Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2015) 37(4): 727–739. First published online June 18, 2015 doi:10.1093/plankt/fbv049

Measurements of nitrogen fixation in the oligotrophic North Pacific Subtropical Gyre using a free-drifting submersible incubation device

DENIZ BOMBAR[1,2†*], CRAIG D. TAYLOR[2,3], SAMUEL T. WILSON[2,4], JULIE C. ROBIDART[1,2,5], ARIEL RABINES[6], KENDRA A. TURK-KUBO[1,2], JOHN N. KEMP[3], DAVID M. KARL[2,4] AND JONATHAN P. ZEHR[1,2]

[1]OCEAN SCIENCES DEPARTMENT, UNIVERSITY OF CALIFORNIA SANTA CRUZ, 1156 HIGH STREET, CA 95064, USA, [2]CENTER FOR MICROBIAL OCEANOGRAPHY: RESEARCH AND EDUCATION, UNIVERSITY OF HAWAII, 1950 EAST-WEST ROAD, HONOLULU, HI 96822, USA, [3]WOODS HOLE OCEANOGRAPHIC INSTITUTION, 266 WOODS HOLE RD. MS# 52, WOODS HOLE, MA 02543-1050, USA, [4]DEPARTMENT OF OCEANOGRAPHY, UNIVERSITY OF HAWAII, 1000 POPE RD., HONOLULU, HI 96822, USA, [5]NATIONAL OCEANOGRAPHY CENTRE, SOUTHAMPTON; EUROPEAN WAY, SOUTHAMPTON SO14 3ZH, UNITED KINGDOM AND [6]J. CRAIG VENTER INSTITUTE, 4120 CAPRICORN LANE, LA JOLLA, CA 92037, USA

[†]PRESENT ADDRESS: BIOLOGISK INSTITUT, MARINBIOLOGISK SEKTION, KØBENHAVNS UNIVERSITET, STRANDPROMENADEN 5, HELSINGØR DK-3000, DENMARK.

*CORRESPONDING AUTHOR: dbombar@bio.ku.dk

Received December 16, 2014; accepted May 26, 2015

Corresponding editor: John Dolan

One challenge in field-based marine microbial ecology is to achieve sufficient spatial resolution to obtain representative information about microbial distributions and biogeochemical processes. The challenges are exacerbated when conducting rate measurements of biological processes due to potential perturbations during sampling and incubation. Here we present the first application of a robotic microlaboratory, the 4 L-submersible incubation device (SID), for conducting *in situ* measurements of the rates of biological nitrogen (N_2) fixation (BNF). The free-drifting autonomous instrument obtains samples from the water column that are incubated *in situ* after the addition of $^{15}N_2$ tracer. After each of up to four consecutive incubation experiments, the 4-L sample is filtered and chemically preserved. Measured BNF rates from two deployments of the SID in the oligotrophic North Pacific ranged from 0.8 to 2.8 nmol N L^{-1} day^{-1}, values comparable with simultaneous rate measurements obtained using traditional conductivity–temperature–depth (CTD)–rosette sampling followed by on-deck or *in situ* incubation. Future deployments of the SID will help to better resolve spatial variability of oceanic BNF, particularly in areas where recovery of seawater samples by CTD compromises their integrity, e.g. anoxic habitats.

KEYWORDS: nitrogen fixation; *in situ* device; Pacific Ocean; diazotroph

available online at www.plankt.oxfordjournals.org

INTRODUCTION

Biological nitrogen (N_2) fixation (BNF), the conversion of N_2 gas to ammonia (NH_3), is performed by a select group of microorganisms, termed diazotrophs. BNF is a key component of the oceanic nitrogen cycle, with estimates of up to 200 Tg nitrogen (N) being fixed per year on a global scale (Karl *et al.*, 2002; Capone *et al.*, 2005; Gruber and Galloway, 2008). However, such estimates have large uncertainties, partly due to an incomplete understanding of the full diversity and ecology of marine diazotrophs (Goebel *et al.*, 2010; Moisander *et al.*, 2010; Farnelid *et al.*, 2011). In the ocean, the major groups of diazotrophs include: (i) the filamentous, non-heterocystous cyanobacterium *Trichodesmium* (Mague *et al.*, 1977; Capone *et al.*, 1997; Capone *et al.*, 2005; Laroche and Breitbarth, 2005), (ii) unicellular, free-living cyanobacteria such as *Crocosphaera watsonii* (UCYN-B) (Hewson *et al.*, 2009; Webb *et al.*, 2009; Bench *et al.*, 2011; Foster *et al.*, 2013) and (iii) cyanobacteria that form symbioses with eukaryotic algae, e.g. the heterocystous genera *Richelia* and *Calothrix* that are associated with diatoms (Villareal, 1990; Janson *et al.*, 1995; Foster and Zehr, 2006) and unicellular *Candidatus* Atelocyanobacterium thalassa (UCYN-A) associated with prymnesiophytes (Zehr *et al.*, 2001; Moisander *et al.*, 2010; Thompson *et al.*, 2012; Bombar *et al.*, 2014).

Although the major abiotic, nutrient and internal controls of BNF activity and its distribution appear to have been identified (Sohm *et al.*, 2011; Voss *et al.*, 2013), the relative strengths with which these different factors govern BNF under different environmental settings remain elusive. These variable controls are likely responsible for the unexplained large spatiotemporal variability in the abundances of diazotrophs in the surface ocean, recently highlighted by high-resolution sampling using a drifting robotic gene sensor (Robidart *et al.*, 2014). It is currently unknown how these abundance fluctuations affect the variability in BNF. Thus, conducting corresponding rate measurements at similarly high spatiotemporal resolution is critical for better understanding the role of diazotrophs in oceanic N cycling.

BNF field measurements are typically conducted using versions of the $^{15}N_2$ tracer gas technique (Montoya *et al.*, 1996). This method requires the collection of seawater using a conductivity–temperature–depth (CTD)–Niskin rosette sampling system, and subsequent on-deck or *in situ* incubations of tracer-amended seawater, lasting anywhere from a few hours to a few days. Seawater samples in traditional BNF studies experience changing pressures, light levels and temperatures upon recovery and seawater transfer, while characterization from cultured representatives and sorted cells suggest that many of these processes affect diazotroph populations (e.g. Thompson *et al.*, 2012). Further, methodological improvements on the $^{15}N_2$ tracer technique have demonstrated that BNF rates have been underestimated in most studies, with the addition of $^{15}N_2$ tracer in a dissolved form more representative of the actual rates (Grosskopf *et al.*, 2012).

Automated sampling devices capable of conducting sampling and incubation *in situ* are a promising approach for resolving variability associated with biogeochemical cycling in the marine environment. Such devices have been developed and successfully employed to increase the spatial and temporal resolution of planktonic primary production rates (Dandonneau and Bouteiller, 1992; Taylor and Howes, 1994), as well as for comparing manipulations performed *in situ* and under "simulated" *in situ* conditions (Gundersen, 1973; Lohrenz *et al.*, 1992). The submersible incubation device (SID) was originally designed for primary productivity measurements (Taylor and Doherty, 1990) and has since been adapted for a range of oceanographic measurements (Lohrenz *et al.*, 1992; Taylor and Howes, 1994; Albert *et al.*, 1995; Edgcomb *et al.*, 2011, 2014; Pachiadaki *et al.*, 2014). Integrating the BNF method protocol with a large capacity submersible *in situ* device (4L-submersible incubation device, 4L-SID) became more feasible with recent developments of the $^{15}N_2$ assimilation technique, which requires dissolving the $^{15}N_2$ gaseous tracer in sterile seawater prior to its addition to the samples (Mohr *et al.*, 2010; Grosskopf *et al.*, 2012). In the present study, a modified version of the 4L-SID was deployed, which is capable of autonomously executing the entire sampling, tracer amendment (predissolved $^{15}N_2$, ^{13}C-bicarbonate), incubation and filtration processes associated with BNF and primary production measurements *in situ*. By conducting the entire sampling and incubation procedure directly in the water column, delays in the onset of the incubations and perturbations of the microbial community assemblages during sampling are minimized. Further, such devices have the potential to help overcome the major hurdle of achieving higher sampling resolution, which could reveal currently unknown heterogeneity in BNF rates and the key environmental factors that control them.

