 24 h and thawed prior to further treatment (thawed subsample). Both fresh and thawed sediment samples were centrifuged at 8600 *g* for 30 min at 4°C to extract pore water. Total water content of sediment ranged from 55.1 to 60.4%. Nutrient concentrations in the extracted pore water (NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}) were measured on a Skalar autoanalyzer following standard protocols (Grasshoff et al. 1983). Immediately after pore water extraction, pigment extraction from the pellets

was carried out. Chlorophyll a (chl *a*) and chlorophyll c (chl *c*) were extracted in 100% methanol overnight at 4°C in darkness (Thompson et al. 1999). Extracts were centrifuged at 2200 *g* for 10 min and measured using a spectrophotometer (Unicam UV/Vis UV2®). Chl *a* and *c* were estimated as described by Ritchie (2008).

Statistical analysis. Differences between treatments or depths for the measured variables were tested by 1-way and 2-way analysis of variance (ANOVA) followed by Tukey's HSD test for multiple pairwise comparisons. Linear regressions and correlations between variables were also performed using the software Statgraphics Plus 5.1.

RESULTS

Effect of freezing on chlorophyll and inorganic nutrient extractions

Mean chlorophyll content (winter and summer data pooled) showed similar vertical distributions (Fig. 1) with highest values at the sediment surface (mean ± SE: 36.7 ± 5.1 µg chl *a* g DW^{-1} and 6.0 ± 1.5 µg chl *c* g DW^{-1}) decreasing rapidly with depth (1-way ANOVA, p < 0.01). Both pigments showed similar vertical distribution with depth and their concentrations were highly correlated (r^2 = 0.99, p < 0.01, Table 1, Fig. 1). Freezing increased the extraction yield of both chl *a* and *c* significantly (~1.2-fold, Fig. 1, Table 1), however the effect of freezing on the extraction yield decreased with depth (2-way ANOVA, p < 0.05).

Mean pore water NO$_2^-$ concentration (winter and summer data pooled) measured in fresh samples decreased significantly with depth within the sediment from 0.7 ± 0.3 µmol l^{-1} at the sediment surface to 0.2 ± 0.1 µmol l^{-1} at 7.5 mm depth (1-way ANOVA, p < 0.05). NO$_3^-$ and PO$_4^{3-}$ also decreased with depth from 1.9 ± 1.4 and 0.9 ± 0.4 µmol l^{-1}, to 0.3 ± 0.2 and 0.2 ± 0.1 µmol l^{-1}, respectively, but changes were not significant due to a large variability in the upper sediment layer. NH$_4^+$ remained constant with depth at about 22.1 ± 1.8 µmol l^{-1} (Fig. 1).

The freeze-thaw procedure prior to the extraction of sediment pore water significantly increased the pore water concentrations of analysed nutrients (2-way ANOVA, p < 0.01). Inorganic nutrients decreased with
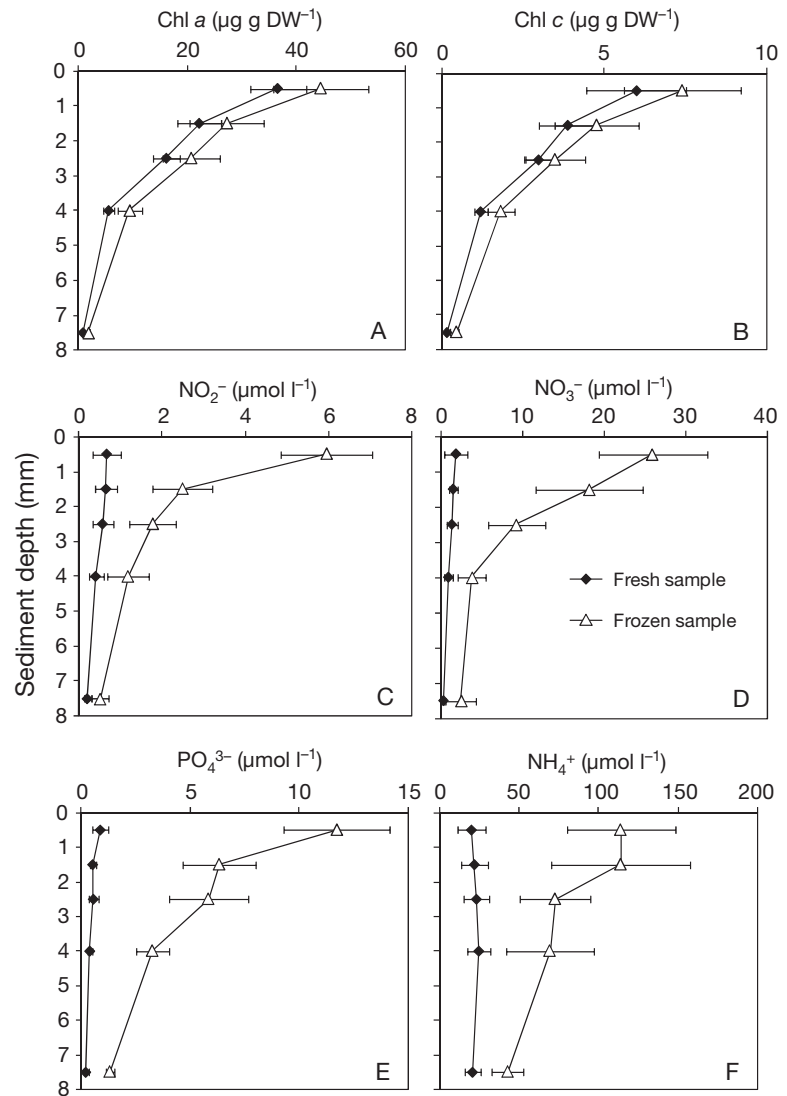

