Ocean acidification impacts on nitrogen fixation in the coastal western Mediterranean Sea

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Abstract

The effects of ocean acidification on nitrogen (N_2) fixation rates and on the community composition of N_2-fixing microbes (diazotrophs) were examined in coastal waters of the North-Western Mediterranean Sea. Nine experimental mesocosm enclosures of ~50 m^3 each were deployed for 20 days during June-July 2012 in the Bay of Calvi, Corsica, France. Three control mesocosms were maintained under ambient conditions of carbonate chemistry. The remainder were manipulated with CO_2 saturated seawater to attain target amendments of pCO_2 of 550, 650, 750, 850, 1000 and 1250 µatm. Rates of N_2 fixation were elevated up to 10 times relative to control rates (2.00 ± 1.21 nmol $L^{-1}d^{-1}$) when pCO_2 concentrations were >1000 µatm and pH_T (total scale) < 7.74. Diazotrophic phylotypes commonly found in oligotrophic marine waters, including the Mediterranean, were not present at the onset of the experiment and therefore, the diazotroph community composition was characterised by amplifying partial *nifH* genes from the mesocosms. The diazotroph community was comprised primarily of cluster III *nifH* sequences (which include possible anaerobes), and proteobacterial (α and γ) sequences, in addition to small numbers of filamentous (or pseudo-filamentous) cyanobacterial phylotypes. The implication from this study is that there is some potential for elevated N_2 fixation rates in the coastal western Mediterranean before the end of this century as a result of increasing ocean acidification. Observations made of variability in the diazotroph community composition could not be correlated with changes in carbon chemistry, which highlights the complexity of the relationship between ocean acidification and these keystone organisms.

1. Introduction

The impact of 250 years of industrial activity is now being detected throughout our environment at scales that range from cellular to regional and even global scales. The change in atmospheric carbon dioxide (CO_2) from ~280 parts per million (ppm) in pre-industrial times to ~399 ppm in 2014 has impacted the Earth system on several scales, not least of which are the warming of the atmosphere and the oceans as a result of an enhanced greenhouse effect (IPCC, 2013). The oceans and atmosphere are intimately linked so that changes to the partial pressure of atmospheric CO_2 result in proportional changes in dissolved CO_2 in the marine environment. As a result of this, the rise of global temperatures has been buffered by the exchange of approximately ~26% of anthropogenic CO_2 into the oceans (Le Quéré *et al.*, 2014) and it is this condition that has resulted in a profound change to ocean carbonate chemistry and the phenomenon of ocean acidification (OA) (Raven *et al.*, 2005). As a consequence, surface seawater pH is on average ~0.1 units lower than it was prior to the industrial revolution, which equates to an increase in acidity of 26%. Earth system models project a global additional decrease in pH by 2100 ranging from 0.06 to 0.32 units (15 – 110% increase in acidity) depending on our future CO_2 emissions (Ciais *et al.*, 2013). Elevated oceanic partial pressure of CO_2 (pCO_2) and the subsequent decrease in pH will have direct and indirect impacts on microbial nutrient cycling and carbon fixation which may fundamentally alter current biogeochemical cycles (Hutchins *et al.*, 2009)

Nitrogen (N_2) fixation is a critical process in the biogeochemical cycling of elements in sub-tropical and tropical, nutrient poor waters (Carpenter & Capone, 2008) which has an equivocal response to OA. Research efforts into the sensitivity of