METHOD

Cruise overview

The BioLINCS cruise (Biosensing Lagrangian Instrumentation and Nitrogen Cycling Systems) was conducted in the North Pacific Subtropical Gyre (NPSG) (24.39–25.13°N, 158.20–157.29°W) in September 2011, aboard the R/V Kilo Moana (Fig. 1). The overall goal of

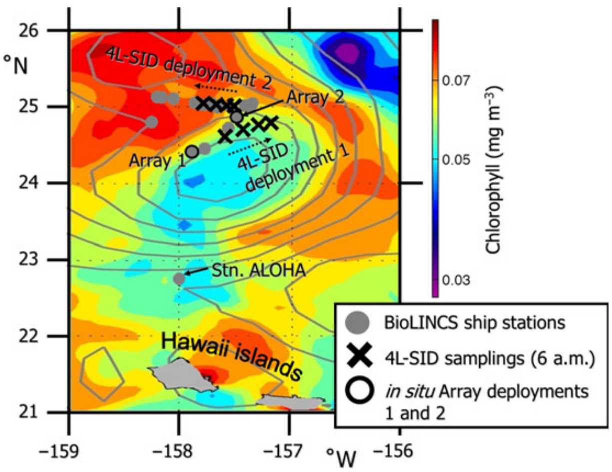

Fig. 1. Sampling stations north of Station ALOHA visited during the BioLINCS cruise (gray dots), locations of the SID on the mornings of all incubation starts (black crosses) and stations at which the *in situ* array was deployed (black circles). The gray contour lines (mesoscale altimetry) indicate the presence of two anticyclonic eddies that influenced the drift paths of the SID (described in the text). Color-coded near-surface chlorophyll concentrations are averages of satellite data from Archiving, Validation and Interpretation of Satellite Oceanographic data (AVISO) and Moderate Resolution Imaging Spectroradiometer (MODIS) Aqua, for 6–20 September 2011.

the scientific cruise was to examine microbial biogeochemical cycling associated with the nitrogen cycle and was an ideal context for implementing the 4L-SID test. To characterize the hydrographic and biogeochemical conditions of the upper water column, vertical profiles were conducted daily using a CTD system coupled to a rosette consisting of 24 × 12 L Niskin bottles. Oxygen (O_2) and fluorescence sensors were calibrated against discrete measurements of dissolved O_2 (Carritt and Carpenter, 1966) and chlorophyll extracted and analyzed by fluorometry (Strickland and Parsons, 1972). Seawater for determination of nutrient concentrations was sampled and analyzed as documented in the online manual for "HOT Laboratory Protocols" (http://hahana.soest.hawaii.edu/hot/protocols/protocols.html). Regional ocean color and sea-level anomaly for the NPSG were analyzed using satellite-derived images from the Moderate Resolution Imaging Spectroradiometer (MODIS).

Operation of the submersible incubation device

Since the original description of the SID in the 1990's (Taylor and Doherty, 1990), there have been several subsequent versions of the SID concept which have adapted the instrumentation (Lohrenz *et al.*, 1992; Taylor and Howes, 1994; Albert *et al.*, 1995; Edgcomb *et al.*, 2014; Pachiadaki *et al.*, 2014). This study is the first time that the 4L-SID has been used for conducting $^{15}N_2$ rate measurements and therefore the entire instrument configuration relevant to quantifying N_2 fixation is outlined here.

The SID, as configured for this study, consists of a hydraulically driven, syringe-like 4-L incubation chamber, an 18-port fluidic distribution valve (FDV) for directing fluid flows, an array of 8 "version 1" Fixation Filter units (FF1s, Taylor *et al.*, 2013) for collection and preservation of incubated particulate samples and a controlling electronics/battery pack (Fig. 2A). The incubation chamber consists of a precision bore borosilicate glass chamber [interior silane treated with SurfaSil™ siliconizing fluid (Thermo Scientific) for biological inertness]. Each end of the incubation chamber is capped with silicone O-ring-sealed polycarbonate end caps and it contains a silicone O-ring-sealed polycarbonate floating piston. The rotor/stator components of the FDV in contact with sample are made of PVC and Teflon® and interfacing tubing between the incubation chamber, FDV and FF1s are made of Teflon®. All interiors were acid-washed and rinsed with deionized water prior to deployment. Communication with the instrument prior to and after each deployment for programming and data retrieval was via a serial RS-232 link with a laptop PC. The 4L-SID was mounted to a free-drifting spar float system (Fig. 2B) for deployments at a fixed depth of 25 m. During the instrument operation *in situ*, the location of the spar float system was constantly monitored via two iridium GPS transponders. The FF1s are unique in-line filter units that each contain an appropriate chemical preservative that is delivered (with no moving parts) through the filter by density-driven laminar convection after completion of filtration (Supplementary data, Fig. S1).

The SID was configured for deployment with the hydraulically driven floating piston flushed against the check valve-containing end cap (Fig. 3A). The space behind the floating piston was filled with deionized water. After deployments in the afternoon, the incubation sequence was programmed to automatically commence the next morning at 0530. To condition the interior of the incubation chamber with environmental sample, ~500 mL of seawater from the depth of deployment was drawn into the chamber via the inlet check valve (ICV, red inset, Fig. 3B) and expelled back into the environment via the FDV "Waste" outlet (Fig. 3A). A total of two flushes were executed. The flushing operation was immediately followed by complete filling of the incubation chamber with sample via the ICV, advancement of the FDV rotor to the first FF1 filter unit and immediate filtration of the entire 4 L to obtain a natural abundance time zero (T_0) particulate sample. The ICV has a large enough internal spacing that will not select against larger

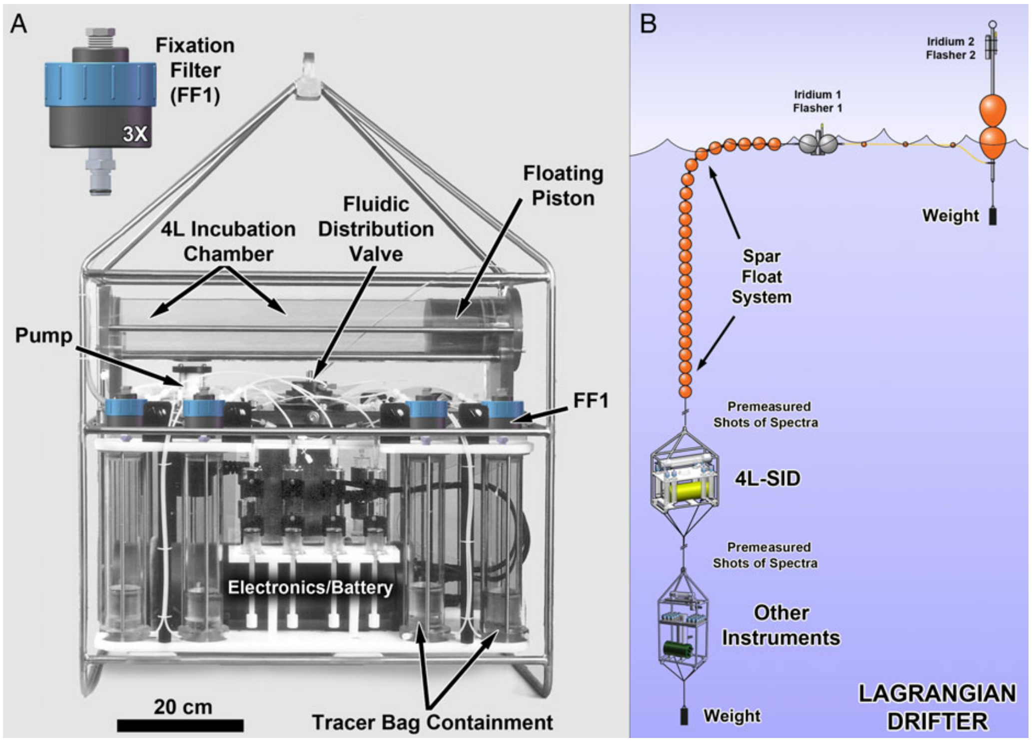

Fig. 2. 4L-SID and Lagrangian drifting buoy. (**A**) Chart showing all components of the 4L-SID including the Fixation Filters "FF1s," stand-alone devices for stopping biological activity and chemically preserving incubated sample. Not shown are the four gas-tight 200-mL polyethylene bags (http://www.pmcbag.com/) that stored $^{15}N_2$-enriched seawater and were individually connected to the 18-port sample distribution valve via Luer locks and 1.6-mm I.D. Teflon tubing. Within each individual piece of connection tubing, 400 μL of a 0.1-M solution of $H^{13}CO_3^-$ were stored, separated from seawater by small bubbles of air (see Fig. 3). (**B**) Lagrangian drifter setup used in this study.