Fig. 1. Vertical profiles of (A) chlorophyll *a*, (B) chlorophyll *c* and pore water (C) nitrite, (D) nitrate, (E) phosphate and (F) ammonium concentrations in the sediment measured in parallel subsamples, 1 fresh untreated and 1 subjected to a single freeze-thaw cycle. Values are mean ± SE (n = 6)

depth exponentially, from 5.9 ± 1.1, 26.0 ± 6.6, 11.7 ± 2.4 and 113.9 ± 33.9 µmol l^{-1}, for NO$_2^-$, NO$_3^-$, PO$_4^{3-}$ and NH$_4^+$, respectively, at the sediment surface to 8 to 37% of surface values at the deepest analysed sediment layer (Fig. 1).

FL-IN concentration showed similar profiles, with the highest concentrations at sediment surface and a progressive decrease with depth (Fig. 2). The highest release of nutrients after the freeze-thaw treatment was observed for FL-NH$_4^+$ ranging from 99.7 ± 26.9 µmol l^{-1} at the sediment surface to 22.1 ± 5.6 µmol l^{-1} at the deepest layer. The lowest release of nutrient was observed for FL-NO$_2^-$, ranging from 5.3 ± 1.1 at

the surface to 0.3 ± 0.1 µmol l^{-1} at the deepest layer. FL-NO_3^- and FL-PO_4^{3-} ranged from 24.1 ± 5.6 and 10.9 ± 2.4 µmol l^{-1} at the surface to 2.2 ± 1.7 and 1.1 ± 0.3 µmol l^{-1} at the deepest layer, respectively.

Table 1. Linear regressions between concentrations of chlorophyll *a* and *c* in frozen and fresh samples, and between freeze-lysable (FL) content of nitrate, nitrite, ammonium and phosphate concentrations and chl *a* content in the sediment after a single freeze-thaw cycle. All regressions were statistically significant ($n = 30$, $p < 0.01$). In all cases, the independent terms were set to be equal to 0 because in a previous regression analysis they were not significantly different from 0

