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ORIGINAL ARTICLE

# Macrofauna regulate heterotrophic bacterial carbon and nitrogen incorporation in low-oxygen sediments

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**Oxygen minimum zones (OMZs) currently impinge upon >1 million km² of sea floor and are predicted to expand with climate change. We investigated how changes in oxygen availability, macrofaunal biomass and retention of labile organic matter (OM) regulate heterotrophic bacterial C and N incorporation in the sediments of the OMZ-impacted Indian continental margin (540–1100 m; $[O_2] = 0.35–15\ \mu mol\ l^{-1}$). *In situ* pulse-chase experiments traced $^{13}C$:$^{15}N$-labelled phytodetritus into bulk sediment OM and hydrolysable amino acids, including the bacterial biomarker D-alanine. Where oxygen availability was lowest ($[O_2] = 0.35\ \mu mol\ l^{-1}$), metazoan macrofauna were absent and bacteria assimilated 30–90% of the labelled phytodetritus within the sediment. At higher oxygen levels ($[O_2] = 2–15\ \mu mol\ l^{-1}$) the macrofaunal presence and lower phytodetritus retention with the sediment occur concomitantly, and bacterial phytodetrital incorporation was reduced and retarded. Bacterial C and N incorporation exhibited a significant negative relationship with macrofaunal biomass across the OMZ. We hypothesise that fauna–bacterial interactions significantly influence OM recycling in low-oxygen sediments and need to be considered when assessing the consequences of global change on biogeochemical cycles.**
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## Introduction

The bathyal continental margins (200–2000 m) are regions of high biogeochemical activity in the deep sea accounting for ~7% of global sea floor area, but recycle up to 30% of oceanic sedimentary organic matter (OM) (Middelburg *et al.*, 1997). Episodic deposition of phytoplankton-derived detritus (phytodetritus) represents a major OM input to the deep-sea floor (Billett *et al.*, 1983), stimulating the faunal and microbial feeding responses that mediate OM cycling (Witte *et al.*, 2003; Moodley *et al.*, 2005, 2011; Buhring *et al.*, 2006). Oxygen minimum zones (OMZs) are geologically stable water bodies where oxygen concentrations are persistently $<22\ \mu mol\ l^{-1}$, occuring in oceanic upwelling regions, where high surface primary productivity combines with poor advective mixing of the water column. At present, OMZs impinge upon >1 million km² of the sea floor, producing depth-dependent gradients in oxygen availability across the continental margins of the eastern Pacific, northern Indian and southwest Atlantic Oceans (Helly and Levin, 2004). OMZ-impacted sediments experience high OM accumulation and burial rates (for example, Cowie *et al.*, 1999). The low-oxygen conditions within OMZs provide suitable habitat for denitrifying and anaerobic ammonia-oxidising bacteria, with between 30% and 50% of marine nitrogen loss occurring in OMZ-impacted regions (reviewed in Francis *et al.*, 2007). In future climate projections, OMZs are predicted to expand with far-reaching implications for global biogeochemical cycles (Stramma *et al.*, 2010). Changes in oxygen availability and sediment geochemistry across an OMZ influence sediment community composition, producing changes in the faunal and bacterial assemblages that control the rates and pathways for OM recycling (Woulds *et al.*, 2007; Andersson *et al.*, 2008; Gooday *et al.*, 2009; Hunter *et al.*, 2011, 2012; Moodley *et al.*, 2011). Therefore, an understanding of the effects of oxygen limitation upon OM availability, sediment community structure and ecosystem processes is required in order to predict the effects of climate change upon global biogeochemical cycles.

The measurement of N dynamics in OMZs has primarily focussed upon quantifying inorganic N production rates and fluxes, from the sediment and through the water column (for example, Schwartz *et al.*, 2009; Ward *et al.*, 2009; Woulds *et al.*, 2009). Little information is available regarding bacterial

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utilisation of organic N within OMZs, yet the lability of sedimentary OM is partially determined by the availability of nitrogenous organic compounds, such as amino acids (Dauwe and Middelburg, 1998; Vandewiele *et al.*, 2009). The ecological mechanisms for OM recycling should, therefore, be considered in terms of both C and N processing (Thingstad, 1987). Stable-isotope labelling is a powerful experimental technique that allows C and N to be traced through an ecosystem. To date, application of this technique in OMZ-impacted sediments has focused upon the fate of organic carbon, within shipboard incubations (for example, Woulds *et al.*, 2007; Andersson *et al.*, 2008; Moodley *et al.*, 2011). The advent of a method to trace $^{15}N$ incorporation into hydrolysable amino acids (HAAs), including the bacteria-specific amino-acid D-alanine, (Veuger *et al.*, 2005, 2007b) makes it possible to empirically assess bacterial N utilisation and de-coupling of C and N in marine sediments (Cook *et al.*, 2007; Veuger and Middelburg, 2007; Evrard *et al.*, 2008).

*In situ* methods overcome many of the challenges associated with working in deep-sea hypoxic environments, ensuring that neither oxygen contamination nor changes in temperature and pressure influence experimental designs (Glud *et al.*, 1994; Hall *et al.*, 2007). In this study, the deep-sea submersible Shinkai 6500 deployed dual-labelling ($^{13}C$:$^{15}N$) pulse-chase experiments at the deep-sea floor. Phytodetrital C and N were traced into sediment bulk OM and HAAs, including the bacterial biomarker D-alanine (D-Ala). We investigate the changes in bacterial C and N incorporation and C:N coupling, across an OMZ-impacted continental margin; testing the hypotheses that bacterial activity is controlled by changes in macrofaunal biomass and the retention of labile OM within the OMZ-impacted sediments of the Indian continental margin.

## Materials and methods

*Study area*

The Arabian Sea OMZ impinges upon the western Indian continental margin between 150 and 1500 m producing depth-dependent changes in oxygen availability, sediment geochemistry and the mega- and macrofauna (Hunter *et al.*, 2011, 2012). The present study was carried out between September and November 2008, during R/V Yokosuka (JAMSTEC, Japan) cruise YK08-11. *In situ* pulse-chase experiments were conducted at four experimental stations (Figure 1) in the OMZ core (540 m) and lower OMZ boundary (800–1100 m) that differed in terms of oxygen availability (Table 1).

