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Nitrogen fixation within the water column associated with two hypoxic basins in the Southern California Bight

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ABSTRACT: We measured pelagic dinitrogen (N_2) fixation by incorporation of $^{15}N_2$ during regular cruises over 4 yr to deep hypoxic basins in the Southern California Bight, USA. N_2 fixation in the photic zone was dominated (80%) by nanoplankton (<10 µm). N_2 fixation rates in surface waters were near the upper range measured for nanoplankton in tropical waters, averaging 5.8 µmol m^{-3} d^{-1} at the San Pedro Ocean Time Series (SPOTS) station, and 2.4 µmol m^{-3} d^{-1} at the Santa Monica Bay Observatory (SMBO) station, with a maximum at SPOTS of 35 µmol m^{-3} d^{-1}. Quantitative polymerase chain reaction (qPCR) assays and nested PCR-based clone libraries targeting the nitrogenase gene *nifH* indicate that the uncultivated unicellular cyanobacterial group A (UCYN-A) is an abundant diazotroph in the photic zone. Although N_2 fixation rates were highest at the surface, mean N_2 fixation averaged 0.07 µmol m^{-3} d^{-1} at depths of 500 and 885 m within hypoxic basin waters (<10% O_2 saturation). When integrated over the aphotic water column, this deep N_2 fixation may account for as much as one-third of the total areal N_2 fixation, estimated at 150 µmol N m^{-2} d^{-1}. These deep hypoxic N_2 fixers were an assemblage of heterotrophic bacteria, including *Alpha-* and *Gammaproteobacteria* and putative sulfate-reducing bacteria. Our results suggest that N_2 fixation could play a role in other hypoxic, high-nitrate waters.

KEY WORDS: Nitrogen fixation · Diazotrophy · Southern California Bight · Hypoxic basin

INTRODUCTION

The inventory of bioavailable (fixed) nitrogen (N) in the ocean is largely regulated by nitrogen fixation, denitrification, and anaerobic ammonium oxidation (anammox) — microbial processes that mediate exchanges with the dominant dinitrogen (N_2) pool. The consistency of atmospheric carbon dioxide (CO_2) levels over the last 10 000 yr does not support large-scale changes in the inventory of fixed N, suggesting that N_2-fixing and N_2-producing processes may be tightly coupled via negative feedbacks (Gruber & Sarmiento 1997, Deutsch et al. 2001, Karl et al. 2002, Gruber 2004). Others have suggested that the sources and sinks of marine N may not be in balance over glacial–interglacial time scales or that high anthropogenic N_2 fixation might have unbalanced the anthropocene (e.g. the post-industrial) N cycle (discussed in Codispoti et al. 2001, Gruber 2004, Codispoti 2007). One proposed mechanism that could regulate the balance between denitrification and N_2 fixation is the suggestion that diazotrophs may be able to out-

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compete other phytoplankton when nitrate (NO_3^-) concentrations are limiting, a condition that could be exacerbated by denitrification (Karl et al. 2002). However, experimental work demonstrates that N_2 fixation can still take place even at relatively high NO_3^- concentrations (Mulholland et al. 2001, Holl & Montoya 2005).

Despite the assertion that sources and sinks of N to the ocean must be in balance, most scaled estimates of marine N sinks greatly exceed estimated sources (Codispoti et al. 2001, Galloway et al. 2004, Gruber 2004, Codispoti 2007). In the effort to address this imbalance, nitrogen fixation, the largest N source term, has received a great deal of attention, and yet its magnitude is not well constrained (Codispoti et al. 2001). Marine N_2 fixation appears to be dominated (ca. 90%) by pelagic processes (Codispoti et al. 2001, Galloway et al. 2004). To address problems with scaling point measurements of N_2 fixation, geochemical estimates of pelagic N_2 fixation have been developed based on observed sub-euphotic deviations in the ratio of dissolved inorganic N to P (phosphate, PO_4^{3-}) relative to that expected from the canonical Redfield ratio (N:P = 16; Redfield et al. 1963). One such measure, N*:

$$N^* = [NO_3^-] - 16\,[PO_4^{3-}] + 2.9\ \mu mol\ kg^{-1} \quad (1)$$

is a measure of the deviation of the ratio $[NO_3^-]$:$[PO_4^{3-}]$ expected from the degradation of average plankton organic matter (Gruber & Sarmiento 1997, Deutsch et al. 2001). The limitation of the tracer N* is that it cannot disentangle the opposing effects of denitrification and N_2 fixation on the dissolved N:P ratio. However, a recent approach examines the deviation of assimilative uptake of PO_4^{3-} relative to the expected NO_3^- uptake, via changes in the parameter P*:

$$P^* = [PO_4^{3-}] - [NO_3^-]/16 \quad (2)$$

from which Deutsch et al. (2007) estimated a global pelagic N_2 fixation rate of ca. 140 Tg yr^{-1}. In contrast, extrapolations of incubation-based measures of known N_2-fixing organisms can account for only ca. 90 Tg yr^{-1} (Galloway et al. 2004). These estimates have been based on measures of the conspicuous colonial planktonic non-heterocystous cyanobacterium *Trichodesmium* (Capone et al. 1997, 2005), until recently assumed to be the most significant known contributor to pelagic N_2 fixation. *Trichodesmium* plays the greatest role in tropical oligotrophic surface waters with temperatures >25°C, but in recent years, other significant contributors to pelagic N_2 fixation have been discovered. The recovery of *nifH* transcripts related to unicellular cyanobacteria (Zehr et al. 2001, Moisander et al. 2010) as well as the detection of N_2 fixation associated with the <10 µm size class of ocean plankton using the $^{15}N_2$ incorporation technique (Montoya et al. 2004) has highlighted the importance of unicellular nanoplankton in pelagic N_2 fixation. Further, the importance of N_2 fixation in subtropical and temperate waters with surface temperatures <20°C (previously excluded from global N budgets) is now supported by both geochemical modeling (Deutsch et al. 2007) as well as direct measures (Holl et al. 2007, Needoba et al. 2007, Rees et al. 2009, Moisander et al. 2010).

The shortfall in the marine N budget, as well as the insufficiency of *Trichodesmium* alone to account for the geochemically estimated N_2 fixation, has led to a renewed search for N_2 fixation by previously overlooked planktonic organisms and in previously overlooked ocean provinces. From analysis of the geochemical parameter P* combined with a general circulation model of the oceans, Deutsch et al. (2007) suggested that regions of denitrification and N_2 fixation in the oceans might be coupled, with some of the highest rates of N_2 fixation predicted to coincide with surface waters affected by the oxygen minimum zones (OMZs) of the Pacific and Indian oceans, which are important sites for denitrification and/or anammox (Hamersley et al. 2007). The proximity of N_2 fixation and denitrification zones suggests the possibility of coupling and a regulation of N_2 fixation by the dissolved N:P ratio (Deutsch et al. 2007).