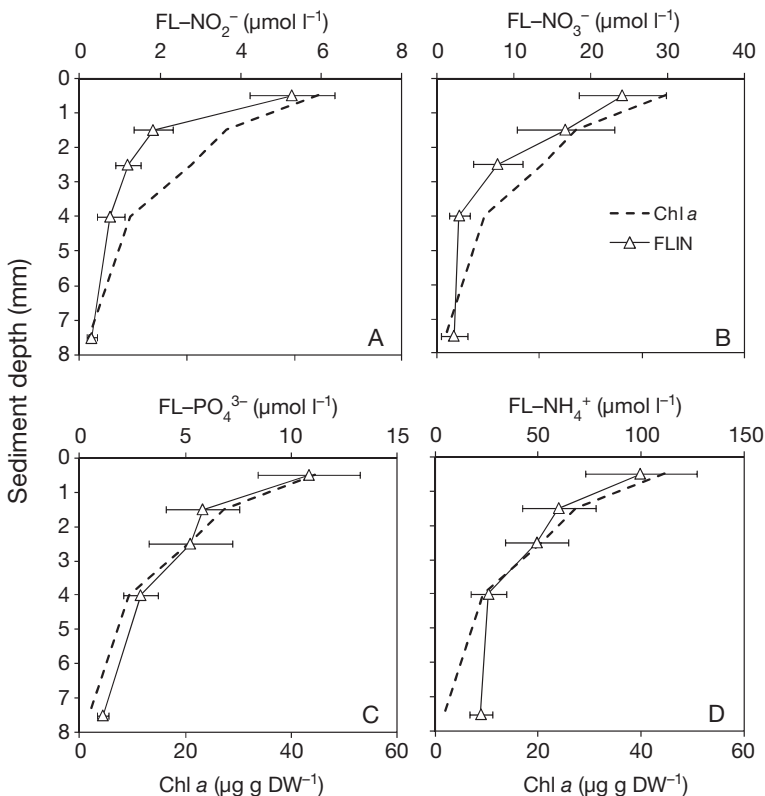| Regression equations	r^2
Chl a^{frozen} = 1.24 chl a^{fresh}	0.91
Chl c^{frozen} = 1.18 chl c^{fresh}	0.89
Chl a^{frozen} = 5.69 chl c^{frozen}	0.99
Chl a^{fresh} = 5.28 chl c^{fresh}	0.93
FL-NO_2^- = 0.09 chl a^{frozen}	0.72
FL-NO_3^- = 0.43 chl a^{frozen}	0.54
FL-PO_4^{3-} = 0.24 chl a^{frozen}	0.88
FL-NH_4^+ = 2.42 chl a^{frozen}	0.94

Fig. 2. Vertical profiles of freeze-lysable (FL) (A) nitrite, (B) nitrate, (C) phosphate and (D) ammonium. Freeze-lysable inorganic nutrients (FL-IN) are defined as the difference in the pore water nutrient concentration between a sediment subsample subjected to a single freeze-thaw cycle and a fresh untreated sample. Values are mean $\pm$ SE ($n = 6$). Chl *a* profile (dashed line) is given for comparison purposes

The general pattern of the vertical profiles of all the FL-IN was similar to that of chl *a* (Fig. 2). FL-IN concentrations were highly correlated with those of chl *a* ($p < 0.01$), although there were clear differences among the nutrients (Table 1). FL-NH_4^+ showed the highest correlation ($r^2 = 0.94$) and FL-NO_3^- the lowest ($r^2 = 0.54$). In contrast, the correlations between the pore water inorganic nutrients and chlorophyll concentrations of fresh subsamples were not significant for NO_3^-, NH_4^+ and PO_4^{3-} ($p > 0.05$), or significant but much weaker in the case of NO_2^- ($r^2 = 0.41$, $p < 0.01$). Inorganic nutrient concentrations in pore water and FL-IN were significantly different (2-way ANOVA, $p < 0.01$), but were weakly correlated except for PO_4^{3-} ($r^2 \leq 0.3$ in all cases, $p < 0.01$).

Winter and summer differences

Chl *a* content in the upper layer of the sediment was higher in winter than in summer (ANOVA, $p < 0.05$), but the difference between seasons decreased with depth (Fig. 3A). Similar differences were found in the chl *c* content between seasons (Fig. 3B). Oxygen supersaturation of the subsurface pore water in the light compared with the dark (Fig. 3C,D) indicates the presence of a photosynthetically active microphytobenthic community in both seasons. However, net primary production (P_n) and dark respiration (R_d) were lower in winter (Table 2).

FL-IN content of the sediment in winter and summer showed the same exponential decrease with depth previously observed for the complete data set (Fig. 4). The concentrations of FL-NH_4^+, FL-NO_2^- and FL-PO_4^{3-} were higher in winter (2-way ANOVA, $p < 0.05$). The difference between the winter and summer samples tended to decrease with depth. However, FL-NO_3^- did not show significant differences between winter and summer (Fig. 4).

The relationships between FL-IN and chl *a* concentration were always significant ($p < 0.01$) when the winter and summer samples were analysed separately, although they varied slightly with season. The highest correlations were observed for FL-NH_4^+ in winter and summer and for FL-PO_4^{3-} in winter (Table 3). Lowest correlations were observed for FL-NO_3^- in winter and for FL-PO_4^{3-} in summer.