*Culture of labelled phytodetritus*

Phytodetritus was produced from an axenic clone of the diatom *Thalassiosira weissflogii* (NCMA, Bigelow Marine Laboratories, East Boothbay, ME, USA), cultured at 16 °C (light: dark = 16:8; salinity = 35; duration = 28 days) in artificial seawater enriched with L1 medium (Guillard, 1975). The medium contained 99% $^{13}C$-bicarbonate ($NaH^{13}CO_3$, Cambridge Isotope Laboratories, Andover, MA, USA), 50% $^{15}N$-sodium nitrate ($Na^{15}NO_3$, Cambridge Isotope Laboratories). Algae were harvested by centrifugation (500 g; 30 min), sonicated (2000 Hz; 5 min); washed three times in ultra-pure water (Milli-Q, Merck Millipore, Billerica, MA, USA) to remove inorganic salts and dissolved organic carbon; and lyophilised (−60 °C; −0.0001 mbar; 24 h) to produce phytodetritus. Phytodetrital labelling was 27.75 atom% $^{13}C$; 33.70 atom% $^{15}N$, with a C:N mass ratio of 4.04, measured via a Flash 1112 elemental analyser linked to a Thermo Delta Plus isotope ratio mass spectrometer (EA-IRMS; Thermo Scientific, Waltham, MA, USA).

*Stable-isotope labelling experiments*

*In situ* pulse-chase experiments were conducted using Oceanlab spreader mesocosms. Each spreader consisted of a transparent polycarbonate tube ($l = 30$ cm; $d = 25$ cm) and acetal plastic lid, which was gently pushed into the sediment to ~10 cm depth. The submersible then pushed a plunger through the lid to release a known dose of isotopically labelled phytodetritus onto the enclosed sediment (Figure 2). At each of the four stations, three replicate spreaders were deployed 2 m apart, by the submersible Shinkai 6500. Each spreader delivered a 650-mg C $m^{-2}$, 160-mg N $m^{-2}$ dose of $^{13}C$:$^{15}N$-labelled phytodetritus onto the enclosed sediments. These doses were equivalent to 0.76% (±0.14%) of the 0–1 cm bulk sediment OM pool at 540 m; 0.82% (±0.22%) at 800 m; and 1.07% (±0.7%) at T2 1100 m. Lids were left in place for a minimum of 2 h, to allow phytodetritus to settle onto the sediment, and then removed by *Shinkai 6500*. This provides semi-enclosed meso-cosms ($h = 20$ cm; $d = 25$ cm), exposing each experimental treatment to the ambient conditions at each station. In contrast to enclosed incubations, $DI^{13}C$ and $DI^{15}N$ fluxes could not be quantified. However, lid-removal mitigates against experimentally-induced anoxia, which may occur in low-oxygen environments (Riedel *et al.*, 2008). Mean current speeds were ~0.1 m $s^{-1}$ across study area (K Oguri *pers. comm.*), unlikely to result in sediment resuspension within the experiments. Experiments were left *in situ* for 4-day incubation periods. Three replicate spreaders were also deployed at stations T2 800 m and T2 1100 m for 7-day incubations. Following elapse of the incubation time each experiment was sampled using 8.2-cm-diameter push cores by Shinkai 6500, and the polycarbonate tubes recovered. Background sediment was sampled by three replicate push cores at each station. Only two spreaders could be sampled at T1 540 m, and two replicate background cores recovered at stations T1 540 m and

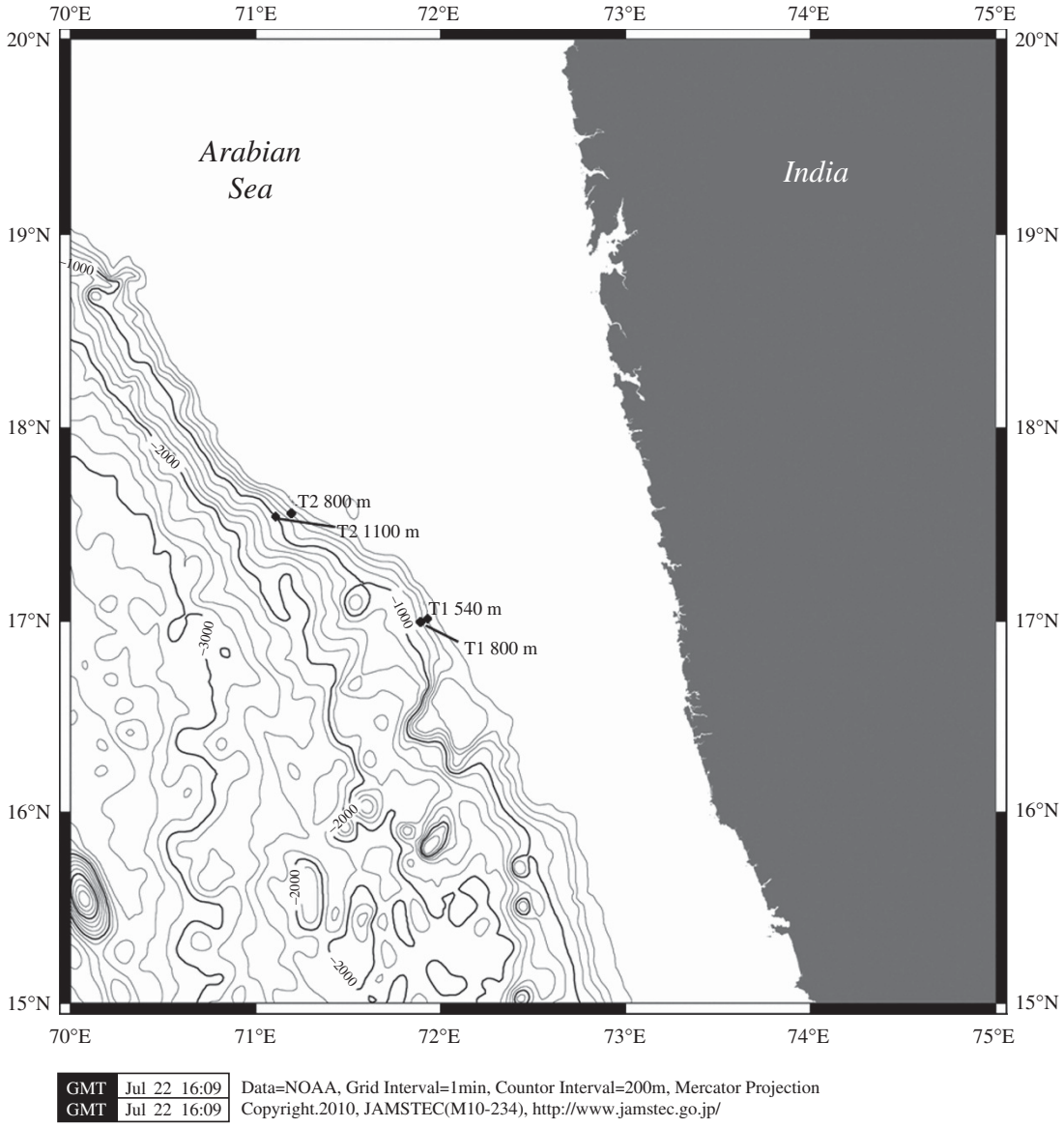


**Figure 1** Bathymetric map of the experimental stations.

T1 800 m. Upon recovery, cores were processed in a constant temperature room, set to sea floor ambient temperatures (Table 1). The upper 2 cm of the sediment cores were sliced into 0–1 and 1–2 cm sections, homogenised and deep frozen ($-80\,^{\circ}C$) for microbial and geochemical analysis.