In the present study, we examined N_2 fixation in the waters adjacent to 2 hypoxic basins in the Southern California Bight. The California Undercurrent transports water bearing the signature of OMZ denitrification (N* < 0) from the eastern tropical North Pacific northward (Codispoti & Richards 1976). Restricted circulation within the basins of the Southern California Bight results in varying degrees of water column hypoxia below the sill depth (Berelson 1991). Upwelling events transport nitrate-rich deep water into the mixed-layer, resulting in periodic algal blooms (Nezlin & Li 2003). Sea surface temperatures (SST) peak up to 22°C in the summer, but fall to ca. 14°C during the winter months (Nezlin & Li 2003).

We measured N_2 fixation via incorporation of $^{15}N_2$ during regular cruises to the waters adjacent to the San Pedro and Santa Monica Basins, near Los Angeles, California (34° N latitude), USA. In the San Pedro Basin, we incubated whole water, size-fractionated particulates by filtration at assay termination, and made measurements in the photic zone as well as deep within the aphotic hypoxic zone. We also characterized the abundance and diversity of the nitrogenase *nifH* gene in the water column through quantitative PCR (qPCR) and construction of *nifH* clone libraries. In the surface waters of Santa Monica Bay, we measured N_2 fixation using trace-metal-clean approaches to study the sensitivity of N_2 fixation to small additions of iron or phosphate.

MATERIALS AND METHODS

Site description and field sampling. We measured hydrographic and chemical profiles and N_2 fixation rates during cruises to 2 sites in the Southern California Bight near Los Angeles, USA. The San Pedro Ocean Time Series (SPOTS) station, near the center of the 900 m deep San Pedro Basin (Fig. 1), was monitored from 14 July 2004 to 13 September 2006 from the RV 'Sea Watch' (Southern California Marine Institute). Hydrographic and chemical profiles were measured on 26 cruises, and N_2 fixation was measured during 15 of these. Nitrogen fixation at SPOTS was measured at the surface, chlorophyll maximum (range 16 to 37 m), 500 m, and 885 m (ca. 1.5 m from bottom). The Santa Monica Bay Observatory (SMBO) station, a site 470 m deep on the margin of the Santa Monica Basin (Fig. 1), was monitored from 11 July 2006 to 7 July 2007 from the RV 'Seaworld' (University of California, Los Angeles, UCLA). Hydrographic and chemical profiles were measured during 25 cruises, and N_2 and C fixation were measured on 8 of these cruises. All reported errors are standard errors (SE).

Hydrographic and chemical measurements. Hydrographic measurements and water samples at SPOTS were collected using a CTD-rosette equipped with 12 Niskin bottles, a Seabird 911 plus CTD with a model 13 oxygen sensor, and a Seapoint chlorophyll fluorometer. *In situ* measurements were calibrated with Winkler titrations (oxygen) and fluorometric measurements of extracted chlorophyll *a* (chl *a*) (Grasshoff et al. 1999) in bottle samples collected at 12 depths throughout the water column. We also identified the diazotrophs present in the photic and aphotic zones on 1 date using the qPCR for the nitrogenase gene *nifH*, as well as generating clone libraries using nested *nifH* PCR. Hydrographic measurements at SMBO were made with a Seabird 19 plus CTD. Water samples were collected at 6 to 12 depths by Niskin bottle. Nitrate and phosphate were analyzed colorimetrically by autoanalysis (Technicon AAII, detection limit 0.1 µmol l^{-1}) (Grasshoff et al. 1999). N* was calculated after Deutsch et al. (2001) (Eq. 1).

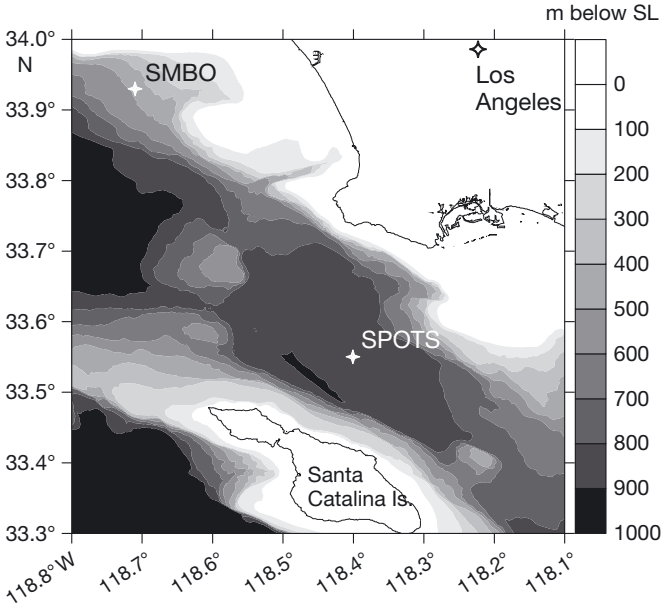

Fig. 1. Bathymetry and location of the 2 sampling stations in the Southern California Bight, USA. SPOTS = San Pedro Ocean Time Series site in the San Pedro Basin. SMBO = Santa Monica Bay Observatory site at the edge of the Santa Monica Basin. SL: sea level

Nitrogen and carbon fixation. Nitrogen fixation was determined using the $^{15}N_2$ incorporation technique (Montoya et al. 2004). Briefly, polycarbonate bottles (4 l at SPOTS, 2.5 l at SMBO) were filled with water collected in Niskin bottles during CTD casts. Water collected from hypoxic depths at SPOTS (500 and 885 m) was introduced by tubing into the bottom of argon-filled bottles to minimize aeration. Bottles were immediately capped with septa, and trace additions of $^{15}N_2$ (98 atom%, Sigma-Aldrich/Isotec; $^{15}N_2$ <1% of background N_2) were introduced through the septum via a gas-tight syringe. Bottles with water from the surface and chlorophyll-maximum depths were incubated in triplicate or quadruplicate in running sea water under ambient light. Chlorophyll-maximum bottles were shaded with mesh screen to simulate *in situ* light levels (ca. 1% of surface). Incubations of water from the 500 and 885 m depths were carried out in duplicate in the dark at 5°C. Background $\delta^{15}N$ and $\delta^{13}C$ of particulate matter were determined on samples collected simultaneously. Duplicate unamended controls were incubated as described above and size-fractionated/filtered as described below; no significant change in the $\delta^{15}N$ and $\delta^{13}C$ of particulate matter was observed after 24 h incubation. The contents of additional duplicate bottles were filtered immediately after adding $^{15}N_2$ (SPOTS and SMBO) and $H^{13}CO_3^{2-}$ (SMBO) as time zero controls. No significant change in $\delta^{15}N$ of particulate matter was seen immediately after tracer injection, and changes in $\delta^{13}C$ were as expected from the necessary delay between tracer addition and filtration. Incubations (24 h) were ended by size-fractionation/filtration through Nuclepore prefilters (Whatman) of poresize 10 µm followed by precombusted GF/F filters (poresize 0.7 µm, Whatman), or, on some dates, through GF/F filters alone. Seston collected on filters of poresize 10 µm was washed with deionized water (DI) onto GF/F filters in preparation for mass spectrometric analysis. GF/F filters were dried for 24 h at 60°C. Mass spectrometric analysis was performed on an elemental analyzer-isotope ratio mass spectrometer (SerCon Integra, University of California, Davis Stable Isotope Facility).