diazotrophic activity to OA have largely been focused on *Trichodesmium*. The first reports showed increased rates of N_2 fixation with partial pressures of CO_2 between 750 and 1250 µatm relative to ambient conditions (Levitan *et al.*, 2007, Barcelos E Ramos *et al.*, 2007, Hutchins *et al.*, 2007, Kranz *et al.*, 2009, Kranz *et al.*, 2010). These experimental investigations were performed under laboratory conditions using a cultured organism, and most of these experiments were performed using replete nutrient conditions. Hutchins *et al.* (2007) performed experiments under enriched and limiting conditions of phosphorus (P) to find that N_2 fixation rates were stimulated by higher levels of CO_2 even in cultures experiencing severe P limitation, despite P being one of the two nutrients most likely to limit N_2 fixation. Spungin *et al.* (2014) found that P limitation actually led to an enhancement of the OA stimulation of N_2 fixation by Trichodesmium. It would appear that limiting quantities of P might enhance diazotrophy, Shi *et al.* (2012) found that N_2 fixation rates of *Trichodesmium* were impaired under conditions of iron depletion. Fu *et al.* (2008) performed similar studies on the unicellular cyanobacterium *Crocosphaera watsonii* to find that under iron replete conditions N_2 fixation rates were enhanced at a pCO_2 of 750 µatm, compared to iron deplete conditions where no effect was observed. A negative impact of OA was also recorded, in *Nodularia spumigena*, a heterocystous diazotroph common to the Baltic sea (Czerny *et al.*, 2009), where cell division rates and nitrogen fixation rates were reduced at CO_2 levels up to 731 ppm. Results from this small number of laboratory studies imply that N_2 fixation can be stimulated by OA, but that there may be a relationship with the nutrient regime and particularly the bioavailable iron concentration (Fu *et al.*, 2008, Shi *et al.*, 2012), and that this may vary between different diazotrophic organisms.

The available evidence of OA impacts on natural communities of diazotrophs is even more limited. Evidence presented by Hutchins *et al.* (2009) and Lomas *et al.* (2012) showed that natural populations of *Trichodesmium* in the Atlantic Ocean were stimulated by increases in CO_2 in a similar manner to those in culture. In contrast, Gradoville *et al.* (2014) found no evidence, during 3 cruises and 11 experiments in the North Pacific, of enhanced N_2 fixation by *Trichodesmium* under elevated levels of CO_2 and further, that there was no change from this under altered conditions of phosphorus, iron or light. Similarly, Law *et al.* (2012) and Böttjer *et al.* (2014) recorded no relationship between CO_2 and N_2 fixation for CO_2 amendments up to 750 and 1100 µatm respectively for natural diazotroph communities dominated by unicellular cyanobacteria in the North and South Pacific.

The Mediterranean is a semi-enclosed sea, which is oligotrophic in nature and, due to its short ventilation period and dense urbanisations close to the coastal areas, is susceptible to anthropogenic driven influences (The Mermex Group, 2011). Recent evidence (Touratier & Goyet, 2011) indicates that all water masses in the Mediterranean Sea are already displaying decreases in pH of 0.05 to 0.14 units (compared to the global mean decrease of 0.1), and thus appears to be one of the regions that is most impacted by acidification (The Mermex Group, 2011).

The Mediterranean has proved enigmatic with respect to the characterisation of its diazotrophy and diazotrophic communities and to date there have been only a limited number of studies which have reported on this. Historically, indirect evidence from nutrient budgets (Bethoux & Copinmontegut, 1986) and stable isotope studies (Pantoja *et al.*, 2002) indicate the potential for nitrogen fixation as an active process. Garcia *et al.* (2006) and Rees *et al.* (2006) provided some of the earliest direct measurements of N_2 fixation for the west and east basins respectively. The high rates