organisms (21 mm diameter annulus with a 1.63-mm spacing, through six 2.38-mm diameter holes, ultimately into the chamber via a 4.76-mm diameter orifice; see Fig. 3B inset). During filling, the ICV exerts low shear stress of 1.2 pascals (Pa); max. 1.7 Pa, at a flow rate of 200 mL/min (Taylor *et al*., 2015). The FDV advanced to the next valve port connected to the first bag of tracer and the chamber then re-filled as described above. The slight negative pressure that developed within the chamber during filling also quantitatively draws the entire $^{15}N_2$ contents from the flexible tracer bag, which also quantitatively sweeps the ^{13}C-bicarbonate contained within the in-line injector coil into the chamber as shown in Fig. 3B (tracer details described below). The gentle turbulence generated from the main bulk of the sample entering the chamber via the ICV completely mixes the tracer with the sample as it enters the chamber (confirmed by dye studies). The 4-L sample was then incubated for a preprogrammed 23.5 h, followed by direction of sample to the next FF1 to obtain the T_{incub} sample (Fig. 3C). Upon completion of the incubation the chamber was flushed 4× as described above to remove tracer. The taper of floating piston and front end cap were machined to the same angle, minimizing the dead volume remaining when the piston meets the front endcap. Assuming an interior dead volume of 4 mL when the chamber is empty, the 4× flushing cycle dilutes the tracer contents by 5.6 orders of magnitude, which is well below background concentrations. A given incubation cycle consumes three ports of the FDV and two FF1s (Fig. 3A). The 4L-SID, as configured, was thus able to conduct four *in situ* incubations.

All filtrations were collected onto 47-mm diameter precombusted glass fiber filters (GFF) and chemically preserved in a pH 2 acid buffer inside the FF1s (Taylor and Doherty, 1990; Taylor and Howes, 1994; Supplementary data, Fig. S1), which terminates biological activity and preserves the sample for at least 1.5 month in warm water (confirmed by a Bermuda Test Bed mooring SID deployment where data agreed well with Bermuda Atlantic Time-Series measurements made at the same depth [C. D. Taylor, unpublished data]). Once the SID was recovered aboard ship, the filters were immediately recovered and dried for 48 h at 60°C in a drying oven and then stored at room temperature until analyzed. In the laboratory, the filters were pelleted and sent for isotopic analysis at the University of California, Davis Stable Isotope

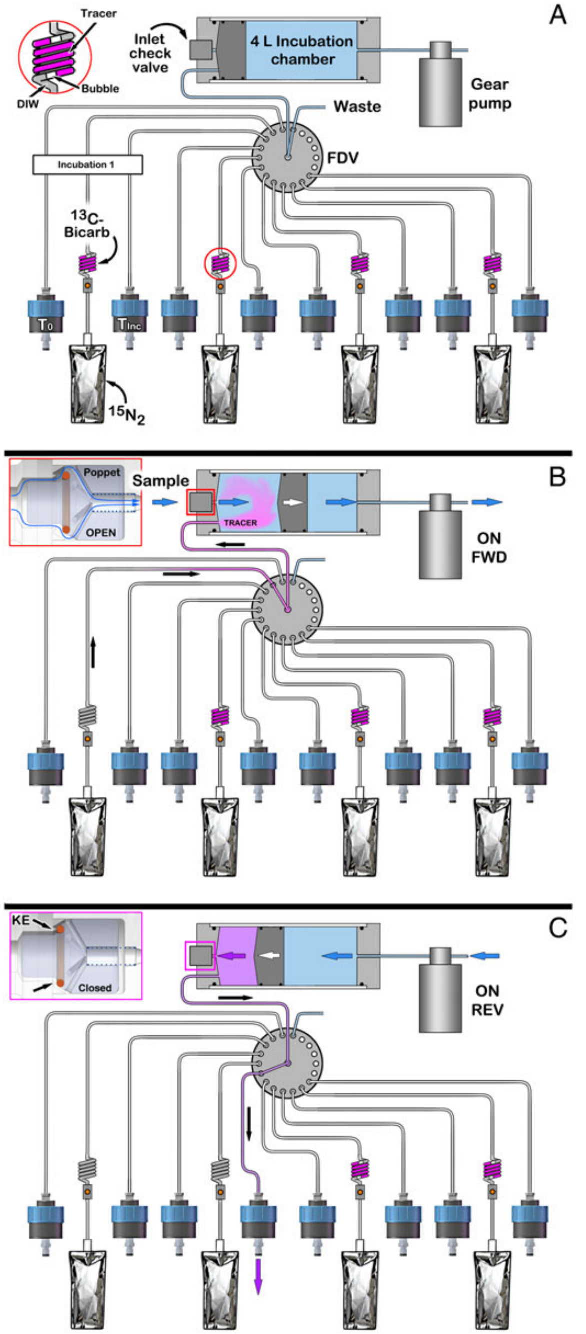

Fig. 3. Diagrammatic illustration of 4L-SID functions. (**A**) Deployed configuration. FDV: 18 port fluidic distribution valve. The inset shows an enlarged view of the ^{13}C-bicarbonate stored in the tracer coil. (**B**) Procurement of sample and introduction of tracer. The inset illustrates the water flow through the inlet check valve (ICV). The poppet is normally closed by a light duty, Teflon® coated spring, except for when sample is drawn into the chamber. When the piston reaches the full extent of its travel in either direction, it is "lugged down" and the reduction in pump rounds per minute sensed by the electronics turns off the pump. (**C**) Delivery of incubated sample through an FF1 filter holder. The inset illustrates the seating of the Poppet O-ring against an annular "Knife Edge" (KE) to prevent loss of sample through the check valve during emptying of the chamber.

Facility. BNF rate calculations followed the protocol of Montoya *et al.* (Montoya *et al.*, 1996). To test for leakage of low molecular weight (LMW) metabolites into the acidic preservative, the remaining preservative contents of the FF1s were also recovered, evaporated onto GFF filters (soaking the GFFs with the preservative and putting them in a drying oven), and these filters were treated as described above for the particulate filters. We found only very low or even undetectable amounts of carbon and nitrogen on these filters, and more importantly, the δ^{13}C and δ^{15}N values were equal or even lower than those of the respective non-tracered T_0 samples. Thus, the SID-derived rates were not underestimated due to loss of tracer to the LMW fraction.

The two tracers added to the SID incubations were $^{15}N_2$ gas to obtain estimates of BNF and ^{13}C-bicarbonate for measurements of primary productivity. $^{15}N_2$ gas (98 atom%; Sigma-Aldrich) was added to seawater samples as "$^{15}N_2$-enriched seawater" which was prepared on land prior to the cruise using sterile-filtered surface seawater from Station ALOHA (10 mL $^{15}N_2$ per liter of seawater; Wilson *et al.*, 2012). The $^{15}N_2$ gas used in this study was from a batch manufactured from 2008 to 2009 by Sigma-Aldrich, and we identified it as not causing severe contamination with other bioavailable inorganic N species (Dabundo *et al.*, 2014). After enrichment, the tracer water was stored in 200-mL gas-tight tri-layer aluminized polyethylene bags (http://www.pmcbag.com/). The bags were individually connected to the 18-port FDV via Luer locks and 1.6-mm I.D. Teflon tubing and coiled in-line tracer loops made of Teflon® tubing, as illustrated in Fig. 3A. A complete 200-mL bag was added to each 4-L incubation, providing a final atom enrichment of 5%. For the ^{13}C additions, 400 μL of a 0.1-M solution of $H^{13}CO_3^-$ were stored within a coiled section of the Teflon tubing (see inset in Fig. 3A). To facilitate loading of the ^{13}C-tracer into the coil using a syringe, small bubbles (volume ~50 μL) were introduced at the beginning and end of the injection. The leading bubble isolates the tracer from the water contained within the tubing leading to the FDV, allowing it to be introduced as a "plug flow" instead of the spreading of tracer by the parabolic laminar flows that would otherwise occur. The trailing bubble provides isolation from the $^{15}N_2$-enriched seawater in the bag. The surface tension of the small bubbles quite effectively confines the tracer within the loop and resists modest vibration.