At SMBO, carbon fixation was determined as the incorporation, over 24 h, of added ^{13}C-bicarbonate into particulate carbon. ^{13}C-bicarbonate was added as $NaH^{13}CO_3$ to a final concentration of 21 µmol l^{-1}, ca. 1% of the mean dissolved inorganic carbon (DIC) background concentration of 1980 µmol l^{-1} (see 'Results' below). The ^{13}C-bicarbonate solution was first passed through a Chelex (Sigma-Aldrich) column to strip out any metal contaminants. Water samples for DIC and alkalinity determination were drawn from Niskin samplers into clean 0.3 l glass bottles, using established gas sampling protocols (Dickson & Goyet 1994), at around 09:30 to 11:00 h local time; DIC was determined immediately after sampling in the UCLA laboratory using the coulometric SOMMA system (Johnson et al. 1993), and alkalinity was determined by open-cell potentiometric titration (Dickson & Goyet 1994).

Molecular analysis. Samples for molecular analysis at the SPOTS station were collected and filtered as described in Goebel et al. (2010) on 27 April 2010. Filters collected for ribonucleic acid (RNA) extraction were placed in RLT buffer (Qiagen) containing β-mercaptoethanol prior to flash freezing. All filters were stored at –80°C until extraction. Deoxyribonucleic acid (DNA) was extracted from both 10 µm and GF/F filters using freeze-thaw cycles, bead-beating, proteinase K treatment, and ribonuclease A digestion as described in Moisander et al. (2008) with modifications. After removal of the filter and centrifugation (5 min at 14 000 rpm, 18 000 × g), the final steps of extraction were automated using a QIAcube and the DNeasy Plant mini kit (Qiagen). DNA extracts were stored at –20°C.

Nested PCR amplification for *nifH* was carried out on DNA extracts using nifH3/nifH4 and nifH1/nifH2 primers as described in Foster et al. (2009a). Prior to cloning, PCR products were gel-purified using the Qiaquick gel extraction kit (Qiagen). *NifH* amplicons were ligated into a pGEM-T vector (Promega) and cloned according to manufacturer's guidelines. Clones were selected randomly and plasmids were extracted using a Montage Plasmid MiniPrep 96 kit (Millipore) then sequenced using the Sanger method at the DNA Sequencing Facility of the University of California, Berkeley. Sequences were quality-trimmed, and vector sequences were removed using Sequencher 4.10.1 software (Gene Codes). Neighbor-joining trees were constructed in ARB (Technical University of Munich) using a publicly-available *nifH* database (Zehr et al. 2003), and bootstrapped in MEGA 4.0 (Tamura et al. 2007). Like sequences were binned into phylotypes using a 97% amino-acid sequence similarity cut-off using DOTUR (Schloss & Handelsman 2005). All sequences were submitted to Genbank under accession numbers HQ660812 to HQ660942.

The abundance of cyanobacterial and proteobacterial diazotrophs was quantified in DNA extracts using Taqman® *nifH* qPCR assays. The uncultivated unicellular cyanobacterial group A (UCYN-A) and *Trichodesmium* spp. were quantified using the primers and probes described in Church et al. (2005a). *Crocosphaera*-like group B (UCYN-B) was quantified as in Moisander et al. (2010). *NifH* from the symbiont *Richelia*, associated with 2 hosts — *Rhizosolenia clevii* (RR) and *Hemiaulus hauckii* (HR) — was quantified using the primers and probes described in Church et al. (2005b) and Foster et al. (2009b), respectively. The proteobacterial diazotrophs, γ-24774A11 and α-24809A06, were quantified as in Moisander et al. (2008). All qPCR assays were conducted as described in Moisander et al. (2010), and each sample was screened for inhibition according to Goebel et al. (2010). Samples where amplification was observed but was below the limit of quantification (8 *nifH* copies per reaction) were designated 'detected not quantified' (DNQ).

Trace nutrient additions. At SMBO, water samples were collected and incubations were made under trace-metal-clean conditions, as described by Mendez (2008). Briefly, water samples for incubations and nutrient measurements were collected using acid-washed Niskin bottles with Teflon-coated external springs and messengers, deployed on a ¼-inch (ca. 6 mm) polyester line from a polyvinylchloride-coated winch. Niskin bottles were rinsed with sea water before use and with DI after use. All bottles or plasticware coming into contact with seawater or amendment solutions were leached for 1 wk in 10% HCl, followed by 1 wk in 1% Ultratrace HCl, followed by a further period of 1 wk in DI to neutralize pH. Incubation bottles were filled on board ship via a hose running from the Niskin bottles to a metal-free laminar flow hood equipped with a high-efficiency particle air filter. ^{13}C-bicarbonate, and iron or phosphate amendments, were added to the incubation bottles under the laminar flow hood before they were capped with septa. Phosphate was added as a KH_2PO_4 solution to a final concentration of 0.43 µmol l^{-1}, a ca. 160% increase over the mean background concentration of 0.26 µmol l^{-1} (see 'Results' below). Iron was added as a solution of $FeSO_4$ in HCl (pH = 2) to maintain solubility. Iron was added to a final concentration of 3.4 nM, a ca. 190% increase over the mean background level of 1.8 nM (Mendez 2008) (see 'Results' below). Measurements of total iron concentrations within bottles before and after the 24 h incubations showed no significant change (Inductively Coupled Plasma Mass Spectrometry, ICP-MS; S. Sañudo-Wilhelmy [University of Southern California, USC] pers. comm.; data not shown). After capping, the bottles were incubated and filtered as described in 'Nitrogen and carbon fixation'.

RESULTS

SPOTS site

The SPOTS site in the San Pedro Basin has a water column characterized by summer thermal stratification, with SST ranging annually from 12° to 23°C (Fig. 2A). The basin is hypoxic (<10% O_2 saturation) below ca. 500 m (Fig. 2D), with a mean bottom-water dissolved O_2 concentration of 11 ± 3 µmol l^{-1} and a temperature of 5.2 ± 0.0°C. In May and June of 2006, a basin-flushing event (Berelson 1991) introduced oxygenated waters into the