reported by Rees *et al*. (2006) have not been repeated and it would seem that the upper limit is of the order of 17 nmol$L^{-1}d^{-1}$ as reported in the annual time-series of measurements made by Garcia *et al.* (2006) at the DYFAMED site in the northwestern basin. Krom *et al.* (2010) has argued that processes peculiar to the eastern basin preclude the budgetary requirement for nitrogen fixation and that P limitation in this region is too severe to allow diazotrophic activity. There is some degree of variability in the rates that have been reported. Low N_2 fixation rates of < 0.15 nmol L^{-1} d^{-1} have been recorded in open waters across both basins (e.g. Ibello *et al.*, 2010; Rahav *et al.*, 2013; Ridame *et al.*, 2011). During the BOUM cruise along a 2000km transect from west to east, the mean rates observed in the western basin were higher than this at 0.63 ± 0.45 nmol L^{-1} d^{-1} (Bonnet *et al.*, 2011), with maximum rates of 1.80 ± 0.19 nmol L^{-1} d^{-1} measured in the vicinity of the plume of the River Rhone. In a further time-series study at DYFAMED, Sandroni *et al.* (2007), recorded rates of between 2 and 7.5 nmol $L^{-1}d^{-1}$, with maximum rates recorded at 10m depth during August. It would seem that the higher rates of N_2 fixation reported (Bonnet *et al.*, 2011, Garcia *et al.*, 2006, Sandroni *et al.*, 2007) are associated with nutrient replete coastal environments. During a Saharan dust addition experiment in coastal waters of Corsica (Ridame *et al.*, 2013) observed increases in rates of N_2 fixation up to ~ 1.3 ± ~1.0 nmol $L^{-1}d^{-1}$ from a background rate of ~0.2 nmol $L^{-1}d^{-1}$ following the addition of Saharan dust to surface waters. During the BOUM cruise the diazotroph community was dominated by picoplanktonic cyanobacteria affiliated to Group A, *Bradyrhizobium* and α proteobacteria (Bonnet et al., 2011). Additionally the filamentous cyanobacterium *Richelia intracellularis* was present at all stations sampled (Bonnet *et al.*, 2011), and also in the coastal eastern basin (Zeev *et al.*, 2008). In other coastal waters the presence of diazotrophs has been related to Archaea,

Proteobacteria and Cyanobacteria (Man-Aharonovich *et al.*, 2007; Le Moal & Biegala, 2009).

The current consensus is that diazotrophy occurs throughout the Mediterranean and similar to other variableswhich include oligotrophy and productivity (The Mermex group, 2011) shows a decreasing trend from west to east. It would appear that coastal regions might support greater rates of N_2 fixation than the open waters of the Mediterranean Sea.

We report here on a mesocosm experiment performed in the Bay of Calvi (BC), Corsica, in the western basin of the Mediterranean during June and July 2012 during which the relationship between OA, N_2 fixation rate and diazotrophic community composition was investigated. This experiment, which is described in detail by Gazeau et al. (sbm, this issue - a), formed a contribution to the European project 'Mediterranean Sea Acidification under changing climate' (MedSeA; http://medsea-project.eu) which was launched in 2011 with the objective to assess uncertainties, risks and thresholds related to Mediterranean acidification at organismal, ecosystem and economical scales.

2. Methods

The Bay of Calvi is situated in the Ligurian Sea, on the northwest coast of Corsica in the Mediterranean Sea (Fig. 1). The bay is subject to little human disturbance and has been described as pristine (Richir & Gobert, 2014) with low river and sewage discharges supplying limited nutrients (Lepoint *et al.*, 2004). The open sea provides the main external source of new nutrients, albeit seasonal, providing deep, nutrient rich waters during the N-NE winds which occur during winter and early

spring and nutrient-poor surface waters during the more common SW winds (Skliris *et al.*, 2001). Chlorophyll levels are typically low (<1µg Chl a L^{-1}) except during the bloom period (February to April). It is these low nutrient, low chlorophyll (LNLC) conditions which account for the oligotrophic description of the waters. The water column is generally well mixed for the majority of the year, with sea surface warming resulting in stratification from May to October (Gazeau, et al. sbm, this issue - a).

Nine mesocosms of 12m depth, ~50 m^3volume were deployed in a water column depth of 25m for a period of 30 days. Six of them (P1 to P6) were subjected to different target levels of pCO_2 (550, 650, 750, 850, 1000 and 1250 µatm respectively) covering the range of atmospheric pCO_2 anticipated for the end of this century and beyond (Bopp *et al.*, 2013, Ciais *et al.*, 2013). The remaining three mesocosms (C1 – C3) were unaltered with a pCO_2 of ~ 450 µatm corresponding to the pCO_2 of surface waters in June and July at BC. An experiment of this scale does not logistically allow the replication of treatments to the extent that might be achieved under laboratory conditions. The replication of controls was considered of paramount importance, particularly due to the grouping of mesocosms in clusters of three, with one control per cluster (Gazeau et al, this issue - a). The creation of a gradient of CO_2 conditions rather than a low number of replicates has several advantages which include providing a more powerful statistical test than equivalent ANOVA-based designs with a small number of replicates (Havenhand *et al.*, 2010).