Complementary $^{15}N_2$ measurements conducted during the cruise

Measurements of BNF were also conducted during the cruise by sampling the water column using the CTD–rosette and incubating the seawater samples either using an *in situ* array or on-deck incubators which simulated *in situ* conditions. The *in situ* incubations were used to obtain vertical profiles of BNF. Seawater was collected from depths of 5, 25, 45, 75, 100 and 125 m into replicate 4.3- L polycarbonate bottles, amended with $^{15}N_2$-enriched water and attached to a free-floating *in situ* array at the appropriate depth for a 24-h period (Church *et al.*, 2009). The on-deck incubations were performed for 24 h using blue shaded incubators cooled with running surface seawater and additional neutral mesh shading to mimic the corresponding light irradiances for each depth. Both sets of BNF measurements were also amended with 400 μL of a 0.1-M solution of $H^{13}CO_3^-$ injected through the septum cap with a syringe. Upon termination of the incubations, the seawater samples were gently filtered through precombusted Whatman GFF filters (0.7-μm nominal pore size) and processed as described for the SID filters.

Quantitative PCR

Different diazotrophs present in the water column were quantified using quantitative PCR (qPCR) enumeration of specific *nifH* gene copies. Water column samples were collected from between 5 and 175 m depth. Once the CTD was recovered, the seawater was immediately drained from the Niskin bottles into acid-washed 4-L polycarbonate bottles. Using peristaltic pumps, 2 L from each depth was filtered in-line through 10 μm (Polyester, Sterlitech, Kent, WA, USA) and 0.2 μm (Supor; Pall Life Sciences, Ann Arbor, MI, USA) pore-size filters, held in 25-mm-diameter Swinnex filter holders (Millipore, Billerca, MA, USA). The filters were placed into sterile 1.5-mL cryotubes containing 0.1 g autoclaved glass beads, frozen in liquid nitrogen and stored at −80°C until processing in the laboratory. DNA extractions were carried out as described previously (Bombar *et al.*, 2013). We used previously designed Taq-Man® primer-probe sets, including cyanobacterial phylotypes UCYN-A and UCYN-B (Moisander *et al.*, 2010), *Trichodesmium* (Church *et al.*, 2005) and two Diatom−Diazotroph Associations (DDAs) (Foster *et al.*, 2007) termed het-1 (*Rhizosolenia-Richelia*) and het-2 (*Hemiaulus-Richelia*). Additionally, we quantified presumed heterotroph diazotroph phylotypes HM210397 (γ-Proteobacteria) and KC013231 (Cluster 3), described in Bombar *et al.* (Bombar *et al.*, 2013). qPCR optimizations and quantifications have been described in detail in Moisander *et al.* (Moisander *et al.*, 2010), Halm *et al.* (Halm *et al.*, 2012) (specifications for phylotype HM210397) and Bombar *et al.* (Bombar *et al.* 2013) (specifications for phylotype KC013231).

RESULTS AND DISCUSSION

The application of *in situ* devices for observing physical, chemical and biological parameters in the ocean is an important approach for better understanding the complex relationships between the physical and chemical environment and microbial distributions and activities (Taylor and Howes, 1994; Johnson *et al.*, 2010; Ottesen *et al.*, 2011; Robidart *et al.*, 2014). This study reports the first successful deployment of an autonomous device capable of conducting sampling, incubation and filtration processes for BNF measurements *in situ*. We evaluate the operation of the SID as an *in situ* instrument for BNF measurement, compare the SID-derived BNF rates with commonly applied sampling and incubation methods and discuss the SID rates in the context of physical, chemical and microbial data obtained during the BioLINCS cruise.

SID operation and measurements of BNF rates

The SID was deployed twice during the 2011 BioLINCS cruise (Fig. 1). During the first deployment (4L-SID Deployment 1) from 9 to 14 September 2011, it followed a 47-km drift path in a north-easterly direction, and during the second deployment (4L-SID Deployment 2) from 16 to 20 September 2011, it drifted 28 km westwards (Fig. 1). During both deployments, the 4L-SID performed four autonomous tracer incubations over a 4-day period at a depth of 25 m in the water column. With respect to the proximity of each incubation event, the 4L-SID sampled approximately every 16 km during Deployment 1 and every 10 km during Deployment 2.

The 4L-SID-derived BNF measurements ranged from 0.8 to 1.9 nmol N L^{-1} day^{-1} (average 1.4 ± 0.5 nmol N L^{-1} day^{-1}) during Deployment 1 and from 1.4 to 2.8 nmol N L^{-1} day^{-1} (average 2.0 ± 0.6 nmol N L^{-1} day^{-1}) during Deployment 2 (Table I). Thus, during each 4-day deployment, a 2-fold variation of BNF was recorded, and BNF rates were overall higher during Deployment 2. The simultaneous rate measurements of ^{13}C primary production by the 4L-SID also revealed higher values for Deployment 2 (344 ± 40 nmol C L^{-1} day^{-1}) compared with Deployment 1 (207 ± 48 nmol C L^{-1} day^{-1}). The higher rates of both ^{13}C primary production and BNF suggest that the difference in BNF rates between the two deployments was not due to methodological errors that were only specific to the BNF measurements (Table I, Fig. 4).

Compared with 4L-SID-derived rates of $^{15}N_2$ assimilation, BNF rates obtained from incubations in the on-deck incubator (with water from 25 m) were higher during Deployment 1 (3.3 ± 0.2 nmol N L^{-1} day^{-1}) and more variable (ranging from 1.4 ± 0.1 to 6.6 ± 1.9 nmol N

Table I: Summary of values of BNF ($^{15}N_2$ fixation) and primary production ($H^{13}CO_3^-$ fixation) obtained from 24-h SID incubations and comparison incubations on-deck or using the in situ *array*

SID			On-deck incubations			*In situ* array		
Date (month/day) in 2011	N_2 fixation (nmol N L^{-1} day^{-1})	Primary production (nmol C L^{-1} day^{-1})	Date (month/day)	N_2 fixation (nmol N L^{-1} day^{-1}) $n = 2$	Primary production (nmol C L^{-1} day^{-1}) $n = 2$	Date (month/day)	N_2 fixation (nmol N L^{-1} day^{-1}) $n = 2$	Primary production (nmol C L^{-1} day^{-1}) $n = 2$
September 10–11	1.9	229	September 10–11	3.3 ± 0.2	322 ± 47	September 8–9	0.9 ± 1.1	381 ± 0
September 11–12	1.6	237		–	–	September 10–11	2.9 ± 0.0	594 ± 68
September 12–13	1.4	226		–	–		–	–
September 13–14	0.8	135		–	–		–	–
September 16–17	2.8	330	September 16–17	1.4 ± 0.1	434 ± 10		–	–
September 17–18	1.4	307		–	–		–	–
September 18–19	1.8	400	September 18–19	6.6 ± 1.9	–		–	–
September 19–20	2.1	338		–	–		–	–

Parallel to the first incubation of each deployment, a comparison incubation in the shipboard incubator was carried out, using water sampled from 25 m at stations close to the SID location. These comparisons, even though they are not perfect control measurements taken in closest proximity to the SID, suggest that the SID-derived rates are of realistic magnitude.

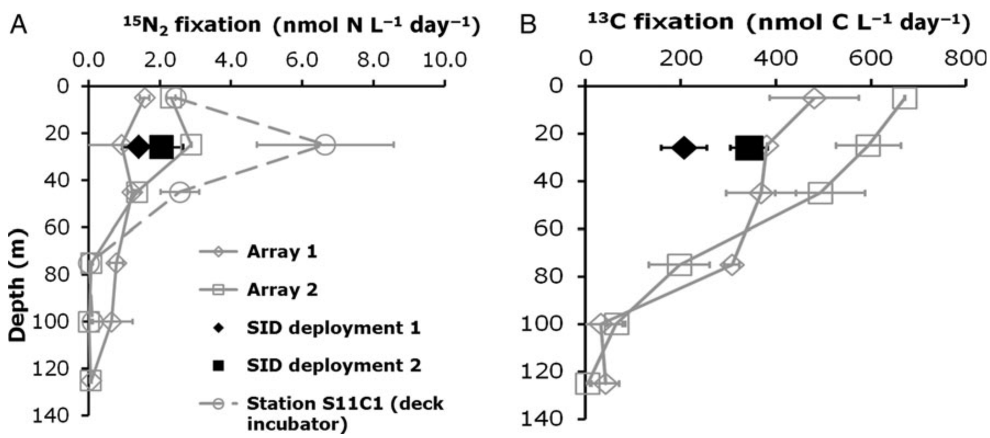

Fig. 4. (**A**) BNF and (**B**) primary production rates obtained from both SID deployments in the context of vertical rate profiles obtained from *in situ* array deployments ($n = 2$). Incubations at Station 11 are shown as well, but were done in the shipboard incubator due to the loss of the *in situ* array. No primary production was measured on Station 11.