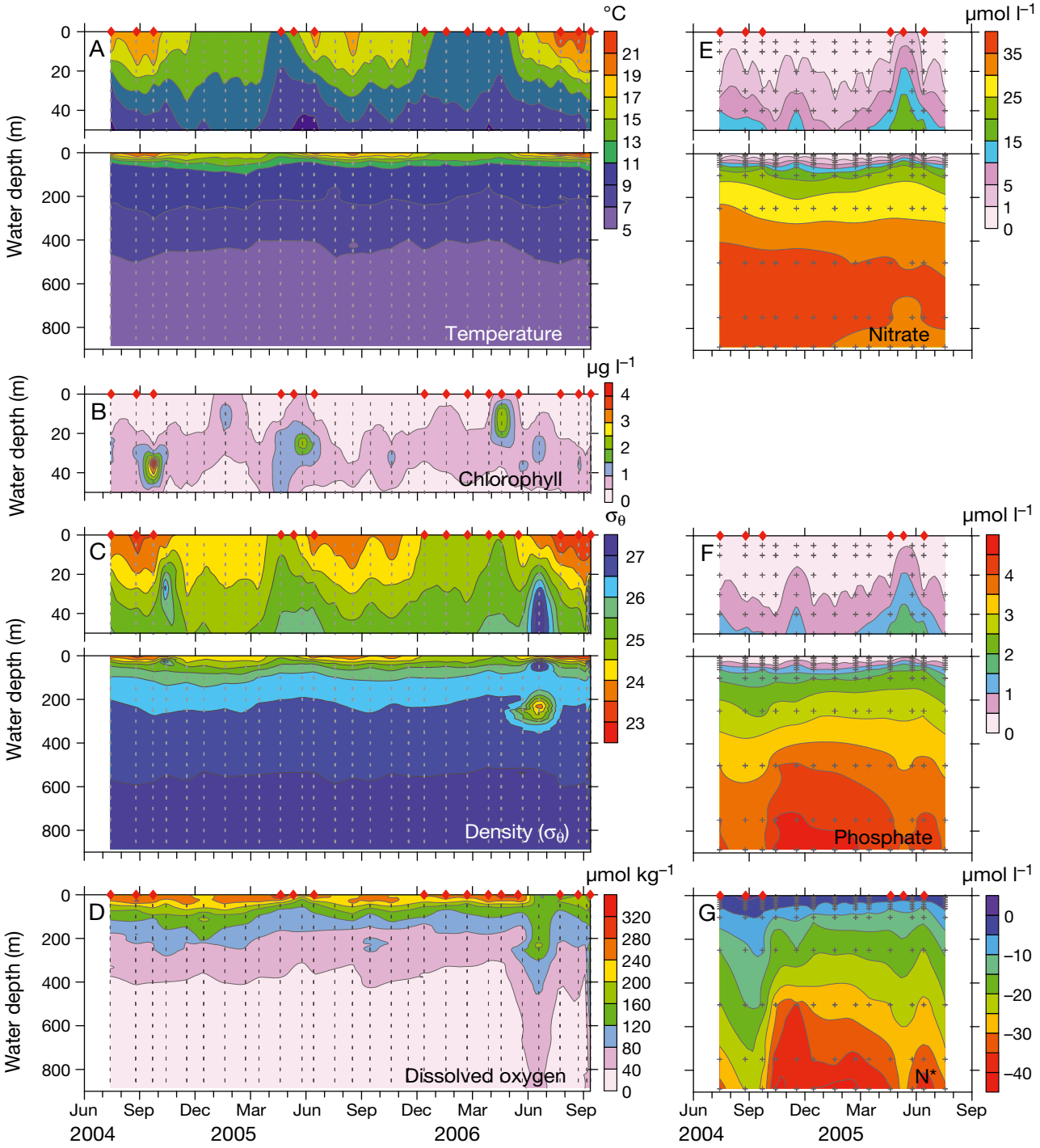

Fig. 2. Hydrographic and chemical properties over a 1- to 2-yr period at SPOTS (see Fig. 1 legend). (A) temperature (°C); (B) chlorophyll *a* (µg l^{-1}); (C) potential density (σ_θ); (D) dissolved oxygen (µmol kg^{-1}); (E) nitrate (µmol l^{-1}); (F) phosphate (µmol l^{-1}); and (G) N^* (µmol l^{-1}). The upper 50 m of the water column is shown in an expanded *y*-axis scale in some of the panels. Sampling dates of continuous profiles are indicated by vertical dashes, while discrete Niskin bottle samples are indicated by crosses. Red diamonds indicate dates of N_2 fixation incubations

basin (Fig. 2D). Surface waters were depleted in nutrients relative to subsurface waters, particularly during stratification, with mean nitrate and phosphate concentrations during the study period of 0.2 ± 0.0 and 0.3 ± 0.0 µmol l^{-1} (maximum 0.4 and 0.4 µmol l^{-1}), respectively (Fig. 2E,F). The depth of maximum chl *a* concentrations ranged from 16 to 37 m, deeper during the summer and shallower in the winter (Fig. 2B). A number of algal blooms were observed, primarily in the spring. N* was negative throughout the water column, indicating the influence of net denitrification on these waters (Fig. 2G).

Nitrogen fixation rates at SPOTS were highest at the surface (mean 5.8 ± 2.6 µmol N m^{-3} d^{-1}; range 0.3 to 36.4 µmol N m^{-3} d^{-1}) (Fig. 3A, Table 1). Nitrogen fixation was dominated by nanoplankton of <10 µm size, which were responsible for 80 ± 5 % of the measured N_2 fixation (Table 1). The highest rates of N_2 fixation were measured in December 2005 and February 2006. With the exception of this episode of high winter N_2 fixation, the pattern of N_2 fixation was significantly and positively correlated with SST (Pearson's product-moment correlation coefficient = 0.54, t_{10} = 2.1, p = 0.03). Nitrogen fixation at the chlorophyll maximum was considerably lower and less variable, averaging 1.0 ± 0.3 µmol N m^{-3} d^{-1} (Fig. 3B). Nitrogen fixation was also detected within the hypoxic water column (at 500 and 885 m) at the lower mean rate of 0.07 µmol N m^{-3} d^{-1} (Table 1, Fig. 3C,D). The contribution of nanoplankton to total N_2 fixation was greater within the hypoxic zone (87 % at 500 m and 100 % at 885 m) than at the surface (80 %) or chlorophyll maximum (77 %). However, the <10 µm size fraction contributed only ca. 70 % of the total particulate N (PN) at the surface and chlorophyll maximum. N_2 fixation by the nanoplankton was therefore greater than would be expected by their contribution to standing stocks of PN alone (see Fig. S1 in the supplement at www.int-res.com/articles/suppl/a063p193_supp.pdf).

Both the uncultivated UCYN-A and the heterocystous symbiont *Richelia* spp. were quantified in the <10 µm fraction within the photic zone using *nifH* qPCR (Table S1 in the supplement at www.int-res.com/articles/suppl/a063p193_supp.pdf). UCYN-A *nifH* abundance decreased dramatically below the photic zone, but was detected at all depths. *Richelia* spp., associated with both *Rhizosolenia* spp. and *Hemiaulus* spp., were quantified in the >10 µm fraction at a depth of 45 m (Table S1). The other cyanobacterial phylotypes, UCYN-B and *Trichodesmium*, were not detected in any samples. An alphaproteobacterial phylotype, α-24809A06, was quantified at low abundance in both size fractions at a depth of 350 m (Table S1).