Table 2. Characteristics of the microphytobenthic community in January and July. Chlorophyll values are integrated for the upper 5 mm of the sediment and expressed as mg m^{-2} (n = 3); net production in light (P_n) and respiration in dark (R_d) as mmol O_2 m^{-2} h^{-1} (n = 20). Values are means ± SE

	Chl a	Chl c	P_n	R_d
Jan	307.2 ± 62.9	55.7 ± 3.9	1.0 ± 0.3	−0.8 ± 0.1
Jul	128.3 ± 21.4	16.8 ± 4.7	3.9 ± 0.4	−1.2 ± 0.1

The content of FL-IN per unit of chl a (FL-NO$_3^-$/chl a, FL-NO$_2^-$/chl a, FL-NH$_4^+$/chl a and FL-PO$_4^{3-}$/chl a) showed clear differences with depth in the sediment, and between seasons for some nutrients (Fig. 5). Two layers were evident, the upper one up to a depth of 3 to 5 mm, characterised by relatively low concentrations of FL-IN per unit of chl a and showing a wide variability of patterns in response to both depth and season among the different inorganic nutrients. The second layer, below a depth of 3 to 5 mm, was characterised by a general increase in all FL-IN/chl a ratios except FL-NO$_3^-$/chl a in July (Fig. 5B).

Table 3. Linear regressions between FL-NH$_4^+$, FL-NO$_2^-$, FL-NO$_3^-$ and FL-PO$_4^{3-}$ concentrations and chl a content (as independent variable) for January and July samples. The intercept was set to 0 in all cases as it was found to be not significantly different from 0 in a previous linear regression analysis. All regressions were significant (n = 15, p < 0.01)

	January		July	
	r^2	Slope	r^2	Slope
FL-NH$_4^+$	0.95	2.48	0.90	2.17
FL-NO$_2^-$	0.74	0.076	0.82	0.138
FL-NO$_3^-$	0.55	0.357	0.73	0.814
FL-PO$_4^{3-}$	0.94	0.248	0.59	0.181

The nutrient concentration in the pore water of fresh samples differed considerably between winter and summer (Fig. 6). The changes involved not only the quantity of the different nutrients, but also the shape of their profiles with depth. NO$_3^-$, NO$_2^-$ and NH$_4^+$ concentrations were higher in winter, whereas PO$_4^{3-}$ concentration was higher in summer except in the upper mm of the sediment (2-way ANOVA, p < 0.01). No significant statistical relationship was found between pore water nutrients and chl a concentrations in either winter or summer, except for NO$_2^-$ in winter (r^2 = 0.40, p < 0.01) and NH$_4^+$ in summer (r^2 = 0.43, p < 0.01). Pore water and FL-IN nutrients in each season were not statistically correlated, except NO$_3^-$ in winter (r^2 = 0.48, p < 0.01) and summer (r^2 = 0.65, p < 0.01).