*Analytical techniques*

The particulate organic carbon, total nitrogen concentrations and isotopic labelling ($^{13}C$ and $^{15}N$) in the sediment bulk OM pool was measured by elemental analyser-IRMS. Before analysis, bulk sediment samples were lyophilised ($-60\,^{\circ}C$; $-0.0001$ mbar; 24 h), acidified with excess 1 mol $l^{-1}$ hydrochloric acid ($HCl_{aq}$) to remove carbonates and dried to constant weight at 60 °C (following Hunter *et al.*, 2011).

$^{15}N$ and $^{13}C$ incorporation into the sediment HAAs and the bacterial biomarker D-alanine (D-Ala) were analysed using the method developed by Veuger *et al.* (2005, 2007b). Briefly, 0.2 g samples of lyophilised sediment were washed in 2 mol $l^{-1}$ $HCl_{aq}$ and Milli-Q to remove soluble material. Washed samples were hydrolysed with 6 mol $l^{-1}$ $HCl_{aq}$ (110 °C; 20 h) and purified by cation-exchange chromatography (DOWEX 50WX8-100 resin, Dow Water & Process Solutions, Midland, MI, USA). HAAs were derivatized with iso-propanol and pentafluoropropionic anhydride; further purified by solvent extraction (chloroform: phosphate buffer, 0.5: 1.0 *v*/*v*); and evaporated to dryness. Relative abundances of $^{15}N$ and $^{13}C$ in derivatized D-and L-amino acids and their concentrations were analysed on a Hewlett Packard 6890 gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) and

**Table 1** Environmental parameters and sediment community description at the four experimental stations identified by transect (T1 or T2) and depth.

| Station | $O_2$ concentration ($\mu mol\,l^{-1}$) | Temperature (°C) | Megafauna density ($ind\,m^{-2}$) | Macrofauna biomass ($mmol\,C\,m^{-2}$) | Sediment fraction (cm) | POC conc. ($mmol\,C\,g^{-1}$) | TN conc. ($mmol\,N\,g^{-1}$) | THAA conc. ($\mu mol\,C\,g^{-1}$) | Bacterial biomass ($\mu mol\,C\,g^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| T1 540 m | 0.35 | 12.1 | 0 | 0 | 0–1 | 2.08 (±0.67) | 0.19 (±0.06) | 39.5 (±5.8) | 47.8 (±30.1) |
| | | | | | 1–2 | 2.28 (±0.21) | 0.19 (±0.01) | 30.9 (±14.0) | 30.3 (±16.5) |
| T1 800 m | 2.2 | 10.1 | 5500 (±2726) | 7.04 (±2.52) | 0–1 | 2.41 (±0.79) | 0.24 (±0.08) | 64.3 (±7.6) | 71.6 (±38.4) |
| | | | | | 1–2 | 2.94 (±0.70) | 0.29 (±0.06) | 66.9 (±3.3) | 49.7 (±34.0) |
| T2 800 m | 2.36 | 9.9 | 4833 (±1126) | 6.29 (±3.53) | 0–1 | 2.76 (±0.39) | 0.27 (±0.05) | 55.3 (±5.1) | 35.4 (±7.8) |
| | | | | | 1–2 | 2.54 (±0.39) | 0.23 (±0.04) | 55.5 (±7.3) | 26.9 (±5.6) |
| T2 1100 m | 15 | 7.2 | 3572 (±1003) | 2.38 (±2.33) | 0–1 | 2.35 (±0.66) | 0.24 (±0.05) | 54.2 (±15.9) | 30.1 (±16.0) |
| | | | | | 1–2 | 2.79 (±0.86) | 0.26 (±0.06) | 47.5 (±3.5) | 18.9 (±8.9) |

Abbreviations: HAA, hydrolysable amino acids; POC, particulate organic carbon; TN, total nitrogen.
Oxygen concentrations, temperature and abundance of epi-benthic megafauna are from Hunter *et al.* (2011). Macrofaunal biomass are from Hunter *et al.* (2012). Concentrations of particulate organic carbon (POC concentration), total nitrogen (TN concentration), hydrolysable amino acids (HAA concentration) and bacterial biomass (estimated from PLFA concentrations) were determined from sediment samples collected.

Thermo Delta Plus IRMS linked via a Thermo type III combustion interface (GC-c-IRMS; Thermo Scientific). HAAs were also extracted from the labelled phytodetritus in order to determine their $^{13}C$ and $^{15}N$ enrichment.

Phospholipid-derived fatty acids (PLFAs) were extracted from spreader and background cores to quantify bacterial biomass. PLFAs were extracted from 1.5 g of lyophilised sediment using a single-phase extraction mixture of chloroform, methanol and citrate buffer (1: 2: 0.8 v/v/v), over 2 h, and fractionated on silicic acid columns (6 ml ISOLUTE SIS PE columns, International Sorbent Technologies Ltd, Ystrad Mynach, UK) by sequential elution with chloroform (neutral lipids), acetone (glycolipids) and methanol (phospholipids). Phospholipids were transmethylated fatty acid methyl esters obtained by alkaline methanolysis, and concentrations were determined by gas chromatography flame ionisation detection (Agilent 6890N, Agilent Technologies, Santa Clara, CA, USA) (following Thornton *et al.*, 2011).

*Data treatment*

Concentrations of carbon and nitrogen within sediment bulk OM and HAAs, and their isotopic ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) were determined by IRMS. Isotope ratios were used to calculate the atom% $^{13}C$ and $^{15}N$ for each sample by

$$X = \left[\frac{R_{sample}}{R_{sample}+1}\right]\times 100 \quad (1)$$

where $X$ is the $^{13}C$ or $^{15}N$ content (in atom%) and $R_{sample}$ is the isotopic ratio of the sample. $^{15}N$ and $^{13}C$ enrichments of both bulk OM and HAAs, expressed per gram dry sediment, were calculated by

$$E = \frac{(X_{sample} - X_{control})}{100}\times S_{conc.} \quad (2)$$

where $E$ is either the concentration of excess $^{13}C$ or $^{15}N$ within a HAA or sediment sample; $X_{sample}$ to be the atom% $^{13}C$ or $^{15}N$ of the sample; $X_{control}$ to be the natural abundance (atom%) of $^{13}C$ or $^{15}N$ of background HAAs or sediment OM; and $S_{conc.}$ is the concentration of HAAs (in $nmol\,g^{-1}$) or OM (in $\mu mol\,g^{-1}$). $^{13}C$ concentrations of the HAAs were corrected for addition of unlabelled carbon during derivatization, as described by Veuger *et al.* (2006). $^{13}C$ and $^{15}N$ enrichments of the bulk OM and HAAs were normalised to phytodetrital C ($_{phyto}C$) and N ($_{phyto}N$) incorporation by

$${}_{phyto}E = \frac{{}_{iso}E}{{}_{iso}F} \quad (3)$$

where ${}_{phyto}E$ is the concentration of $_{phyto}C$ or $_{phyto}N$, ${}_{iso}E$ is the excess $^{13}C$ and $^{15}N$ concentration of the sample and ${}_{iso}F$ is the $^{13}C$ or $^{15}N$ fraction of the phytodetritus ($^{13}C = 0.2775$; $^{15}N = 0.3370$).