NifH clone libraries were generated from DNA extracts from depths of 5, 750 and 885 m. In the surface samples, only 3 phylotypes were recovered: 2 UCYN-A phylotypes and a gammaproteobacterium closely related to γ-24774A11 (Fig. 4). The UCYN-A phylotype, SPOTS_45702A2, was the most highly recovered in this study and was found in both size fractions at the surface, as well as at depths of 750 and 885 m. The *nifH* sequences recovered from the hypoxic water samples were an assemblage of heterotrophs, most of which were recovered only once. Phylotypes belonged primarily to Clusters I and III, but included a single Cluster IV phylotype (Fig. 4). The most highly recovered

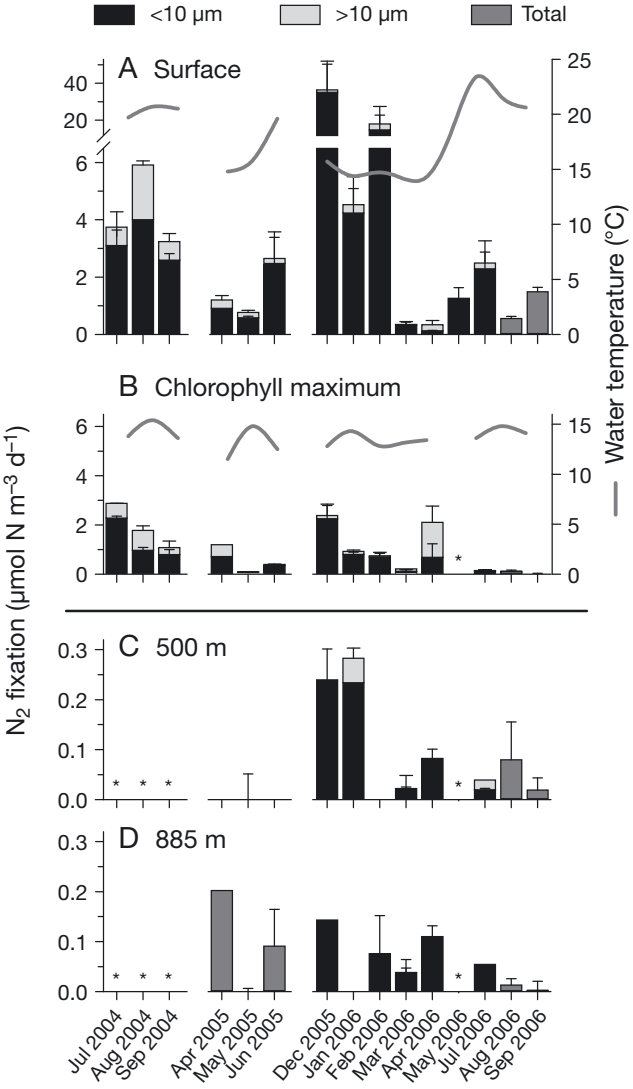

Fig. 3. N_2 fixation at SPOTS (see Fig. 1 legend) by particle size fraction at 4 depths: (A) surface (2.5 m); (B) chlorophyll maximum (range 16 to 37 m; see Fig. 2B); (C) 500 m; (D) 885 m. N_2 fixation rates for the <10 µm particulate fraction are indicated by black bars, the >10 µm fraction by light gray, and total N_2 fixation (unfractionated incubations) by dark gray. Water temperatures are indicated by gray lines in panels A and B. Mean temperatures at 500 m and 885 m were 6.6 and 5.2°C, respectively. Note the differing *y*-axis scales in panels C and D vs. A and B. An asterisk (*) indicates that no data were collected on that date. Error bars are SE

Table 1. Mean particulate N and N_2 fixation at SMBO and SPOTS (see Fig. 1). Measurements at SPOTS are size-fractionated into <10 µm and >10 µm pools. Chl. max. = chlorophyll maximum. SE in parentheses

Site	Depth	Total	<10 µm	>10 µm	% <10 µm
Particulate N (µmol l^{-1})					
SMBO	Surface	1.6 (0.3)			
SPOTS	Surface	1.1 (0.2)	0.86 (0.13)	0.39 (0.07)	70 (3)
SPOTS	Chl. max.	2.0 (0.8)	1.4 (0.5)	0.80 (0.38)	67 (3)
SPOTS	500 m	0.23 (0.03)	0.12 (0.04)	0.10 (0.04)	56 (16)
SPOTS	885 m	0.25 (0.04)	0.15 (0.05)	0.08 (0.03)	61 (14)
N_2 fixation (µmol m^{-3} d^{-1})					
SMBO	Surface	2.4 (1.6)			
SPOTS	Surface	5.8 (2.6)	5.5 (2.7)	0.75 (0.27)	0 (5)
SPOTS	Chl. max.	1.0 (0.3)	0.82 (0.21)	0.34 (0.12)	77 (6)
SPOTS	500 m	0.07 (0.03)	0.10 (0.04)	0.01 (0.01)	87 (10)
SPOTS	885 m	0.07 (0.02)	0.08 (0.02)	0.00 (0.00)	100 (0)

non-cyanobacterial phlyotype, SPOTS_45708A38, accounted for 8% of all the sequences recovered in this study, and was most closely related to an uncultivated alphaproteobacterium from the South China Sea (GenBank accession no. EU052627; Moisander et al. 2008). SPOTS_45708A198, SPOTS_45712A16 and SPOTS_45706A170 had high amino acid sequence identity with an uncultivated gammaproteobacterium from an intertidal microbial mat (GenBank accession no. AF046847; Olson et al. 1999), and together also accounted for 8% of all sequences. A number of phylotypes recovered belonged to Cluster III, of which a majority of the cultivated representatives are strict anaerobes. When considered together, Cluster III sequences accounted for nearly a third (26%) of the sequences recovered from SPOTS. Several of these were closely related to putative sulfate-reducing bacteria (e.g. Cluster 3E sequences, Fig. 4).

SMBO site

The SMBO site also exhibited summer thermal stratification, although SSTs were slightly lower than at SPOTS (12° to 21°C) (Fig. 5A). Although this site was at the edge of the Santa Monica Basin (Fig. 1), bottom waters were hypoxic (Fig. 5C). Four distinct chl *a* blooms were observed in the fall of 2006 (Fig. 5B). Four upwelling events took place during the winter and spring of 2007, as can be seen by the intrusion of denser and cooler water to the surface (Fig. 5A,D). These upwelling events also brought NO_3^- and PO_4^{3-} to the surface (Fig. 5E,F). As at SPOTS, nitrate and phosphate were usually depleted at the surface (means 0.7 and 0.3 µmol l^{-1} and maxima 10.9 and 1.0 µmol l^{-1}, respectively). N^* was also negative, although not as strongly as at SPOTS (Fig. 5G).

N_2 fixation at SMBO averaged less than half of that at SPOTS, at 2.4 ± 1.6 µmol N m^{-3} d^{-1} (Fig. 6, Table 1). In September 2006, an episode of elevated N_2 fixation attained a rate of 14.9 µmol N m^{-3} d^{-1}. Carbon fixation at SMBO averaged 6.0 ± 2.3 mmol m^{-3} d^{-1} (maximum 14.6 mmol m^{-3} d^{-1}), in excellent agreement with the recent partial pressure of carbon dioxide (pCO_2)-based estimate of net primary production (7 mmol m^{-3} d^{-1}) by Leinweber et al. (2009). There was also no significant difference in N_2 or C fixation between control treatments and treatments with added P or Fe (repeated measures ANOVA; N_2 fixation: $F_{2,8}$ = 0.97, p = 0.42; C fixation: $F_{2,68}$ = 0.17, p = 0.84) (Fig. S2 in the supplement at www.int-res.com/articles/suppl/a063p193_supp.pdf).