pCO_2 levels were achieved by additions of CO_2 saturated seawater. Saturated seawater was prepared by bubbling 100% CO_2 directly into 25L carboys containing 5 mm filtered (in order to remove fish and jellyfish, whilst leaving the mesozooplankton community intact) seawater, which was collected from close to the mesocosm anchorage at the near surface (1 – 2 m). Between 50 and 500L of CO_2 saturated

seawater was added using a diffusing system to individual mesocosms in order to achieve a homogenous distribution of target levels of pH/pCO_2. Additions were performed over a four day period in order to minimise stress to the biological community. Conditions of pH/pCO_2 were not modified further once target levels were reached to minimise disturbance to the mesocosms and to allow the system to modify its environment. Due to the relatively low proportional addition of saturated seawater to mesocosms (0.1 to 1% of volume), impacts of this addition were considered to be insignificant and were not monitored. For comparison, selected variables are presented from within and outside of mesocosms on Day 0 and Day 20 in Table 1.

A limited number of samples (for diazotroph analysis) were collected outside of the mesocosms prior to the experiment on Day-3. The experimental sampling began on the 24th June (Day 0) as targeted levels of OA were reached. Sampling of individual mesocosms was achieved using an integrating water sampler (Hydrobios) which collected a 5 litre sample over the full depth of the mesocosm. CTD profiles were performed within and outside of mesocosms on a daily basis using a Seabird 19plusV2 with sensors for dissolved oxygen, salinity, temperature, fluorescence, pH and light (PAR) irradiance.

2.1. Carbonate Chemistry and chlorophyll *a*

Dissolved Inorganic Carbon (DIC, C_T) was determined daily using an automated infra-red inorganic carbon analyser (AIRICA). C_T measurements were performed, at 25 °C, on 1200 µL samples directly poisoned after sampling with a saturated solution of mercuric chloride ($HgCl_2$). The system was calibrated ($r^2 \geq$ 0.999) using 1100, 1200 and 1300 µL samples of a certified reference material (A.

Dickson, Batch 117, which had values of: salinity = 33.503, C_T = 2009.99 µmol kg^{-1}, total alkalinity (A_T) = 2239.18 µmol kg^{-1}). Precision of all measurements performed in triplicate (n = 240) was better than ± 3.5 µmol kg^{-1}. Accuracy and stability tests of the system over the experimental period (n = 38) found a mean offset from the certified reference of -0.56 µmol kg^{-1}, which is well within the excepted limit of ± 2 s.d.

A_T was determined using a Metrohm Titrando titrator following the procedure described in Dickson et al. (2007; SOP 3b). This parameter was measured daily from June, 24 (day 0) to June, 27 (day 3) and every second day from June, 27 to July, 16 (day 20) because of its low variability. Measurements were performed on triplicate 50 mL samples at 25 °C, which had been filtered through GF/F filters and poisoned with $HgCl_2$. In coastal waters samples are filtered in order to remove any inorganic debris or calcified organisms which might interfere with A_T analysis. The electrode was calibrated every second day on the total scale using TRIS buffer solutions with a salinity of 35.0. Precision (± 1 s.d.) was better than 5.5 µmol kg^{-1} (n = 170). Accuracy and stability tests of the system over the experimental period (n = 41) found a mean offset from the certified reference of -1.61 µmol kg^{-1}, which is well within the excepted limit of ± 2 s.d. A_T values on non-sampling days were estimated as the mean value (± 1 s.d.) of the previous and subsequent day.

The carbonate chemistry was calculated with the R package seacarb (Gattuso *et al.* 2015), using in situ values of temperature, salinity, C_T and A_T. The standard deviation of integrated parameters were accounted for through the application of a Monte-Carlo procedure. For each determination, one thousand values were randomly chosen between the mean ± 1 s.d. for each measured parameter (C_T and A_T). Mean values ± 1.s.d. of seawater pH_T (total scale) and pCO_2 were calculated for each of these 1000 iterations.