Incorporation of $_{phyto}C$ and $_{phyto}N$ into the bacterial biomarker D-Ala is expressed relative to that in the amino acid L-alanine (L-Ala), as the D/L-Ala ratio calculated by

$$D/L - \mathrm{Ala} = \left(\frac{I_{D\text{-}Ala}}{I_{L\text{-}Ala}}\right) \quad (4)$$

where $I_{D\text{-}Ala}$ is the $_{phyto}C$ or $_{phyto}N$ incorporation by D-alanine and $I_{L\text{-}Ala}$ is the $_{phyto}C$ or $_{phyto}N$ incorporation by L-Ala. D-Ala data were not corrected for racemisation (formation of D-Ala from L-Ala during sample hydrolysis). Instead a racemisation background D/L-Ala ratio of 0.015 was measured for the phytodetritus (axenic *T. weissflogii*), and is applied in the calculation of the bacterial $_{phyto}N$ and $_{phyto}C$ incorporation as

$${}_{bact}\mathrm{Incorporation} = \frac{[\mathrm{Excess}_{D/L\text{-}Ala} - 0.015]}{[\mathrm{bact}_{D/L\text{-}Ala} - 0.015]}\times 100 \quad (5)$$

Where ${}_{bact}$Incorporation is the bacterial contribution (%) to label incorporation in the HAAs (%) and $\mathrm{Excess}_{D/L\text{-}Ala}$ is the D/L-Ala ratio for excess $_{phyto}C$ or $_{phyto}N$.

D/L-Ala ratios of 0.05 and 0.1 were used as minimum and maximum estimates of the bacterial contribution ($\mathrm{bact}_{D/L\text{-}Ala}$) (Veuger *et al.*, 2007b). These estimates are displayed graphically.

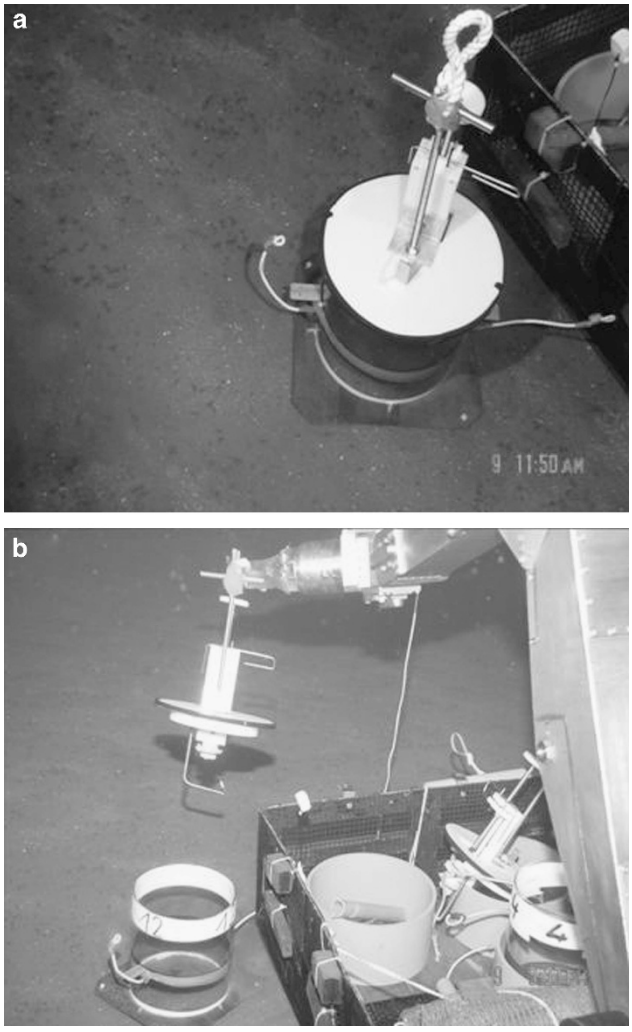


**Figure 2** Deployment of a spreader mesocosm by Shinkai 6500: (**a**) A spreader placed onto the sediment surface; (**b**) the submersible recovers the spreader lid, leaving the mesocosm *in situ*. All images were taken by UW during Shinkai 6500 dive SK1106 and are used courtesy of the Japan Agency Marine Science and Technology.

Bacterial biomass was calculated from the most common bacterial PLFAs across the four stations, *i*15:0, *ai*15:0, *i*16:0 and 16:1ω7c (Boschker and Middelburg, 2002), following Middelburg *et al.* (2000)

$$I_{\text{biomass}} = \sum \left( \frac{I_{\text{PLFA}}}{[a \times b]} \right) \quad (6)$$

where $I_{\text{biomass}}$ is the bacterial biomass; $I_{\text{PLFA}}$ is the concentration of a bacteria-specific PLFA; $a$ is the PLFA concentration of bacteria in hypoxic sediments (~0.038) (Brinch-Iversen and King, 1990); and $b$ is the fraction of bacterial PLFAs recovered from sediment samples at each station (T1 540 m, 0.28; T1 800 m, 0.25;T2 800 m, 0.22; T2 1100 m, 0.21).

Relationships between bacterial C incorporation ($_{\text{phyto}}$C D/L-Ala), and changes in macrofaunal biomass and $_{\text{phyto}}$C retention in the HAAs were tested for significance by robust median-based regression (Sen, 1968), using the *mblm* package (Komsta, 2007) in *R 2.14.1* (R Development Core Team, 2009). Bacterial N incorporation data ($_{\text{phyto}}$N D/L-Ala) were not amenable to statistical analysis because of missing values.

## Results

*Sediment geochemistry and microbial biomass*

Environmental conditions at each station are described by Hunter *et al.* (2011) and summarised in Table 1. HAAs made up 1.3–3.0% of the sediment organic C content and 15–30% of the sediment N content, across all stations. Highest values were recorded in 0–1 cm sediment layers at T1 800 m (HAA-C ~ 3.1%; HAA-N ~ 30%). Minimal values were observed in the 1–2 cm sediment layers at T1 540 m (HAA-C ~ 1.3%; HAA-N ~ 15%). Mean bacterial biomass ranged from 19 to 72 μmol $g^{-1}$. Sediment HAA concentrations and bacterial biomass exhibited high within-station variability but no trends across the study area (Table 1).