As a preliminary assessment of areal rates of N_2 fixation for SPOTS, we divided the water column into a photic zone, which extended from the surface to the chlorophyll maximum, and an aphotic zone, that extended from the chlorophyll maximum to the bottom. Photic zone N_2 fixation was estimated as the depth-integrated mean of the measured surface and chlorophyll maximum N_2 fixation, while aphotic zone N_2 fixation was estimated as the depth-integrated mean of the N_2 fixation measurements at 500 m and 885 m. While we believe that this is a conservative estimate of areal N_2 fixation, because it supposes that N_2 fixation immediately below the chlorophyll maximum is equal to the much lower N_2 fixation rates we measured at 500 m and 885 m, we acknowledge that the N_2 fixation rate between the chlorophyll maximum and a depth of 500 m is poorly constrained. Estimated in this way, N_2 fixation integrated over the entire water column averaged 150 µmol N m^{-2} d^{-1} (maximum 760 µmol N m^{-2} d^{1}). Although volumetrically, aphotic-zone N_2 fixation rates are low relative to the photic zone (Fig. 3), because of its greater vertical extent, the aphotic water column may be responsible for a significant proportion (ca. 50%, or 55 µmol N m^{-2} d^{-1}) of the photic areal N_2 fixation (98 µmol N m^{-2} d^{-1}) (Fig. S3 in the supplement at www.int-res.com/articles/suppl/a063p193_supp.pdf).

DISCUSSION

Nitrogen fixation by nanoplankton

We studied N_2 fixation over a 4 yr period in waters in, and adjacent to, 2 hypoxic basins in the Southern California Bight. These sites were characterized by nega-

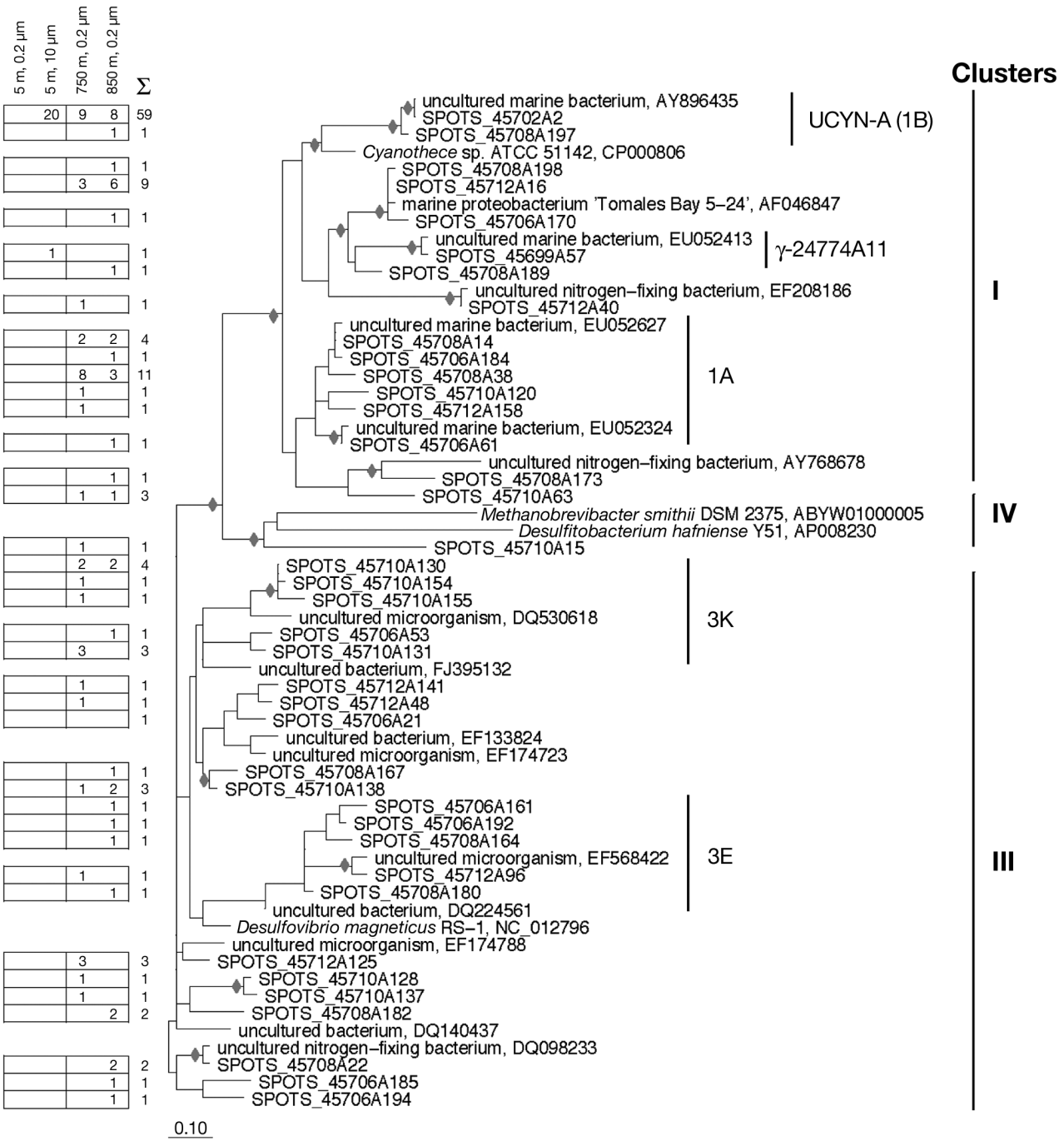

Fig. 4. Neighbor-joining tree of partial *nifH* amino acid sequences (with GenBank accession numbers if available) recovered from depths of 5, 750 and 885 m at SPOTS (see Fig. 1 legend). Branch lengths were computed using the Kimura correction in ARB software, and bootstrapping was performed in MEGA 4.0. Nodes supported by bootstraps >70% (1000 replicates) are marked with a diamond. The number and distribution of clones recovered for each phylotype is indicated in the table to the left of the tree. Clusters (I, II,and IV) and sub-clusters (1A, 1B, 3K, and 3E), according to *nifH*-based phylogeny, are shown to the right of the tree. Reference amino acid sequences are followed by GenBank accession nos

tive N* and subsurface hypoxia, similar to the waters associated with the OMZs of the Pacific Ocean, which geochemical modeling has suggested may be hot spots of N_2 fixation (Deutsch et al. 2007). Nitrogen fixation at the surface and chlorophyll-maximum depth was primarily (mean 80%) associated with unicellular diazotrophs of <10 µm particle size (Table 1). This is higher than the contribution of unicellular diazotrophs to total N_2 fixation at Stn ALOHA near Hawaii, USA, and in the tropical North Atlantic, which, in several