Samples for pigments analyses (including total Chl a shown here) were taken every day. Two litres of sampled seawater were filtered onto GF/F. Filters were directly frozen with liquid nitrogen and stored at -80 °C pending analysis at the Laboratoire d'Océanographie de Villefranche (France). Filters were extracted at -20 °C in 3 mL methanol (100%), disrupted by sonication and clarified one hour later by vacuum filtration through GF/F filters. The extracts were rapidly analysed (within 24 h) by high performance liquid chromatography (HPLC) with a complete Agilent Technologies system. The pigments were separated and quantified as described in Ras *et al.* (2008).

2.2. Nitrogen Fixation

Seawater samples were collected every second day from each mesocosm before sunrise into a10 L dark carboy and returned to the laboratory within one hour of collection. Samples were re-distributed into a single 2.4 L polycarbonate bottle per mesocosm. Bottles were filled and sealed excluding all air bubbles with a drilled cap fitted with a Teflon backed septa. 2.4 mL of ^{15}N-N_2 (98 atom%, Sigma-Aldrich; Lot #SZ1423V) were added to each bottle, which was incubated in-situ for 24 h at 6 m depth close to the mesocosms. Incubations were terminated by gentle filtration onto pre-combusted (12 hours at 450°C) 25 mm GF/F filters (Whatman) which were then dried at 50°C for 24 h and stored on silica gel until return to Plymouth Marine Laboratory (PML) where particulate nitrogen and ^{15}N atom% were measured using continuous-flow stable isotope mass-spectrometry (PDZ-Europa 20-20 and GSL; (Owens & Rees, 1989)), with rates determined according to Montoya *et al.* (1996). Instrument precision was better than 0.23% CV based on urea standards ((Iso-

Analytical Ltd) in the range 0.25 – 2.0 µmol N, which were analysed during three sample runs of the mass spectrometer (mean ± 1 s.d. = 0.3659 atom% ± 0.0008, n=27). The mean particulate N content of samples was 0.84 µmol. The detection limit for N_2 fixation rate was calculated from the determined ^{15}N significant enrichment level and the lowest observed particulate nitrogen concentration (Ridame *et al.*, 2013) and was estimated at 0.042 nmol $L^{-1}d^{-1}$.

It has been recognised that rates of N_2 fixation determined in this manner may prove to be somewhat of an underestimate due to an unequal dissolution with incubation time of the ^{15}N-N_2 bubble (Mohr *et al.*, 2010). Absolute rates may remain the same (Rees, unpublished) or may alter by between 1.4 (Mulholland *et al.*, 2012) and up to 6 times (Groszkopf *et al.*, 2012, Wilson, 2012) with modified methodology. At the time that this experiment was performed we decided on an approach similar to that taken by others (Langlois *et al.*, 2012; Law *et al.*, 2012; Ridame *et al.*, 2013) and considered that rates determined using a bubble addition could be considered conservative, but that this would not impact on the relative changes between OA treatments investigated. In Groszkopf *et al.* (2012), the differences noted between these two methodological approaches are considered to be due to regional variability which is likely a function of the diazotrophic community. Whilst the data we present here indicates that a diverse community of diazotrophs was present, it was quite different from the Atlantic community and none of the specific reasons noted by Grosskopf (e.g. *Trichodesmium* buoyancy) would indicate that a varied response should be expected in this study.