L^{-1} day^{-1}) during Deployment 2 (Table I). There are several factors which might contribute to the difference in 4L-SID and on-deck measurements of $^{15}N_2$ assimilation. The first is the potential for sampling different populations of diazotrophs by the SID and the shipboard CTD–rosette. The distance between the ship and the SID when the comparative samples were taken was 5.4 and 3.9 km for 4L-SID Deployment 1 and Deployment 2, respectively (Fig. 1). Another potential source of variability is the method of incubation itself. The on-deck incubators are a best-effort to mimic temperature and light levels equivalent to a depth of 25 m in the water column, but have a few limitations. For example, near-surface seawater intake is used for cooling which has a slightly higher temperature than 25 m water, and the existence of variability in light intensity due to shading among incubation bottles or from ship structures.

Potential perturbations of the natural abiotic conditions during sampling and incubations are a well-known problem (Feike *et al.*, 2012), and highlight the necessity for using *in situ* devices especially when longer incubations are required. This theory seems to be supported by the BNF measurements obtained using an *in situ* array which performed incubations at 25 m, the results of which are better aligned with the 4L-SID Deployment 1 values (Table I, 0.9–2.9 nmol N L^{-1} day^{-1}). Unfortunately, corresponding *in situ* array incubations to match Deployment 2 were not obtained due to the loss of the array at sea. In contrast, the available rates of ^{13}C primary production (Table I) do not mirror the high similarity of BNF observed between the SID and the *in situ* array measurements, with higher rates obtained by the *in situ* array compared with the SID. However, overall both the 4L-SID-derived BNF and primary production rates compare favorably with values obtained using traditional methods during the same oceanographic expedition and during time-series measurements conducted at nearby Station ALOHA. BNF and ^{14}C primary production (*in situ* array incubations) rate measurements are conducted on a nearly monthly basis at Station ALOHA, situated ~100 km to the south of the BioLINCS expedition region [Hawaii Ocean Time-series (HOT) and other funding programs (Grabowski *et al.*, 2008; Church *et al.*, 2009)]. BNF rates at 25 m depth ranged from 1 to 5 nmol N L^{-1} day^{-1} during the summer months (July–September) between 2004 and 2007 (Church *et al.*, 2009, $^{15}N_2$ bubble addition protocol). During a more recent cruise in June 2014, BNF rates were measured daily for 7 days and produced values of 5.9 ± 1.1 nmol N L^{-1} day^{-1} (range 3.7–7.2 nmol N L^{-1} day^{-1}) (S. T. Wilson, unpublished data, $^{15}N_2$ added as predissolved in sterile-filtered seawater). The average rate of ^{14}C primary production for the month of September during the years 1989–2011 is 463 ± 126 nmol C L^{-1} day^{-1}, which is quite similar to values obtained using different incubation techniques during our cruise (Table I). We can only speculate on the reasons for the comparably high primary production from *in situ* array 2 (11 September 2011, 594 ± 68 nmol C L^{-1} day^{-1}) or the relatively low values from SID deployment 1 (207 ± 48 nmol C L^{-1} day^{-1}). Interestingly, CTD chlorophyll fluorescence measurements were relatively high at 25 m during sampling for *in situ* array 2 (0.20 ± 0.00 μg L^{-1}) compared with any measurements at 25 m at four stations in proximity to 4L-SID samplings during deployment 1 (0.13 ± 0.04 μg L^{-1}). Given the high spatiotemporal variability in prokaryotic distributions and abiotic conditions for our particular expedition (Fig. 5, also see Robidart *et al.*, 2014), the observed range in primary production appears realistic. Without more specific comparison experiments, at this point it is difficult to claim that one or the other method delivers more trustworthy rate measurements. Most importantly, the overall similar range of BNF and primary production values obtained using the different methods supports the efficacy of the SID for *in situ* rate measurements.

Hydrographic and biogeochemical background

4L-SID Deployment 1 was on the northern edge of an anticyclonic eddy (Fig. 1). During the second deployment, the SID drifted west between the primary eddy and a smaller adjoined anticyclonic eddy (Fig. 1). The drift paths for both 4L-SID deployments followed the clockwise circulation patterns of the two anticyclonic eddies revealed by sea surface altimetry (Fig. 1) and shipboard acoustic doppler current profiler measurements (Robidart *et al.*, 2014). These mesoscale eddies introduced small-scale physical and chemical heterogeneity in the area, which clearly affected microbial distributions, especially of diazotroph cyanobacteria (Robidart *et al.*, 2014).

Several hydrographic and biogeochemical conditions may have influenced the 4L-SID measurements. Low average wind speeds of 4.8 m s^{-1} for Deployment 1 and 6.9 m s^{-1} for Deployment 2 contributed to a shallow mixed layer depth (MLD) during both deployments, but with an average of 17 m (range 10–27 m) during Deployment 1 and 30 m (range 14–57 m) for Deployment 2 (based on 0.03 density offset from 10 m criterion) (De Boyer Montégut *et al.*, 2004). Accordingly, along the 25-m depth horizon seawater temperatures averaged 25.9°C (range 25.7–26.1°C) during Deployment 1 and 26.2°C (range 26.1–26.2°C) during Deployment 2 (Fig. 5). Therefore, possibly the majority of sampling conducted by the 4L-SID during Deployment 1 was beneath the mixed layer, and thus below the main accumulation of diazotrophs, which could partly explain why lower rates were found compared with Deployment 2. However, with the available qPCR data (samples from 5, 25, 45 m etc.) we cannot resolve whether the MLD had an influence on the vertical distribution of diazotrophs that would explain the variations in BNF.

Nutrient concentrations in the upper 100 m were mostly low, which is common in oligotrophic oceanic gyres (Fig. 5). However, some of the $NO_2^- + NO_3^-$ (low-level nitrogen, LLN) and phosphate concentrations near the surface equaled concentrations much deeper in the water column at around 125 m (Fig. 5D, near the "apex"), which is atypical for NPSG waters (Robidart *et al.*, 2014). Along the 25-m-depth horizon where the 4L-SID was situated, LLN concentrations ranged from 2–6 nmol L^{-1} and phosphorus concentrations ranged from 20 to 137 nmol L^{-1} for the complete 12-day oceanographic expedition (Fig. 5). During Deployment 1, the 4L-SID encountered remarkably steep gradients in salinity and phosphate concentrations (Fig. 5B and D). In turn, waters sampled