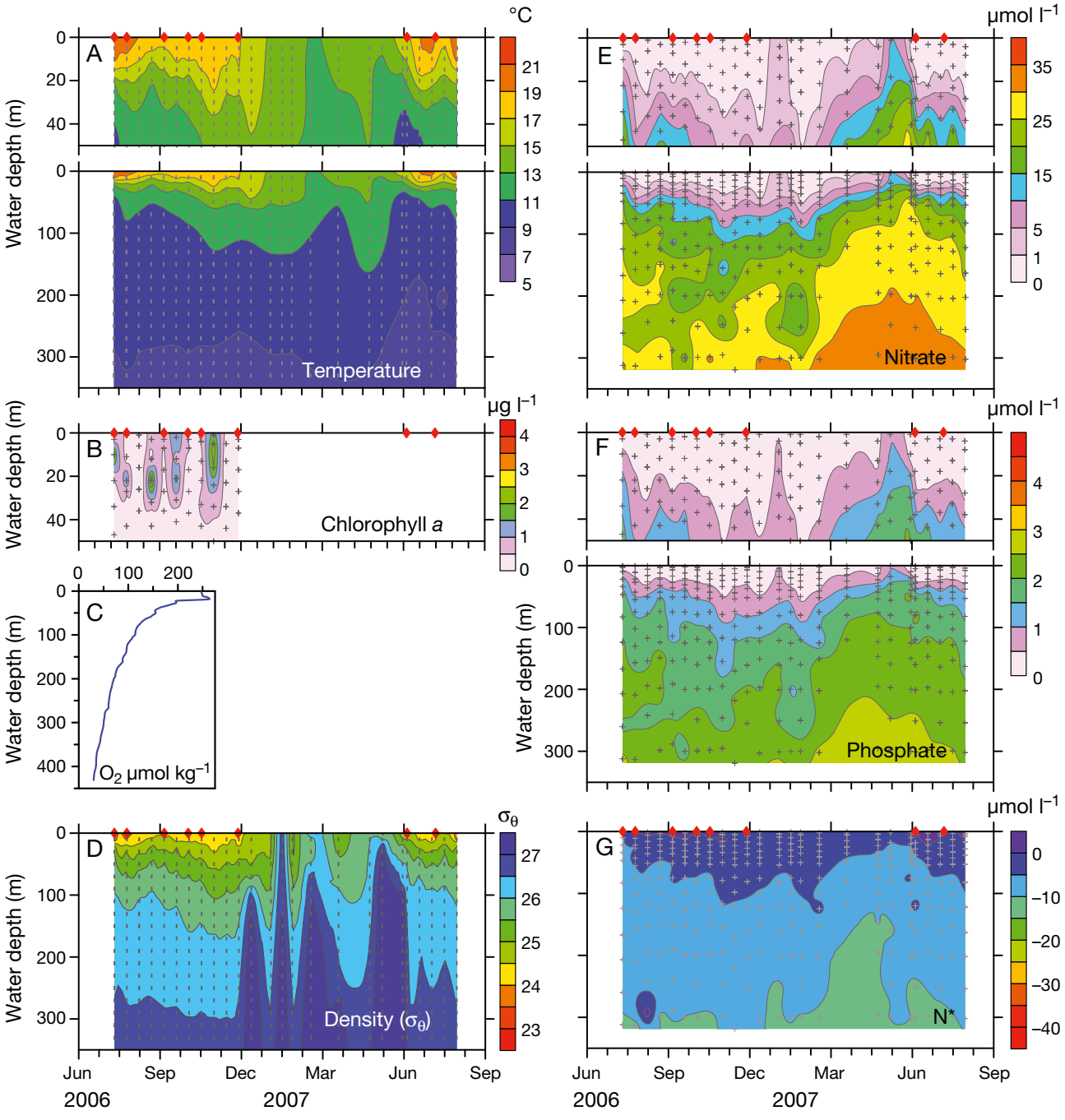

Fig. 5. Hydrographic and chemical properties over a period of 1 yr at SMBO (see Fig. 1 legend). Details are given in the legend of Fig. 2; in Fig. 5, the sample profile for dissolved oxygen is from 11 Jul 2006

studies, ranged from 3 to 64 % (Dore et al. 2002, Falcón et al. 2004, Grabowski et al. 2008). However, in the eastern temperate North Pacific, at the same latitude as our coastal site but in the open ocean, nanoplankton were responsible for nearly 100 % of the measured N_2 fixation (Needoba et al. 2007).

Nitrogen fixation rates attributable to nanoplankton at our sites averaged near the high end of the range of rates previously measured in the tropical Pacific and Atlantic Oceans. The mean surface N_2 fixation rate by nanoplankton at SPOTS was 5.5 µmol m^{-3} d^{-1} (maximum 35 µmol m^{-3} d^{-1}), significantly higher than the average of ca. 0.3 µmol m^{-3} d^{-1} measured at Stn ALOHA (Dore et al. 2002) and in the temperate North Pacific (Needoba et al. 2007), and also higher than the maximum rate of 3.6 µmol m^{-3} d^{-1} measured in

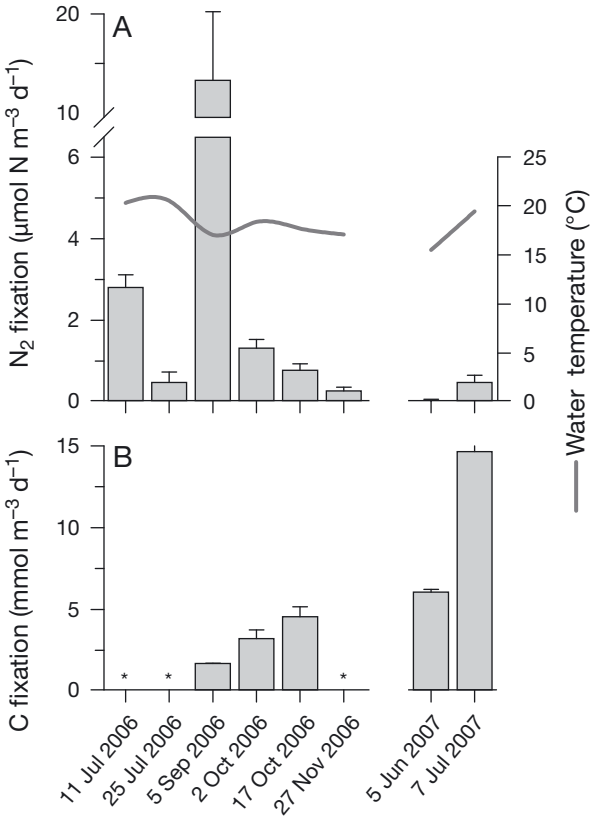

Fig. 6. (A) Nitrogen fixation and (B) carbon fixation in surface waters at SMBO (see Fig. 1 legend). Surface water temperature is indicated by the gray lines in panel A. An asterisk (*) indicates no data for that date. Error bars are SE

several tropical sites (Zehr et al. 2001, Dore et al. 2002, Montoya et al. 2004, Falcón et al. 2004, Holl et al. 2007, Grabowski et al. 2008). However, our maximum rates from the Southern California Bight (36 µmol m^{-3} d^{-1}) were similar to the rates reported by Montoya et al. (2004) from a transect across the eastern North Pacific at ca. 30° N latitude, which ranged up to 44 µmol m^{-3} d^{-1}. Only results from a transect across the northern coast of Australia (maximum 1490 µmol m^{-3} d^{-1}) were significantly higher than our findings (Montoya et al. 2004). The areal rates of photic zone N_2 fixation that we measured in the Southern California Bight averaged 98 µmol m^{-2} d^{-1}, within the range reported earlier for *Trichodesmium* using the acetylene reduction technique (reviewed in Montoya et al. 2004).