DISCUSSION

Effect of freezing and thawing on chlorophyll and nutrient extractions

Freezing and thawing of sediment samples increased the extraction yield of all measured variables, both pigments and nutrients (Fig. 1, Table 1). Freeze-thaw cycles have been used to improve the extraction efficiency from sediment samples of nucleic acids (More et al. 1994), dissolved organic matter (Otero et al. 2007), as well as pigments (Wasmund et al. 2006). The cell lysis induced by freezing also results in the release of dissolved intracellular inorganic nutrients to the sediment pore water (Lomstein et al. 1990, Sayama 2001). This was evident in our study, as nutrient pore water concentrations increased up to 120-fold after a single freeze-thaw cycle (Fig. 1). There-
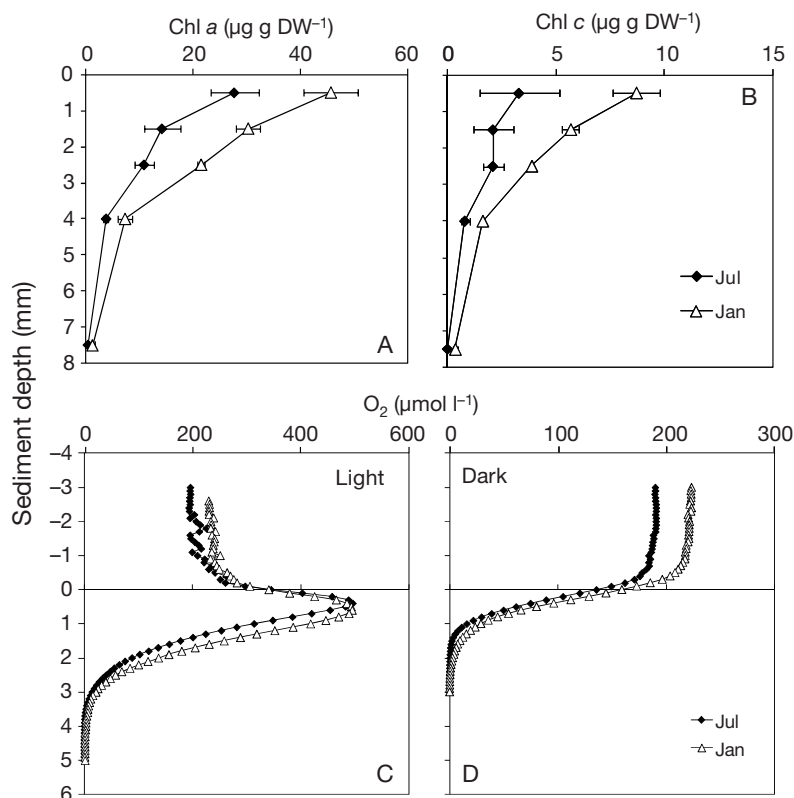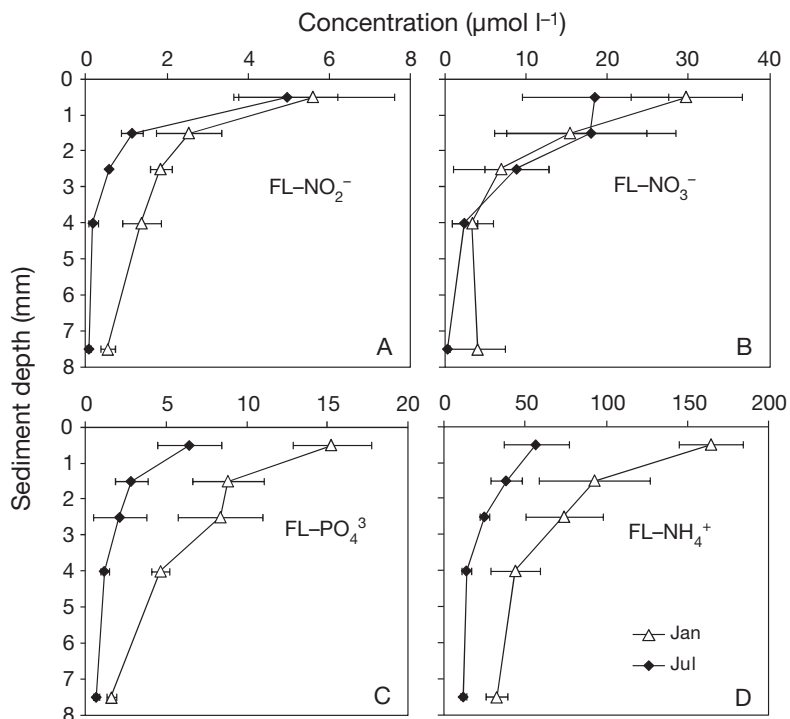

Fig. 3. Vertical distribution of (A) chl a and (B) chl c in the sediment surface after freezing and thawing in January and July. Mean oxygen profiles in the sediment–water interface (C) in the light (PFD = 800 µmol m^{-2} s^{-1}) and (D) in the dark in January and July. Values are mean ± SE (n = 3)

Fig. 4. Vertical profiles of FL-IN: (A) nitrite, (B) nitrate, (C) phosphate and (D) ammonium in January and July. Values are mean ± SE (n = 3)

inorganic nutrients in benthic microalgae as far as we know. Nonetheless, the range of FL-NO_3^-/chl *a* reported here (Fig. 5) is consistent with published data for phytoplanktonic communities. Bode et al. (1997) found that the amount of intracellular NO_3^- normalised to chl *a* was 4.3 ± 2.5 nmol NO_3^- µg^{-1} chl *a* in a coastal upwelling system off northern Spain. In more productive systems, larger intracellular concentrations of NO_3^-, 7.1 ± 6.7 and 8.4 ± 9.5 nmol NO_3^- µg^{-1} chl *a* were found by Dortch et al. (1984) in Dabob Bay and by Pettersson (1991) in the Skagerrak, respectively.