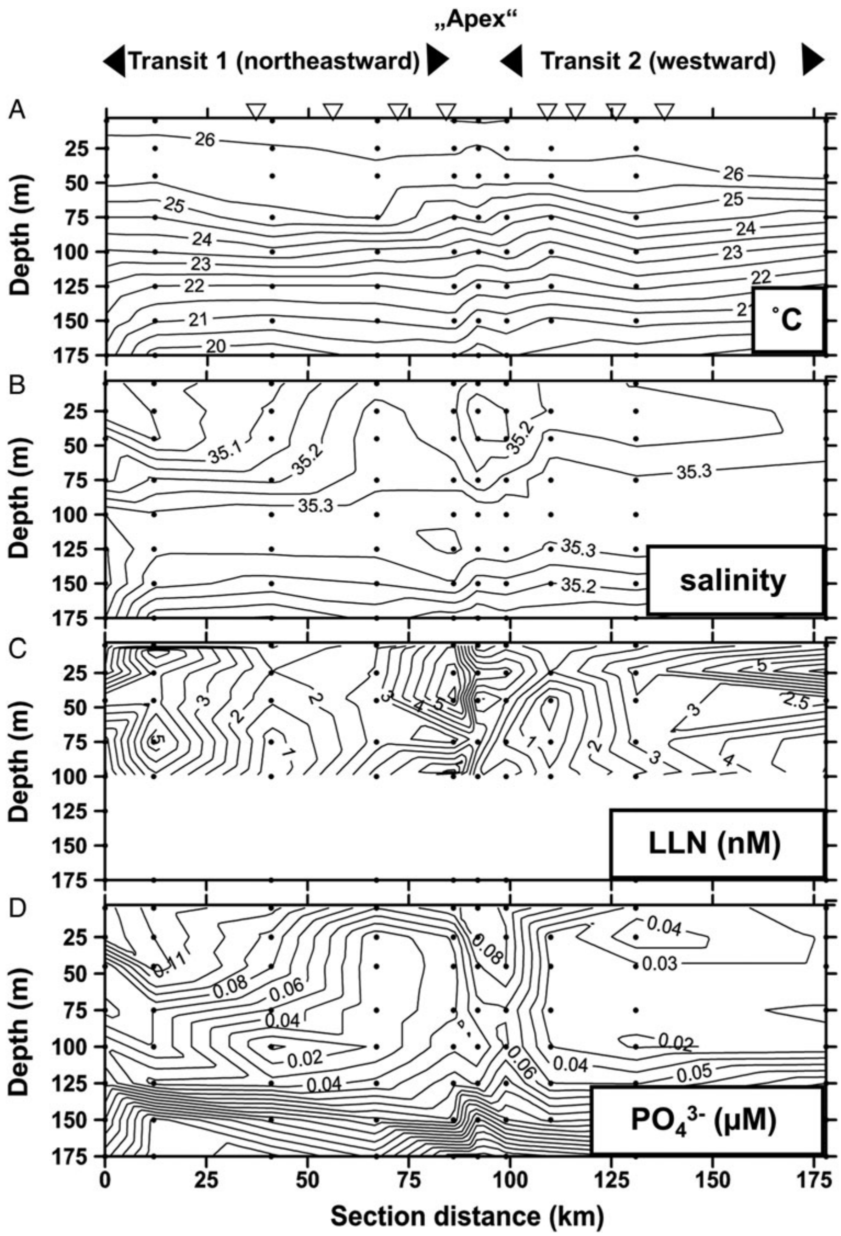

Fig. 5. Contour plots showing (**A**) temperature, (**B**) salinity, concentrations of (**C**) low-level nitrogen (LLN = $[NO_3^- + NO_2^-]$) above 100 m depth, and (**D**) phosphate measured on shipboard stations during the BioLINCS cruise. The data are plotted versus depth and total section distance, i.e. distance covered by shipboard stations along the northeast- and following westward transit. The triangles at the top axis indicate where the SID took samples along the section distance during its two consecutive deployments (four samplings per deployment).

during Deployment 2 were relatively rich in chlorophyll (Fig. 1). Overall, these data suggest that different water types were sampled during 4L-SID Deployments 1 and 2.

The highest BNF and primary production rates were measured nearest to the "apex" of the cruise transit (Fig. 5), where nutrient concentrations were elevated. Possibly, diazotrophs in this region were stimulated by the nutrients and were able to respond with higher BNF, although it is not clear whether nutrient concentrations were elevated due to influx from depth, atmospheric deposition (Kim *et al.*, 2014), or whether there was low demand in the microbial community which led to its accumulation within the surface layer. The 4L-SID drifts in a Lagrangian manner and can provide unbiased samples describing the variability in BNF within a complex setting of small spatiotemporal fluctuations in abiotic parameters. In order to pinpoint specific abiotic/biotic parameters responsible for observed variations in BNF rates, future versions of the SID need to include additional oceanographic sensors, like a CTD package including O_2, nitrate and optical sensors (see next section).

The qPCR quantifications of *nifH* gene copies suggest that unicellular cyanobacterial diazotrophs (*Candidatus* Atelocyanobacterium thalassa, "UCYN-A," and *Crocosphaera watsonii*, "UCYN-B") were the most abundant diazotrophs present in the water column throughout the sampled area (Fig. 6A). In the region near the apex, *nifH* genes of these organisms attained concentrations of $\sim 2.0 \times 10^8$ copies m^{-2}, which was 96% of all quantified *nifH* gene copies. *Trichodesmium* sp. was the next most abundant diazotroph (up to 3.0×10^7 *nifH* gene copies m^{-2}); heterocystous symbionts of diatoms (het-1 and het-2) as well as heterotrophic bacteria were present at much lower abundances (Fig. 6B). While *nifH* gene copy inventories appeared to co-vary for unicellular cyanobacterial diazotrophs (UCYN-A and UCYN-B, Fig. 6A), a different pattern was observed for the remaining five phylotypes, i.e. *Trichodesmium*, heterocystous cyanobacterial symbionts het-1 and het-2, and the two heterotroph diazotrophs (Fig. 6B). The *nifH* gene abundances in this latter group also co-varied, but appeared to be relatively more abundant at stations parallel to 4L-SID deployment 1 (Fig. 6B). This group includes the diazotrophs typically assigned to the >10-μm size fraction (*Trichodesmium* and heterocystous cyanobacteria), and was up to 42% of *nifH* inventories in the "Transit 1" area (Fig. 6C). These data suggest that the eddy-induced advection and mixing in the area had clear effects on the distribution of different diazotrophs. While such data cannot be used to infer which diazotrophs were responsible for the measured BNF rates, it is noteworthy that the overall higher rates obtained during 4L-SID deployment 2 coincided with a generally lower abundance of diazotrophs in the >10-μm size fraction.

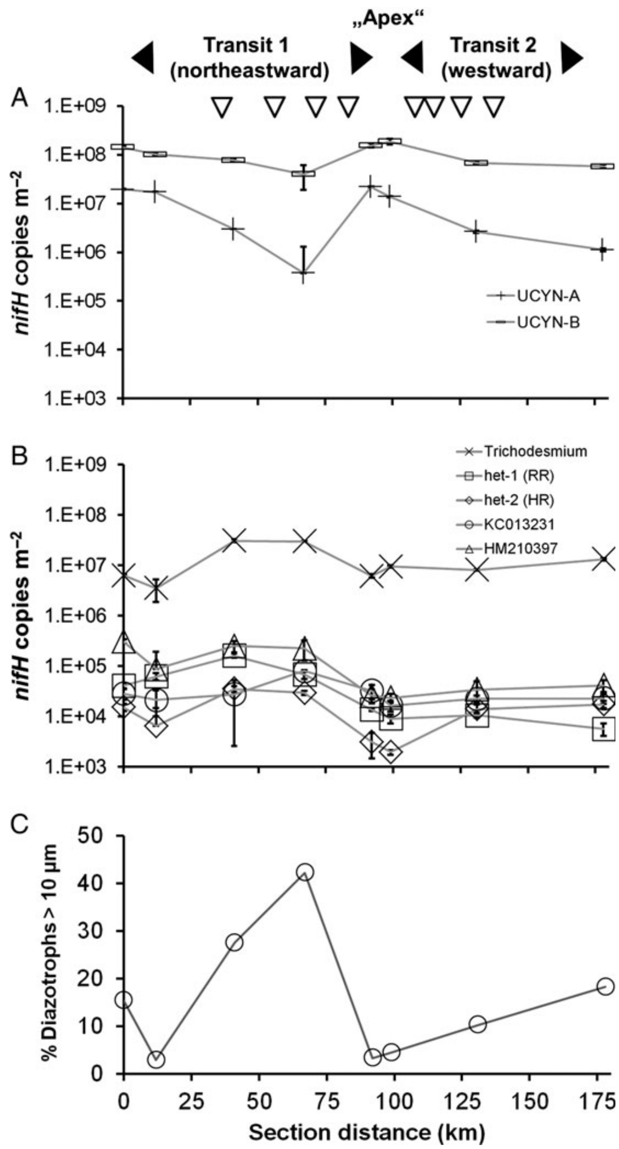

Fig. 6. Depth-integrated inventories of *nifH* gene copies of diazotroph microorganisms (quantified by qPCR) on ship stations in close proximity to the SID drift paths. Section distance on the *x*-axis is the total distance covered by shipboard stations along the northeast and following westward transit. (**A**) *nifH* inventories of UCYN-A (*Candidatus* Atelocyanobacterium thalassa) and UCYN-B (*Crocosphaera watsonii*). (**B**) *nifH* inventories of *Trichodesmium* sp., het-1 and het-2 [Diatom–Diazotroph associations between the diazotroph cyanobacteria *Richelia intracellularis* and two different diatom species, *Rhizosolenia-Richelia* (RR) and *Hemiaulus-Richelia* (HR)] and heterotroph phylotypes HM210397 (Halm *et al.*, 2012) and KC013231 (Bombar *et al.*, 2013). (**C**) Percentage of the total *nifH* gene copies m^{-2} of diazotrophs that are usually found in the >10 μm size fraction (*Trichodesmium*, het-1, het-2). The triangles at the top axis indicate where the SID took samples along the section distance during its two consecutive deployments (four samplings per deployment).