*Phytodetrital C and N within sediment bulk OM and amino-acid pools*

$_{\text{phyto}}$C and $_{\text{phyto}}$N labels within bulk OM and HAA pools were primarily restricted to the 0–1 cm sediment layers (Figure 3). Only at the core of the OMZ (T1 540 m) were $_{\text{phyto}}$C and $_{\text{phyto}}$N detectable within the 1 – 2-cm HAA pool, after 4 days (Figures 3c and d). Higher concentrations of $_{\text{phyto}}$C and $_{\text{phyto}}$N were retained in the bulk OM and HAAs at stations on transect 1 (T1 540 m, T1 800 m) compared with transect 2 (T2 800 m, T2 1100 m). No changes in $_{\text{phyto}}$C or $_{\text{phyto}}$N retention are observed in sediment bulk OM or HAAs between 4 and 7 days (Figures 3a and b).

*Bacterial incorporation of phytodetrital C and N*

Incorporation of $_{\text{phyto}}$C and $_{\text{phyto}}$N into D-Ala were highest after 4 days at the core of the OMZ (T1 540 m): ranging from 0.036 to 0.045 in 00–01 cm sediments; and 0.039–0.048 in 01–02 cm sediments. Here, bacterial incorporation accounted for 30–90% of the $_{\text{phyto}}$C and $_{\text{phyto}}$N retained within the HAA pool (Figure 4). In the OMZ lower boundary (800–1100 m), $_{\text{phyto}}$C and $_{\text{phyto}}$N D/L-Ala ratios were close to the racemisation background, with bacterial contribution to $_{\text{phyto}}$C in the total HAAs accounted for only 6–11%. Bacterial incorporation of $_{\text{phyto}}$N was low across the OMZ lower boundary (800–1100 m), with the exception of one incubation at T2 800 m, where bacteria accounted for 22–54% of the $_{\text{phyto}}$N in HAAs. After 7 days, D/L-Ala ratios show bacterial incorporation accounted for maxima of 28 and 22% of the $_{\text{phyto}}$C in the HAA pool at T2 800 m and T2 1100 m. After 7 days, bacterial $_{\text{phyto}}$N

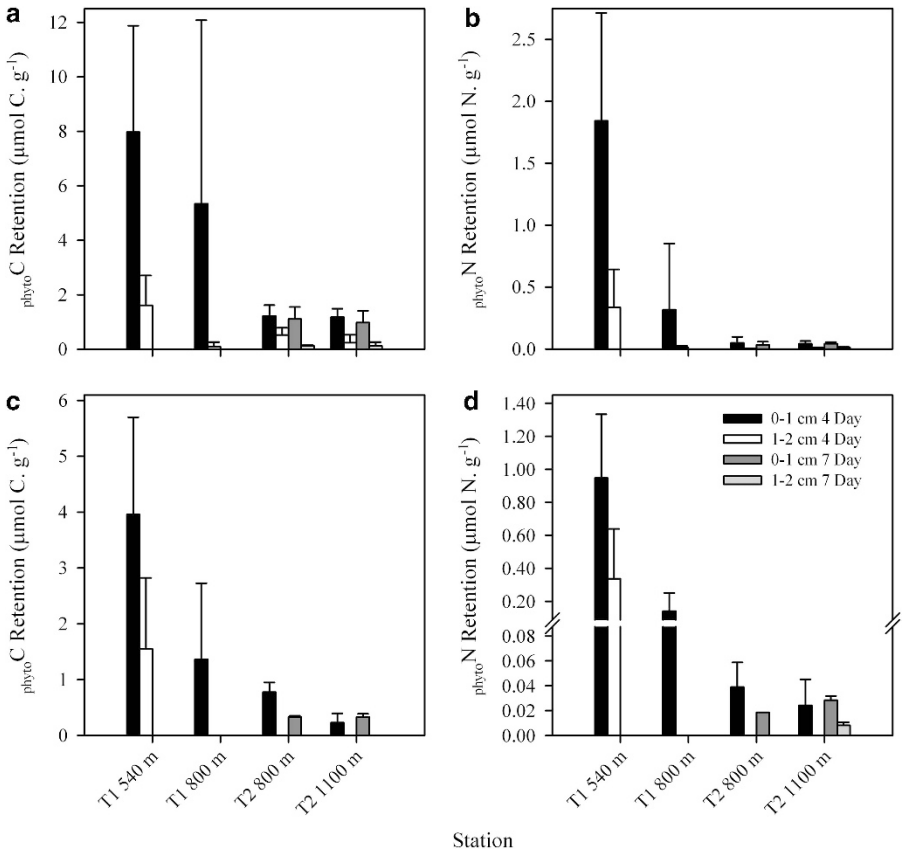


**Figure 3** Mean (± data ranges) phytodetrital carbon (**a**, **c**) and nitrogen (**b**, **d**) retention in the sediment bulk OM (**a**, **b**) and HAAs (**c**, **d**), over 4 and 7 days.

incorporation accounted for between 23% and 50% of the $_{phyto}$N within the sediment HAA pool, at T2 1100 m (Figure 4).

A significant negative relationship between bacterial $_{phyto}$C incorporation and changes in macrofaunal biomass occurred across the study area (Figure 5). The estimated $R^2$ value indicates that this relationship explained approximately 26% of the variation in bacterial $_{phyto}$C incorporation. No relationship was observed between bacterial $_{phyto}$C incorporation and retention of $_{phyto}$C within the sediment HAA pool ($P = 0.991$).

*De-coupling of phytodetrital C and N*

The $_{phyto}$C:$_{phyto}$N ratios of the sediment bulk OM exhibited little difference between stations and incubation times (Figure 6a). Values ranged between 8 and 12 and were consistently greater than the C:N ratio of the bulk phytodetrital material added (4.04). In the OMZ core, the $_{phyto}$C:$_{phyto}$N ratios of the sediment HAA pool was substantially lower than that of the added phytodetrital HAAs, revealing a preferential retention of $_{phyto}$N within the HAAs (Figure 6b). $_{phyto}$C:$_{phyto}$N ratios were higher at the OMZ boundary stations. At oxygen concentrations $\sim 2\,\mu mol\,l^{-1}$ $_{phyto}$C:$_{phyto}$N ratios ranged across the C:N ratio of the phytodetrital HAAs (16.65), with peak values of 18.8 and 21.8 after 4 days, and a value of 18.8 recorded after 7 days. At oxygen concentrations of $15\,\mu mol\,l^{-1}$, $_{phyto}$C:$_{phyto}$N ratios were lower than the C:N ratio of the added phytodetritus, indicating enhanced $_{phyto}$N retention within sediment HAAs. Between 4 and 7 days changes in $_{phyto}$C:$_{phyto}$N ratios at T2 1100 m, suggest a progressive loss of $_{phyto}$N (Figure 6b).