Our results support the significance of nanoplankton in N_2 fixation in cooler subtropical and temperate waters (<25°C). Furthermore, it confirms earlier indications that these cooler regions support populations of UCYN-A, in contrast to the *Trichodesmium* populations found in warmer waters, and is the first report of this diazotroph from the Southern California Bight. Our findings also agree with previous reports of UCYN-A in waters with elevated nitrate levels (e.g. Needoba et al. 2007, Rees et al. 2009, Moisander et al. 2010), again in contrast to *Trichodesmium*. The emerging picture is of a wider latitudinal range for pelagic N_2 fixation than has been assumed, particularly for the less temperature- and nitrate-sensitive nanoplankton, which may play a greater role than colonial diazotrophs in temperate waters.

Nitrogen fixation in the hypoxic water column

We also detected N_2 fixation within the hypoxic, high-nitrate waters of the San Pedro basin at depths of 500 and 885 m. To our knowledge these represent the deepest N_2 fixation measurements reported yet and the only ones from hypoxic waters. N_2 fixation was significantly greater than zero in 12 out of 22 samples in which N_2 fixation was measured (Fig. 3). Although these rates were lower than our photic-zone rates (<0.3 µmol m^{-3} d^{-1}), they were similar to rates reported earlier from surface waters at Stn ALOHA near Hawaii (Zehr et al. 2001, Dore et al. 2002, Falcón et al. 2004, Montoya et al. 2004, Codispoti 2007, Grabowski et al. 2008) and to a site in the eastern subtropical North Pacific (Needoba et al. 2007). Further, when integrated over the water column (from the chlorophyll-maximum depth to the bottom), they may contribute as much as 55 µmol N m^{-2} d^{-1}, ca. one-third of the total estimated areal N_2 fixation rate (150 µmol N m^{-2} d^{-1}) for this site. Our results within the hypoxic basins of the Southern California Bight were in contrast to other studies that measured N_2 fixation at depth, which found that nearly all of the areal N_2 fixation took place near the surface (Falcón et al. 2004, Holl et al. 2007, Needoba et al. 2007). N_2 fixation took place in the hypoxic waters of our study despite nitrate concentrations >32 µmol l^{-1}. However, the Basin waters were deficient in nitrate relative to phosphate, with strongly negative N^* values ranging from −15 to −42 (Fig. 2). Particles <10 µm in size always accounted for >87% of the hypoxic N_2 fixation, and as these particles likely have very low sinking rates (<1 m d^{-1}; Bienfang 1985), any phototrophic diazotrophs sinking from the mixed layer would likely have exhausted their energy resources by the time they reached these depths.

Phylogenetic characterizations of the deep hypoxic nanoplanktonic *nifH* gene revealed an assemblage of Clusters I and III heterotrophic diazotrophs. Previous studies have reported diverse heterotrophic, and in some cases anaerobic, *nifH* sequences from abyssopelagic waters (e.g. Hewson et al. 2007), and although in addition to detecting *nifH* sequences — our study

measured N_2 fixation rates directly — it is still unclear which heterotrophic diazotrophs might be responsible for the measured rates. The nested PCR approach necessary to amplify low-abundance *nifH* from the marine environment can be vulnerable to contamination (see discussion in Turk et al. 2011, and references therein). Although none of the sequences recovered from SPOTS were closely related to known contaminants, it is virtually impossible to identify a contaminant based on sequence alone. More quantitative molecular methods (e.g. qPCR) will be required to determine which of these phylotypes are ecologically relevant. The results from the present study represent a first step in investigating which heterotrophic diazotrophs are actively fixing N_2 in deep hypoxic waters at SPOTS.

Controls on N_2 fixation

Relatively few measurements of N_2 fixation have been made in subtropical or temperate waters. While our coastal measurements at 34° N latitude were significantly higher than those made at the same latitude in the open Pacific (Needoba et al. 2007), they were similar to measurements of total N_2 fixation made in the English Channel (maximum 20 µmol m^3 d^1; Rees et al. 2009). The high N_2 fixation rates measured in our study, as well as in the English Channel, confirm that the assumption in global N budgets that N_2 fixation is significant only in waters with surface temperatures >20°C needs to be re-examined (Galloway et al. 2004, Holl et al. 2007, Needoba et al. 2007). The significance of the high nanoplanktonic N_2 fixation rates in high-latitude, temperate waters that we report is corroborated by the recent report of wide distribution of N_2-fixing cyanobacteria in similar latitudes and water temperatures (Moisander et al. 2010).

Contrary to expectations that N_2 fixation should be maximal during summer stratification, when phytoplankton growth depletes surface water N and P (e.g. Dore et al. 2002, Falcón et al. 2004), we found the highest rates of nanoplanktonic N_2 fixation during December and February (Fig. 3). This observation suggests that nanoplanktonic N_2 fixation may play a significant role year-round, rather than being restricted to periods of intense stratification. In contrast to earlier studies that found a correlation between PN and N_2 fixation (Dore et al. 2002), episodes of elevated N_2 fixation were not associated with episodes of high PN at either SPOTS or SMBO (data not shown), nor was N_2 fixation correlated with gross carbon fixation at SMBO (Fig. 6). These observations indicate that N_2 fixation and overall production were uncoupled at our sites.

Our 3 yr record of N_2 fixation at SPOTS suggests that surface N_2 fixation is often correlated with SST (Fig. 3) However, the high N_2 fixation rates measured in the winter of 2005 to 2006 are anomalous, because surface temperatures were low during this time. Neither can these anomalously high N_2 fixation rates be explained by changes in any of the other hydrochemical parameters measured. The relationship of surface N_2 fixation rates to SST at SPOTS also does not explain the 2-fold lower mean N_2 fixation rates at SMBO, because mean temperatures were lower at SMBO, while nitrate and phosphate concentrations were similar (Figs. 2 & 4). The factors controlling N_2 fixation within the Southern California Bight, and particularly the winter N_2 fixation peaks, remain unclear.

Additions of iron and phosphate to sea water during incubations at SMBO had no appreciable effect on N_2 fixation rates (Fig. S2). Limitation of phytoplankton growth by iron has been reported for nearby sites in the California Current System (Firme et al. 2003, King & Barbeau 2007). However, the dissolved iron concentrations measured in these studies were < 0.46 nmol l^{-1}, compared to the mean concentration of 1.8 mmol l^{-1} measured at SMBO (Mendez 2008). Similarly, PO_4^{3-} concentrations at SPOTS and SMBO averaged 0.3 µM, which were greater than ambient concentrations where PO_4^{3-} stimulation of N_2 fixation has been observed (Mills et al. 2004, Grabowski et al. 2008). However, we did not eliminate possible co-limitation by Fe and PO_4^{3-} (Mills et al. 2004).

Our study of N_2 fixation rates within the Southern California Bight supports earlier observations that N_2 fixation rates by nanoplankton can be high in temperate waters. Further, we report significant N_2 fixation rates within deep, hypoxic waters with high concentrations of nitrate. Although these rates were much lower than at the surface, when integrated over the water column, they might contribute as much as one-third of the total areal rate of N_2 fixation. These observations suggest that N_2 fixation might also play a role within the large OMZs of the Pacific Ocean. It is possible that an expansion of the range of active pelagic N_2 fixation into temperate and hypoxic waters may address the imbalance between denitrification and N_2 fixation in scaled global estimates.

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