Recommendations for future SID deployments

In its current configuration, the SID was successfully deployed and recovered on two occasions, providing daily BNF and primary production measurements in the surface waters of the oligotrophic open ocean. The SID concept can contribute more environmentally relevant rates of BNF to inform global flux calculations, and this technology has been validated in this study and others (Taylor and Howes, 1994; Pachiadaki *et al.*, 2014; Edgcomb *et al.*, 2014) for expeditions in various marine provinces. The SID could be especially helpful for studies in "delicate" habitats (e.g. anoxic habitats), where the seawater samples are severely compromised when they are brought onboard the research vessel for on-deck incubations (Feike *et al.*, 2012; Edgcomb *et al.*, 2014).

The current SID technology would be improved by conducting simultaneous replicate measurements. Furthermore, an increased number of FF1s would permit more samples to be processed and longer deployment periods. The ability to also collect replicate samples for metagenomic and metatranscriptomic analysis will enable investigators to link biological function with the identity and activity of the prokaryotic key players present in the water column at the exact time of sampling (Robidart *et al.*, 2014). To this end, a new SID-implemented fixation filter (FF3, Taylor *et al.*, 2015) capable of chemically preserving particulate microbial samples in a manner compatible with subsequent metagenomic and metatranscriptomic study (Edgcomb *et al.*, 2014) has just been developed. Finally, the addition of further oceanographic sensors, like an ISUS for NO_3^- measurements (Johnson and Coletti, 2002), and the ability for adaptive sampling in response to thresholds in environmental parameters, would allow the SID to sample along environmental gradients. A relatively new version microbial sampling-SID (MS-SID, Edgcomb *et al.*, 2014; Pachiadaki *et al.*, 2014; with a host of sensors [CTD, turbidity sensors, oxygen optode]), that was not available during this study, is now in hand and possesses the ability of collecting/*in situ* chemically preserving up to 48 incubated samples and/or larger volume microbial samples as well as 24 samples in gas-tight bags. At present, a dual incubation chamber SID is in advanced development (Vent-SID, laboratories of C. Taylor and S. Sievert, WHOI).

CONCLUSIONS

The development of a device for conducting the entire sampling, incubation and filtration processes of $^{15}N_2$ rate measurements *in situ* could help in the future to obtain higher resolution coverage of direct estimates of oceanic BNF. The two 4-day deployments conducted during the September 2011 BioLINCS cruise were successful with respect to instrument operation and obtaining BNF rates comparable with those achieved by traditional CTD–rosette sampling and incubation. Overall, the SID offers increased sampling resolution of BNF measurements and a platform for conducting *in situ* sampling of oceanic water columns where on-deck incubations are not feasible. This device will help in identifying driving factors of BNF *in situ*, and could be used to test important hypotheses about the regulation of BNF within the oceanic nitrogen cycle.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

ACKNOWLEDGEMENTS

We thank M. Hogan and T. Cote for help in cruise organization, the personnel onboard R/V Kilo Moana for technical assistance and John Ryan for help with Fig. 1.

FUNDING

This study was supported by Gordon and Betty Moore Foundation (GBMF) Marine Investigator Awards (J.P.Z. and D.M.K.), the MEGAMER facility (supported by GBMF), the grant from the NSF Emerging Frontiers Program (Center for Microbial Oceanography: Research and Education, grant DBI-0424599) and grants NSF OCE-1061774 to V. Edgcomb and C.T.

REFERENCES

Albert, D. B., Taylor, C. and Martens, C. S. (1995) Sulfate reduction rates and low molecular weight fatty acid concentrations in the water column and surficial sediments of the Black Sea. *Deep Sea Res. Part I*, **42**, 1239–1260.

Bench, S. R., Ilikchyan, I. N., Tripp, H. J. and Zehr, J. P. (2011) Two strains of *Crocosphaera watsonii* with highly conserved genomes are distinguished by strain-specific features. *Front. Microbiol.*, **2**, 261.

Bombar, D., Heller, P., Sanchez-Baracaldo, P., Carter, B. J. and Zehr, J. P. (2014) Comparative genomics reveals surprising divergence of two closely related strains of uncultivated UCYN-A cyanobacteria. *ISME J.*, **8**, 2530–2542.

Bombar, D., Turk-Kubo, K. A., Robidart, J., Carter, B. J. and Zehr, J. P. (2013) Non-cyanobacterial nifH phylotypes in the North Pacific subtropical gyre detected by flow-cytometry cell sorting. *Environ. Microbiol. Rep.*, **5**, 705–715.

Capone, D. G., Burns, J. A., Montoya, J. P., Subramaniam, A., Mahaffey, C., Gunderson, T., Michaels, A. F. and Carpenter, E. J. (2005) Nitrogen fixation by *Trichodesmium* spp.: an important source of new nitrogen to the tropical and subtropical North Atlantic Ocean. *Global Biogeochem. Cycles*, **19**, GB2024: 1–17.

Capone, D. G., Zehr, J. P., Paerl, H. W., Bergman, B. and Carpenter, E. J. (1997) *Trichodesmium*, a globally significant marine cyanobacterium. *Science*, **276**, 1221–1229.

Carritt, D. E. and Carpenter, J. H. (1966) Comparison and evaluation of currently employed modifications of the Winkler method for determining dissolved oxygen in seawater; a NASCO report. *J. Mar. Res.*, **24**, 286–318.

Church, M. J., Jenkins, B. D., Karl, D. M. and Zehr, J. P. (2005) Vertical distributions of nitrogen-fixing phylotypes at Stn ALOHA in the oligotrophic North Pacific Ocean. *Aquat. Microb. Ecol.*, **38**, 3–14.

Church, M. J., Mahaffey, C., Letelier, R. M., Lukas, R., Zehr, J. P. and Karl, D. M. (2009) Physical forcing of nitrogen fixation and diazotroph community structure in the North Pacific subtropical gyre. *Global Biogeochem. Cycles*, **23**, GB2020.

Dabundo, R., Lehmann, M. F., Treibergs, L., Tobias, C. R., Altabet, M. A., Moisander, P. H. and Granger, J. (2014) The contamination of commercial $^{15}N_2$ gas stocks with ^{15}N-labeled nitrate and ammonium and consequences for nitrogen fixation measurements. *PLoS ONE*, **9**, e110335.

Dandonneau, Y. and Bouteiller, A. L. (1992) A simple and rapid device for measuring planktonic primary production by *in situ* sampling, and ^{14}C injection and incubation. *Deep Sea Res. Part I*, **39**, 795–803.

De Boyer Montégut, C., Madec, G., Fischer, A. S., Lazar, A. and Iudicone, D. (2004) Mixed layer depth over the global ocean: an examination of profile data and a profile-based climatology. *J. Geophys. Res. Oceans*, **109**, C12003.

Edgcomb, V., Orsi, W., Taylor, G. T., Vdacny, P., Taylor, C., Suarez, P. and Epstein, S. (2011) Accessing marine protists from the anoxic Cariaco Basin. *ISME J.*, **5**, 1237–1241.

Edgcomb, V. P., Taylor, C., Pachiadaki, M. G., Honjo, S., Engstrom, I. and Yakimov, M. (2014) Comparison of Niskin vs. *in situ* approaches for analysis of gene expression in deep Mediterranean Sea water samples. *Deep Sea Res. Part II*. doi: 10.1016/j.dsr2.2014.10.020

Farnelid, H., Andersson, A. F., Bertilsson, S., Al-Soud, W. A., Hansen, L. H., Sorensen, S., Steward, G. F., Hagstrom, A. *et al*. (2011) Nitrogenase gene amplicons from global marine surface waters are dominated by genes of non-cyanobacteria. *PLoS One*, **6**, e19223.

Feike, J., Juergens, K., Hollibaugh, J. T., Krueger, S., Jost, G. and Labrenz, M. (2012) Measuring unbiased metatranscriptomics in suboxic waters of the central Baltic Sea using a new *in situ* fixation system. *ISME J.*, **6**, 461–470.

Foster, R. A., Subramaniam, A., Mahaffey, C., Carpenter, E. J., Capone, D. G. and Zehr, J. P. (2007) Influence of the Amazon River plume on distributions of free-living and symbiotic cyanobacteria in the western tropical north Atlantic Ocean. *Limnol. Oceanogr.*, **52**, 517–532.

Foster, R. A., Sztejrenszus, S. and Kuypers, M. M. M. (2013) Measuring carbon and N_2 fixation in field populations of colonial and free-living unicellular cyanobacteria using nanometer-scale secondary ion mass spectrometry1. *J. Phycol.*, **49**, 502–516.