## Discussion

*Methodological considerations*

Previous studies have used isotopic labelling of HAAs as a proxy for microbial $^{13}$C and $^{15}$N incorporation (Tobias *et al.*, 2003; Veuger *et al.*, 2007a), assuming sediment HAAs to account for between 50% and 60% of bacterial biomass (Cowie and Hedges, 1992). However, low oxygen mediates the preservation of biomolecules resulting in a relatively large amino-acid pool within OMZ-impacted sediments (Cowie *et al.*, 1999; Vandewiele *et al.*, 2009). In the present study, HAA concentrations were used as an indicator of labile OM availability and bacterial incorporation of $_{phyto}$C and $_{phyto}$N were traced using the bacteria-specific amino acid D-Ala. Unlike its stereoisomer L-Ala, which is present in all organisms, D-Ala is

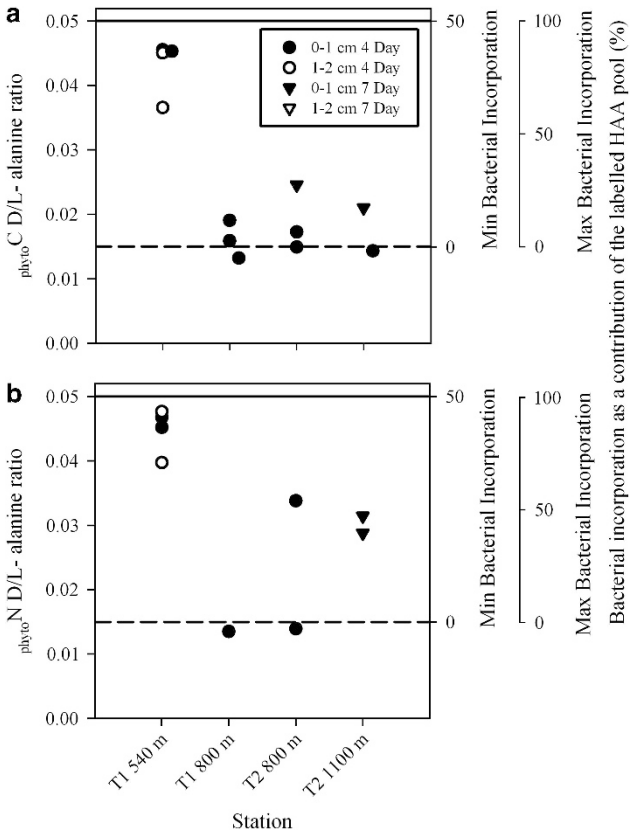


**Figure 4** D/L-Ala ratios and corresponding minimum and maximum estimates of contribution of bacterial assimilation to total labelled HAA pools of (**a**) phytodetrital carbon ($_{phyto}$C in D-Ala/ $_{phyto}$C in L-Ala) and (**b**) phytodetrital nitrogen ($_{phyto}$N in D-Ala/$_{phyto}$N in L-Ala). Dashed line indicates the racemisation background. Missing data points occur where $_{phyto}$C or $_{phyto}$N D-alanine concentrations fell below detectable thresholds.

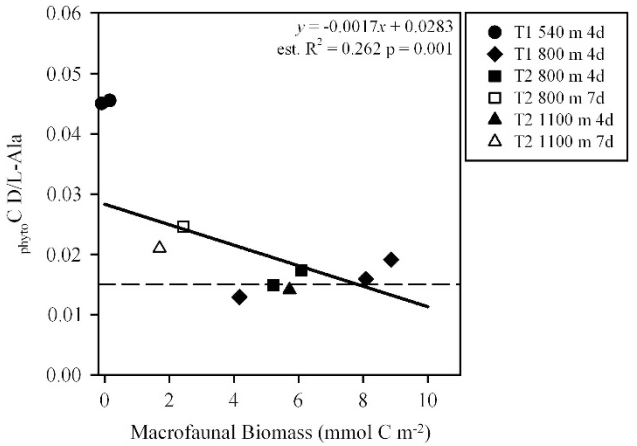


**Figure 5** Robust linear regression of $_{phyto}$C D/L-Ala ratios, in 0–1 cm sediment, against macrofaunal biomass. Dashed line indicates the racemisation background.

specific to bacteria. Thus, $^{13}$C and $^{15}$N labelling of D-Ala provides direct evidence of bacterial incorporation of $_{phyto}$C and $_{phyto}$N (Veuger *et al.*, 2005). Ratios between D and L-Ala subsequently reflect the

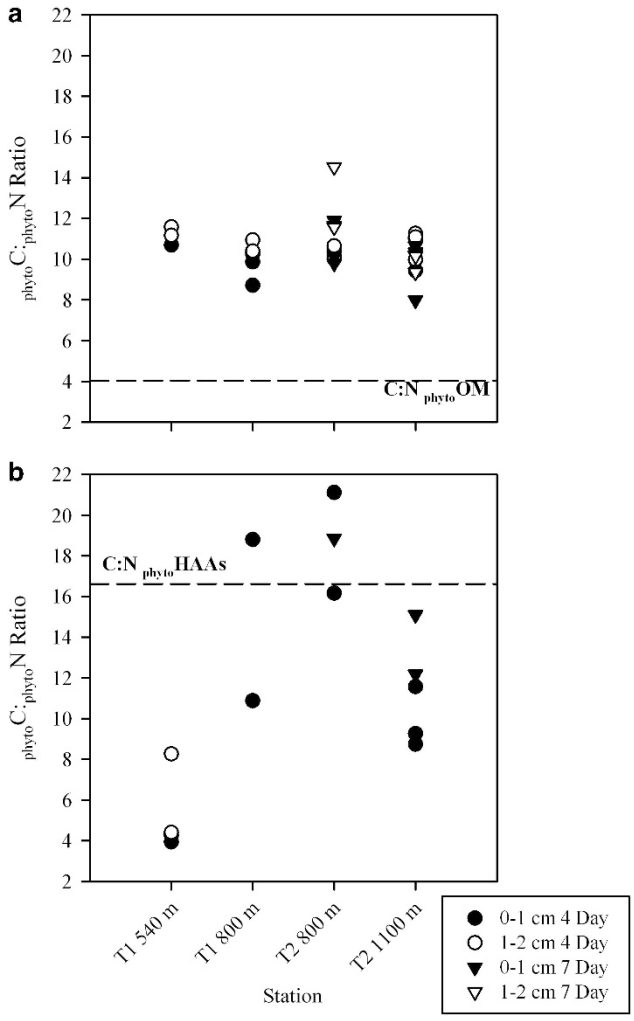


**Figure 6** $_{phyto}$C:$_{phyto}$N ratio of (**a**) the sediment bulk OM and (**b**) hydrolysable amino-acid pools. Dashed line indicates the C:N mass ratios of (**a**) the bulk phytodetrital OM and (**b**) the phytodetrital HAAs.

fraction of the total labelled HAA pool incorporated into bacterial biomass. Therefore, D/L-Ala ratios provide biomass specific indices of bacterial $_{phyto}$C and $_{phyto}$N (Veuger and Middelburg, 2007; Veuger *et al.*, 2007a). While D-Ala is bacteria-specific, it also forms by the racemisation of L-Ala during the hydrolysis and extraction of amino acids. As a result, D/L-Ala ratios were plotted against a racemisation background value obtained from D/L-Ala ratio of the $^{13}$C:$^{15}$N-labelled phytodetritus (following Kaiser and Benner, 2005; Veuger *et al.*, 2007b).