Chlorophyll concentrations in our experiments were within the typical range of intertidal sediments (MacIntyre et al. 1996). The microphytobenthic community was likely dominated by diatoms, as suggested by the strong correlation between the chl *a* and *c* contents. Diatoms are usually the dominant microphytobenthic taxa in marine intertidal sediments (MacIntyre et al. 1996). Diatom cells contain large vacuoles in which NO_3^- can be stored
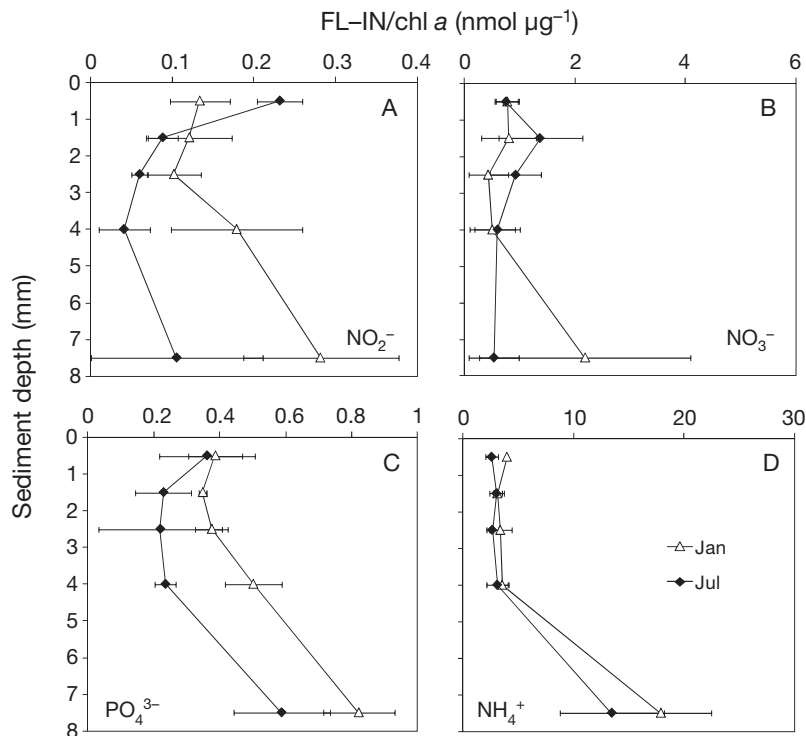
fore, freezing of sediment samples for preservation is unacceptable if the variable of interest is the concentration of inorganic nutrient in the pore water.

Lomstein et al. (1990) found a 5-fold increase in the FL content for NH_4^+ and 18-fold for NO_3^- in the sediment after the sedimentation of the spring phytoplankton bloom. They suggested that freezing could release intracellular NH_4^+ and NO_3^- from the sedimented diatom cells. The strong statistical correlations between the concentration of FL-IN and the chl *a* and *c* content in the sediment of our area suggest that a large fraction of the FL-IN is closely associated with the microphytobenthic biomass (Table 1, Fig. 2). These results indicate that benthic microalgae store inorganic nutrients, as has been reported for planktonic diatoms (Dortch 1982, Thoresen et al. 1982, Dortch et al. 1984, Bode et al. 1997). Unfortunately, no information is available in the literature on the intracellular content of

Fig. 5. Ratio of FL-IN per unit of chl *a*: (A) FL-NO_2^-/chl *a*, (B) FL-NO_3^-/chl *a*, (C) FL-PO_4^{3-}/chl *a* and (D) FL-NH_4^+/chl *a*. Nutrient and chl *a* are expressed per gram of fresh weight, being the resulting ratio in nmol FL-IN/µg chl *a*. Values are mean ± SE (n = 3)
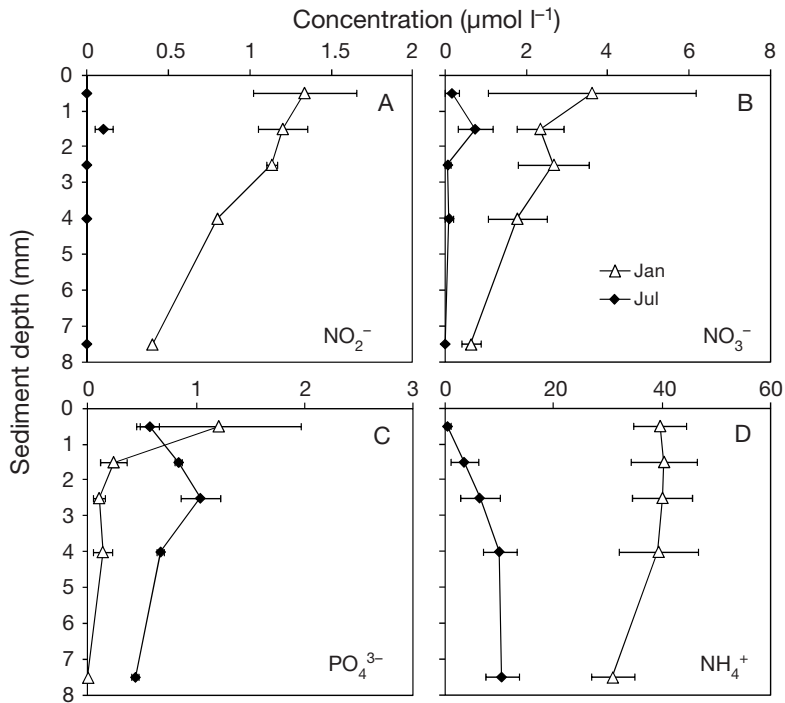
Fig. 6. Vertical profiles of (A) nitrite, (B) nitrate, (C) phosphate and (D) ammonium concentrations in the pore water in January and July. Values are mean ± SE (n = 3)