Foster, R. A. and Zehr, J. P. (2006) Characterization of diatom-cyanobacteria symbioses on the basis of *nifH*, *hetR*, and 16S rRNA sequences. *Environ. Microbiol.*, **8**, 1913–1925.

Goebel, N. L., Turk, K. A., Achilles, K. M., Paerl, R. W., Hewson, I., Morrison, A. E., Montoya, J. P., Edwards, C. A. *et al*. (2010) Abundance and distribution of major groups of diazotrophic cyanobacteria and their potential contribution to N_2 fixation in the tropical Atlantic Ocean. *Environ. Microbiol.*, **12**, 3272–3289.

Grabowski, M. N. W., Church, M. J. and Karl, D. M. (2008) Nitrogen fixation rates and controls at Stn ALOHA. *Aquat. Microb. Ecol.*., **52**, 175–183.

Grosskopf, T., Mohr, W., Baustian, T., Schunck, H., Gill, D., Kuypers, M. M., Lavik, G., Schmitz, R. A. *et al*. (2012) Doubling of marine dinitrogen-fixation rates based on direct measurements. *Nature*, **488**, 361–364.

Gruber, N. and Galloway, J. N. (2008) An earth-system perspective of the global nitrogen cycle. *Nature*, **451**, 293–296.

Gundersen, K. (1973) In situ determination of primary production by means of a new incubator ISIS. *Helgol. Wiss. Meeresunters.*, **24**, 465–475.

Halm, H., Lam, P., Ferdelman, T. G., Lavik, G., Dittmar, T., Laroche, J., D'hondt, S. and Kuypers, M. M. M. (2012) Heterotrophic organisms dominate nitrogen fixation in the South Pacific Gyre. *ISME J.*, **6**, 1238–1249.

Hewson, I., Poretsky, R. S., Beinart, R. A., White, A. E., Shi, T., Bench, S. R., Moisander, P. H., Paerl, R. W. *et al*. (2009) *In situ* transcriptomic analysis of the globally important keystone N_2-fixing taxon *Crocosphaera watsonii*. *ISME J.*, **3**, 618–631.

Janson, S., Rai, A. N. and Bergman, B. (1995) Intracellular cyanobiont richelia-intracellularis—ultrastructure and immuno-localization of phycoerythrin, nitrogenase, Rubisco and glutamine-synthetase. *Mar. Biol.*, **124**, 1–8.

Johnson, K. S. and Coletti, L. J. (2002) In situ ultraviolet spectrophotometry for high resolution and long-term monitoring of nitrate, bromide and bisulfide in the ocean. *Deep Sea Res. Part I*, **49**, 1291–1305.

Johnson, K. S., Riser, S. C. and Karl, D. M. (2010) Nitrate supply from deep to near-surface waters of the North Pacific subtropical gyre. *Nature*, **465**, 1062–1065.

Karl, D., Michaels, A., Bergman, B., Capone, D., Carpenter, E., Letelier, R., Lipschultz, F., Paerl, H. *et al*. (2002) Dinitrogen fixation in the world's oceans. *Biogeochemistry*, **57/58**, 47–98.

Kim, I.-N., Lee, K., Gruber, N., Karl, D. M., Bullister, J. L., Yang, S. and Kim, T.-W. (2014) Increasing anthropogenic nitrogen in the North Pacific Ocean. *Science*, **346**, 1102–1106.

Laroche, J. and Breitbarth, E. (2005) Importance of the diazotrophs as a source of new nitrogen in the ocean. *J. Sea Res.*, **53**, 67–91.

Lohrenz, S. E., Wiesenburg, D. A., Rein, C. R., Arnone, R. A., Taylor, C. D., Knauer, G. A. and Knap, A. H. (1992) A comparison of *in situ* and simulated *in situ* methods for estimating oceanic primary production. *J. Plankton Res.*, **14**, 201–221.

Mague, T. H., Mague, F. C. and Holm-Hansen, O. (1977) Physiology and chemical composition of nitrogen fixing phytoplankton in the central North Pacific Ocean. *Mar. Biol.*, **41**, 213–227.

Mohr, W., Grosskopf, T., Wallace, D. W. R. and Laroche, J. (2010) Methodological underestimation of oceanic nitrogen fixation rates. *PLoS One*, **5**, e12583. doi:10.1371/journal.pone.001258.

Moisander, P. H., Beinart, R. A., Hewson, I., White, A. E., Johnson, K. S., Carlson, C. A., Montoya, J. P. and Zehr, J. P. (2010) Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. *Science*, **327**, 1512–1514.

Montoya, J. P., Voss, M., Kahler, P. and Capone, D. G. (1996) A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. *Appl. Environ. Microbiol.*, **62**, 986–993.

Ottesen, E. A., Marin, R., Preston, C., Young, C. R., Ryan, J. P., Scholin, C. and Delong, E. (2011) Metatranscriptomic analysis of autonomously collected and preserved marine bacterioplankton. *ISME J.*, **5**, 1881–1895.

Pachiadaki, M. G., Taylor, C., Oikonomou, A., Yakimov, M. M., Stoeck, T. and Edgcomb, V. (2014) In situ grazing experiments apply new technology to gain insights into deep-sea microbial food webs. *Deep Sea Res. Part II*. doi:10.1016/j.dsr2.2014.10.019.

Robidart, J. C., Church, M. J., Ryan, J. P., Ascani, F., Wilson, S. T., Bombar, D., Marin, R., Richards, K. J. *et al*. (2014) Ecogenomic sensor reveals controls on N2-fixing microorganisms in the North Pacific Ocean. *ISME J.*, **8**, 1175–1185.

Sohm, J. A., Webb, E. A. and Capone, D. G. (2011) Emerging patterns of marine nitrogen fixation. *Nat. Rev. Microbiol.*, **9**, 499–508.

Strickland, J. D. H. and Parsons, T. R. (1972) *A Practical Handbook of Seawater Analysis*. Vol., Fisheries Research Board of Canada, Ottawa.

Taylor, C. D. and Doherty, K. W. (1990) Submersible Incubation Device (SID), autonomous instrumentation for the *in situ* measurement of primary production and other microbial rate processes. *Deep Sea Res. Part I*, **37**, 343–358.

Taylor, C. D., Doherty, K. W. and Honjo, S. (2013). *Fixation Filter Assembly.* US Patent #8,426,218.

Taylor, C. D., Edgcomb, V. P., Doherty, K. W., Engstrom, I., Shanahan, T., Pachiadaki, M. G., Molyneaux, S. J. and Honjo, S. (2015) Fixation filter, device for the rapid *in situ* preservation of particulate samples. *Deep Sea Res. Part I*, **96**, 69–79.

Taylor, C. D. and Howes, B. L. (1994) Effect of sampling frequency on measurements of seasonal primary production and oxygen status in near-shore coastal ecosystems. *Mar. Ecol. Prog. Ser.*, **108**, 193–203.

Thompson, A. W., Foster, R. A., Krupke, A., Carter, B. J., Musat, N., Vaulot, D., Kuypers, M. M. M. and Zehr, J. P. (2012) Unicellular cyanobacterium symbiotic with a single-celled eukaryotic alga. *Science*, **337**, 1546–1550.

Villareal, T. A. (1990) Laboratory culture and preliminary characterization of the nitrogen-fixing *Rhizosolenia-Richelia* symbiosis. *Mar. Ecol.*, **11**, 117–132.

Voss, M., Bange, H. W., Dippner, J. W., Middelburg, J. J., Montoya, J. P. and Ward, B. (2013) The marine nitrogen cycle: recent discoveries, uncertainties and the potential relevance of climate change. *Philos. Trans. R. Soc. B*, **368**, 20130121.

Webb, E. A., Ehrenreich, I. M., Brown, S. L., Valois, F. W. and Waterbury, J. B. (2009) Phenotypic and genotypic characterization of multiple strains of the diazotrophic cyanobacterium, *Crocosphaera watsonii*, isolated from the open ocean. *Environ. Microbiol.*, **11**, 338–348.

Wilson, S. T., Böttjer, D., Church, M. J. and Karl, D. M. (2012) Comparative assessment of nitrogen fixation methodologies, conducted in the oligotrophic North Pacific Ocean. *Appl. Environ. Microbiol.*, **78**, 6516–6523.

Zehr, J. P., Waterbury, J. B., Turner, P. J., Montoya, J. P., Omoregie, E., Steward, G. F., Hansen, A. and Karl, D. M. (2001) Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. *Nature*, **412**, 635–638.