It was not feasible to determine sediment OM stocks *a priori*, and so a fixed phytodetritus dose (650 mg C m$^{-2}$) was used. Between stations, differences in relative phytodetritus dose between stations were small and unlikely to have an impact upon experimental results. The use of semi-enclosed mesocosms potentially exposed the experimental treatments to sediment resuspension and intrusion by epi-benthic megafauna. While this may

introduce additional error sources into the experiments, it ensures each experiment approximates the natural disturbances within the sea-floor environmental regime. Megafaunal abundances were co-linear to macrofaunal biomass. Therefore, we cannot exclude potential roles for megafauna–macrofauna and megafauna–bacterial interactions as regulators of bacterial activity.

*Phytodetrital C and N retention and bacterial assimilation across the OMZ-impacted Indian margin*

In the present study, $_{phyto}$C and $_{phyto}$N retention within the sediment bulk OM and HAA pools changed across the study area, reflecting differences in the availability of oxygen and the presence of macrofauna and megafauna (Table 1). Sedimentary $_{phyto}$C and $_{phyto}$N concentrations were highest in the OMZ core, after 4 days. In this region, oxygen limitation and the absence of mega- and macrofauna are important factors promoting OM preservation (for example, Woulds *et al.*, 2007; Jeffreys *et al.*, 2009 and references therein). In contrast, $_{phyto}$C and $_{phyto}$N retention in the OMZ boundary (800–1100 m) was variable, with high $_{phyto}$C and $_{phyto}$N concentrations in bulk OM and HAA pools at T1 800 m, compared with the relatively low values at T2 800 m and T2 1100 m. This increased variability occured concomitantly with the presence of macrofauna (Table 1) and oxygen concentrations high enough to support nitrification but still sufficiently low to sustain denitrification and anaerobic ammonia-oxidising pathways (Devol, 1978; Jensen *et al.*, 2011). D/L-Ala ratios demonstrate that in the OMZ core, bacterial assimilation accounted for 30–90% of the $_{phyto}$C and $_{phyto}$N labelling of the HAAs. This occurs concomitantly with peaks in $_{phyto}$C and $_{phyto}$N retention in the bulk OM and HAA pools (Figures 3 and 4), and oxygen concentrations $\sim$0.2% of those outside the OMZ (Hunter *et al.*, 2011). In the OMZ boundary, bacterial $_{phyto}$C and $_{phyto}$N incorporation were low after 4 days, despite oxygen levels between 6 and 42 times higher than those OMZ core. However, increased $_{phyto}$C and $_{phyto}$N incorporation between 4 and 7 days indicates retardation of bacterial feeding activity.

Besides the difference in oxygen, there were differences in temperature between stations (Table 1). While ambient temperature is an important control on deep-sea bacterial metabolism (Moodley *et al.*, 2005), Andersson *et al.*, (2008) observed little change in bacterial activity over a similar temperature range at the OMZ-impacted Pakistan margin. In the present study, the primary factor controlling bacterial $_{phyto}$C and $_{phyto}$N incorporation appears to be oxygen availability, either directly or indirectly. A direct effect of oxygen is unlikely because anaerobic bacteria predominate within the sediments of the OMZ-impacted Indian margin (Gonsalves *et al.*, 2011), while facultative anaerobes can sustain aerobic metabolism at oxygen concentrations $\sim$3 nmol l$^{-1}$ (Stolper *et al.*, 2010). Therefore, consistent with Moodley *et al.* (2011) and Andersson *et al.* (2008), we propose that oxygen availability did not directly influence bacterial activity.

Bacterial C and N incorporation were indirectly controlled by changes in oxygen availability through its influence upon benthic macrofaunal assemblages. Hunter *et al.* (2012) report that macrofauna consumed up to 455 μmol C m$^{-2}$, and 25 μmol N m$^{-2}$, after 4 days at 800 and 1100 m stations. These observations, in combination with the loss of $_{phyto}$C and $_{phyto}$N from the sediment HAAs indicate that fauna were active in processing phytodetrital OM. Absence of fauna at T1 540 m, however, prevent us from directly testing the effects of faunal activity upon the bacteria. However, macrofaunal biomass is strongly correlated to faunal OM uptake (for example, Middelburg *et al.*, 2000; Woulds *et al.*, 2007), and it is likely that differences in macrofaunal activity regulate bacterial $_{phyto}$C and $_{phyto}$N incorporation. Bacterial activity can also be limited by labile OM availability (Arrieta and Herndl, 2001; Nausch and Kerstan, 2001) with episodic OM depositions driving the feeding response of deep-sea sediment communities (Witte *et al.*, 2003; Moodley *et al.*, 2005, 2011). While bacterial activity decreased concomitantly with decreases in retention of $_{phyto}$C and $_{phyto}$N in the sediment HAA pools, this exhibited no significant relationship. This may be partially accounted for by high within-station variation in the sediment HAA pools and limited replication within the present study. Nevertheless, changes in bacterial feeding responses were attributed to differences in macrofaunal biomass across the OMZ. It has been shown experimentally that macrofauna feed more efficiently upon concentrated phytodetritus depositions than bacteria, making OM available to the microbial assemblages through excretion of faecal material (van Nugteren *et al.*, 2009). Thus, stimulation of macrofaunal feeding responses may also result in retardation of phytodetrital C and N incorporation by the bacteria, consistent with increases in bacterial incorporation of $_{phyto}$C and $_{phyto}$N, which may be observed between 4 and 7 days.

*De-coupling of phytodetrital C and N in OMZ-impacted sediments*

Excess $_{phyto}$C:$_{phyto}$N ratios of the bulk sediment and HAAs at the four stations reveal differences in C:N coupling across the oxygen gradient (Figure 6). At the OMZ core $_{phyto}$C:$_{phyto}$N ratios of the sediment HAA pool were substantially lower than that of the added phytodetrital HAAs, indicating stronger $_{phyo}$N retention probably because of preferential bacterial incorporation of $_{phyto}$N. By contrast, $_{phyto}$C:$_{phyto}$N ratios of bulk sediment OM changed little between stations, indicating a preferential loss of $_{phyto}$N, from the bulk sediment OM pool, at all stations. The large

difference between the C:N ratios of the bulk sediment OM and added phytodetritus suggest the presence of a $^{15}$N-labelled pool of dissolved inorganic nitrogen within the isotopically labelled phytodetritus. As such, the preferential loss of $_{phyto}$N from the bulk OM pool is likely to be an experimental artefact caused by DI$^{15}$N leaching from phytodetrital cells.