$FL-NO_3^-$/chl a ratio is likely to be more variable than $FL-NH_4^+$/chl a in the sediment photic layer (Fig. 5).

In addition to microphotoautotrophs, other components of the microbenthic community might also contribute to the release of FL-IN. The KCl-lysable fraction of NH_4^+, defined as the NH_4^+ extracted with KCl after freezing, has been shown to be around 80 times larger than KCl-exchangeable NH_4^+, being also positively related to bacterial abundance (Aller 1994). High concentrations of $FL-NO_3^-$ were found in hypertrophic sediments colonised by the sulphur-oxidising bacteria *Beggiatoa* sp., which are capable of accumulating large amounts of NO_3^- (up to 100 mmol l^{-1}) in their vacuoles (McHatton et al. 1996, Sayama 2001). Bacteria are able to store large amounts of PO_4^{3-} as polyphosphate, and bacteria-associated PO_4^{3-} represented a large fraction (up to 80%) of total P in lake sediments (Gachter et al. 1988). Large vacuolated sulphur-oxidising bacteria are absent in the typical intertidal sediments of Cádiz Bay, due to the relatively low organic matter content and the absence of free sulphide in the sediment (García-Robledo et al. 2008, Corzo et al. 2009). Therefore, a large contribution of bacteria to the $FL-NO_3^-$ pool is unlikely. However, a possible contribution of bacteria and micro- and meiofauna to the $FL-PO_4^{3-}$ and, particularly, $FL-NH_4^+$ pools cannot be ruled out due to the general covariance of microphytobenthos abundance and primary production with bacterial and micro- and meiofaunal abundance (Middelburg et al. 2000). In addition, different types of phytodetritus might also contribute to the FL-IN pool of sediment (Lomstein et al. 1990, Corzo et al. 2009).

A proportion of the FL-IN could have an abiotic source as well. The freeze-thaw treatment could, to some extent, extract inorganic nutrients adsorbed to the sediment matrix, such as NH_4^+ and PO_4^{3-}, usually referred to as the exchangeable pool (Blackburn & Henriksen 1983, Slomp et al. 1998). The non-specific binding of NO_3^- to the sediment matrix is considered unlikely in marine sediment (Lomstein et al. 1990), although it does occur in soils (Katou et al. 1996). Our results indicate that changes in the concentration of chl a explained 94, 88, 72 and 54% of variability of $FL-NH_4^+$, $FL-PO_4^{3-}$, $FL-NO_2^-$ and $FL-NO_3^-$, respectively, suggesting a strong relationship with MPB biomass. Nonetheless, the relative importance of the intracellular and exchangeable pools might change depending on the taxonomic composition of microbenthic commu-

(Dortch et al. 1984, Stolte & Riegman 1995, Lomas & Glibert 2000). PO_4^{3-} can be stored intracellularly as orthophosphate, most likely in the vacuole, or as polyphosphate in the cytoplasm (Miyata et al. 1986, Mateo et al. 2006). In addition, the existence of a phosphate pool adsorbed to the cell surface has been shown in marine phytoplankton (Fu et al. 2005). On the other hand, diatoms do not usually store large amounts of NH_4^+ and NO_2^- intracellularly, most probably due to their toxicity (Dortch 1982, Lomas & Glibert 2000). The differences in the capacity of benthic microalgae to use different species of inorganic nitrogen and phosphate for intracellular storage likely explain the differences observed in the relationships between the FL-IN and MPB biomass, both on an annual scale and when the relationships were analysed separately in summer and winter (Tables 1 & 3). The highest statistical correlations between FL-IN and chl a were observed for $FL-NH_4^+$ and the lowest for $FL-NO_3^-$. The high statistical correlation between $FL-NH_4^+$ and chl a is unexpected since pelagic diatoms are unable to store NH_4^+ intracellularly. However, they can store large amounts of inorganic N in the form of NO_3^- (Dortch 1982, Lomas & Glibert 2000). If the intracellular NO_3^- pool in benthic diatoms can be used as a dynamic cell reservoir, the relationship between $FL-NO_3^-$ and chl a is likely to be complex and variable, depending on MPB growth rate and N-availability in the environment. Therefore, the