At the OMZ core, high bacterial $_{phyto}$C and $_{phyto}$N incorporation, demonstrates the importance of bacteria in OM recycling pathways. Organic C is available in a variety of forms (lipids; carbohydrates; amino acids), but the main sources of organic N are amino acids. The $_{phyto}$C:$_{phyto}$N ratios of the HAAs at this station demonstrate preferential $_{phyto}$N incorporation by the bacteria, at this station. Previous studies emphasise the importance of extracellular amino-acid oxidation as a pathway for N incorporation in marine systems (Pantoja and Lee, 1994; Mulholland *et al.*, 2003; Veuger and Middelburg, 2007). In the extracellular amino-acid oxidation pathway bacteria liberate $NH_4^+$ from amino acids outside their cell walls (Pantoja and Lee, 1994). While we cannot determine the importance of direct and indirect N uptake pathways, extracellular amino-acid oxidation represents an efficient method for microbial N incorporation because the energetic costs of transporting $NH_4^+$ across the cell membrane are lower than those of amino acid transport (Berman *et al.*, 1999). Thus, extracellular amino-acid oxidation represents a mechanism by which deep-sea bacteria could preferentially incorporate nitrogen from episodic OM depositions.

In the OMZ boundary, excess $_{phyto}$C:$_{phyto}$N ratios of the sediment HAAs reveal differences in C:N coupling linked to the changes in oxygen availability and the presence of fauna. At the low-oxygen stations (800 m, $[O_2] = 2.20–2.36\ \mu mol\ l^{-1}$) $_{phyto}$C:$_{phyto}$N ratios indicate reduced biological transformation of the HAAs remaining within the sediment, characterised by a weak $_{phyto}$N loss. Concomitantly, the significant relationship between bacterial activity and faunal biomass, with reduced $_{phyto}$C and phytoN retention within faunated sediments, suggests that the macrofauna regulate labile OM availability. Macrofaunal grazing has been observed to control the release of dissolved organic nitrogen in marine sediments (Gilbert *et al.*, 1991) and provides pathways for OM mineralisation, to DIC and $NH_4^+$ (for example, Woulds *et al.*, 2007, 2009). In marine systems, macrofaunal invertebrates are ammonotelic, mineralising relatively large quantities of organic N to $NH_4^+$, compared with the N retained in their body tissues (Wright, 1995). Macrofaunal feeding may provide the mechanism for the loss of $_{phyto}$N loss observed at 800 m, while faunal–bacterial interactions limit bacterial activity. Potentially, this may facilitate utilisation of the phytodetrital N by heterotrophic denitrifying bacteria (Devol, 1978); and both aerobic and anaerobic ammonia-oxidising microbes (Pitcher *et al.*, 2011), whose distributions overlap in oxygen concentrations between 1 and 3 $\mu mol\ l^{-1}$. This hypothesis is supported by observations of enhanced inorganic N fluxes and nitrite production following deposition of phytodetritus at the 800 m stations (C Woulds and H Suga, personal communication).

At the higher oxygen station (1100 m; $[O_2] = 15\ \mu mol\ l^{-1}$) increased retention of $_{phyto}$N within the sediment THAAs after 4 days, occurred concomitantly with low bacterial activity level. Between 4 and 7 days, net $_{phyto}$N loss was observed from sediment HAAs, as bacterial $_{phyto}$C and $_{phyto}$N incorporation increased. This suggests that bacterial activity is retarded, where the OMZs influence upon the sediment is weaker. Lower macrofaunal biomass at this station (Table 1) may retard $_{phyto}$N loss by remineralisation, potentially reducing faunal–bacterial competition for the phytodetrital N source. This would allow increased niche differentiation between macrofaunal and bacterial assemblages (van Nugteren *et al.*, 2009). We propose that macrofaunal activity makes OM available for bacterial utilisation and provides pathway for gradual $_{phyto}$N loss. This is consistent with previous observations from abyssal sediments (>4000 m), where faunal activity mediated bacterial C incorporation (Witte *et al.*, 2003). These effects may be enhanced by megafaunal activity, with high densities present at both 800 and 1100 m. Epi-benthic megafauna are known to have an important role controlling availability of labile OM in OMZ-impacted sediments (for example, Smallwood *et al.*, 1999; Jeffreys *et al.*, 2009), potentially contributing to $_{phyto}$N loss across study area.

Bacterial activity may also be regulated by changes in other faunal assemblages across the OMZ, such as the foraminifera and metazoan meiofauna. Both these assemblages exhibit strong zonation across OMZs, with foraminiferal distributions controlled by sediment OM availability and high foraminiferal densities observed where metazoans are absent (for example, Gooday *et al.*, 2009). By contrast, meiofaunal densities are positively correlated to macrofauna densities, potentially relying on macrofaunal bioturbation to provide suitable microhabitats (Cook *et al.*, 2000). Both foraminfera and meiofauna may graze upon bacteria (for example, Nomaki *et al.*, 2006; Pascal *et al.*, 2008). However, they may also enter into symbiotic relationships with heterotrophic bacteria; for example, in low-oxygen sediments bacterial metabolism provides an inorganic N source for nitrate-respiring foraminifera (Risgaard-Petersen *et al.*, 2006). Interactions between the bacteria, foraminifera, and both metazoan meio- and macrofauna are potentially complex. Therefore, targeted experiments are necessary to disentangle these trophic linkages.

The present study demonstrates that macrofaunal responses to oxygen limitation regulate the heterotrophic bacterial C and N cycling pathways in marine sediments. While macrofaunal presence

was linked to lower retention of phytodetrital OM and preferential loss of organic N from sediments, the relationship between bacterial $_{phyto}$C incorporation and the retention of phytodetrital OM within the sediment was non-significant. We hypothesise that macrofaunal regulation of sediment bacterial activity is complex, potentially occurring via niche-partitioning and interactions with other faunal groups, such as the megafauna, foraminifera and meiofauna. OMZ expansion is likely to have implications upon sea floor biogeochemical processes, with reduced oxygen availability associated with increased nitrogen loss from the sediments. Furthermore, exclusion of fauna at extreme low-oxygen levels is associated with a shift to OM processing by heterotrophic bacteria and retention of organic N within sediments. These hypotheses require testing over longer time periods within a manipulative experimental framework, to further explore faunal–bacterial interactions.

## Conflict of interest

The authors declare no conflict of interest.

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## Author contributions

Experiments were designed by UW, and conducted by UW and WRH. Analytical work was carried out by WRH and BV. WRH, BV and UW all contributed to the writing of the manuscript.

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