nity and, therefore, it could change with depth within the sediment. The significant increase of FL-NH$_4^+$/chl a and FL-PO$_4^{3-}$/chl a with depth below the photosynthetic layer suggests the existence of a FL-IN pool not associated with chl a, the relative importance of which increases with depth, as MPB biomass decreases (Fig. 5). Lomstein et al. (1990) observed that the proportions of the KCl-extractable and FL pools changed with depth within the sediment. The KCl-extractable NH$_4^+$ and NO$_3^-$ were 50 and 25 % of the FL concentrations, respectively, in the upper mm of the sediment, while they were similar or slightly higher below 5 mm depth (Lomstein et al. 1990).

Seasonal differences

Comparison of winter and summer samples revealed large differences in the FL and pore water pools of inorganic nutrients. The general absence of statistical correlations between the pore water nutrient concentration in the sediment and FL-IN and chl a indicate that the FL-IN and the pore water pools are largely unrelated or at least not connected in a simple way. The only exception was NO$_3^-$ in July, which correlated significantly with FL-NO$_3^-$ ($p < 0.01$). Interestingly, however, the slope of the regression equation changed from 4.42 in January to 23.60 in July, suggesting a larger accumulation of intracellular NO$_3^-$ for a very low external nitrate concentration in the pore water (results not shown). The content of FL-NO$_3^-$ normalised by chl a (FL-NO$_3^-$/chl a) in the upper sediment layer also indicates a slightly higher content of intracellular nitrate in the microphytobenthic biomass in July than in January, although the differences were not significant due to high variability in the data (Fig. 5).

Microphytobenthic biomass, as estimated by chl a, was higher in winter (Table 2); however, the microphytobenthic community was more productive in summer, resulting therefore in a higher production/biomass ratio (P/B). This higher P/B ratio indicates a more rapid turnover and, therefore, a higher gross growth rate of MPB during this season. The high productivity and growth rate of MPB in summer also coincided with lower concentrations of NO$_3^-$, NH$_4^-$ and NO$_2^-$ in the pore water compared to winter. The assimilation of NO$_3^-$ and NH$_4^+$ by MPB has been shown to play an essential role in the availability of these compounds at the sediment surface and their net fluxes to the water column (Sundbäck & Miles 2002). Therefore, the lower concentration of inorganic nitrogen compounds in the pore water in summer could be attributed to their consumption by MPB.

The FL-IN pool was always higher in winter except in the case of FL-NO$_3^-$. Therefore, a larger fraction of the sediment inorganic nutrients was immobilised in the microphytobenthic biomass in winter when MPB biomass, as estimated by chl a, was higher as well. However, the relative amount of FL-IN per unit of chl a in the sediment photic layer did not show significant changes with the season (Fig. 5). This is an unexpected result since, according to the cell quota model (Droop 1968), the intracellular content of nutrients in summer should be higher since P_n was significantly higher in this season. In addition, the storage of inorganic nutrients would provide MPB competitive advantage over bacteria, affecting the cycling of nutrients in the sediment.

CONCLUSIONS

The statistical relationship between the concentration of FL inorganic nutrients and chl a content in the sediment indicates that the main source of these nutrients is most likely the intracellular pool of MPB. Freezing and thawing of sediment samples breaks MPB cells, releasing their intracellular content into the pore water and increasing the efficiency of chlorophyll extraction. Nonetheless, other components of the sediment biological community like bacteria and meiofauna might be responsible for a fraction of the released nutrient. In addition, part of the FL-NH$_4^+$ and FL-PO$_4^{3-}$ might have been released from the exchangeable pool, adsorbed to the sediment matrix. The relative importance of the different biotic and abiotic sources of FL-IN is likely to change with the taxonomic composition of the benthic community and with the characteristics of the sediment. Therefore, if the aim is to measure nutrient concentrations in the pore water, the extraction should be done from fresh samples.

The independence and higher concentration of the FL-IN pool with respect to pore water concentration also indicated that MPB can actively accumulate nutrients. These intracellular nutrients are a variable and dynamic pool which could influence both microphytobenthic primary production and the sediment–water fluxes. In addition, the relationships between biomass (estimated here by chl a), FL-IN and pore water nutrients seem to be very dynamic, changing with depth within sediment and between summer and winter seasons.

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