A further complication to the determination of N_2 fixation rates was recently introduced by Dabundo *et al.* (2014). Here it was indicated that there were instances where commercially supplied cylinders of ^{15}N-N_2 were contaminated with ^{15}N

labelled nitrate and ammonium with obvious potential for overestimation of N_2 fixation rates. The ^{15}N-N_2 used during this experiment (Sigma-Aldrich; Lot #SZ1423V) was not investigated in the Dabundo paper and was not tested for contaminants. However, we are confident that contamination by ^{15}N nitrate and ammonium was either extremely low or entirely absent. The same cylinder was used during a second mesocosm experiment performed in the Bay of Villefranche (BV), France during February and March 2013 (Gazeau et al, subm this issue - a). During the BV experiment mean nitrate uptake rates in control mesocosms were determined at ~30 $nmolL^{-1}h^{-1}$, which would suggest comparable rates of ammonium uptake by the occupying microbial community for this time of year. N_2 fixation rate determinations at BV performed in an identical manner to this investigation (BC) returned mean rates of 0.1 $nmolL^{-1}d^{-1}$, which equate to ~0.03% of nitrate uptake rates (assuming 12 hour day length).

2.3. DNA extraction, quantitative PCR (qPCR) and PCR amplification of nitrogenase genes (*nifH*)

To characterise the diazotrophic community composition throughout the mesocosm experiments, 10 L samples were collected from each mesocosm every 4 d, and filtered onto Sterivex^{TM} filters (Millipore, Billarica, MA, USA) using gentle peristaltic pumping. Samples were preserved by sealing the Sterivex^{TM} after introducing 1.4 mL RNA later (Qiagen, Valencia, CA, USA). Samples were frozen at -80°C before being transported on dry ice to the UK and then shipped to the University of California, Santa Cruz, USA on dry ice.

Samples from mesocosms C1, P3, P5, and P6 on days 1, 5, 9, 13, 17, and 20, as well as samples from outside the mesocosms at the initiation of the experiment (day -3), were chosen for molecular analyses. Nucleic acids were extracted using the Qiagen All Prep kit with several modifications. After removal of RNAlater, filters were transferred from the Sterivex^{TM} cartridge into sterile tubes with a 1:1 mix of 0.1:0.5 mm glass beads (BioSpec Products, Bartlesville, OK, USA) and 600 μL of RLT Plus buffer with β-mercaptoethanol. Cells were lysed using three freeze-thaw cycles and four minutes of agitation using a mini-beadbeater-96 (BioSpec Products).

Manufacturer's guidelines were followed after lysis for both DNA and RNA extraction. Extracted RNA was archived at -80°C, and DNA was stored at -20°C until analysis. DNA extracts were quantified using the Quant-it^{TM}PicoGreen® DNA assay kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's guidelines.

DNA extracts were analysed for the presence of diazotrophic phylotypes previously characterized in oligotrophic environments, including the Mediterranean, using quantitative PCR targeting the *nifH* gene. All samples were screened for the presence of the unicellular cyanobacterial (UCYN) groups A1 (Church *et al.*, 2005), A2 (Thompson *et al.*, 2014), and B (Moisander *et al.*, 2010), the *Rhizosolenia*-associated heterocyst-forming cyanobacteria, *Richelia* (Het-2; (Foster & Zehr, 2006) and two proteobacterial phylotypes γETSP1 and γETSP3 (Turk-Kubo *et al.*, 2014). In addition, the samples taken from outside the mesocom at the initiation of the experiment were screened for *Trichodesmium* sp. (Church *et al.*, 2005), the *Hemiaulus*-associated heterosyst-forming cyanobacteria, *Richelia* (Het-1; (Church *et al.*, 2005), and proteobacterial phylotypes γ-24774A11 ((Moisander *et al.*, 2008) and γ ETSP2 (Turk-Kubo *et al.*, 2014). All aspects of these qPCR assays, including reaction set-up, thermocycle parameters, and calculation of *nifH* gene copies from

standard curves are detailed in (Goebel *et al.*, 2010). Due to the large volumes of water filtered, the limit of detection (LOD) and limit of quantitation (LOQ) for these qPCR reactions were 2 and 10 *nifH* copies L^{-1}, respectively. Samples with *nifH* copies that fell between the LOD and LOQ are designated as 'detected not quantified' (DNQ).

Partial *nifH* gene fragments were PCR amplified from C1, P3, P5, and P6 mesocosms on days 1 and 5, as well as the day -3 samples, as described in Turk-Kubo *et al.* (2014). Briefly, degenerate nested PCRs were carried out on DNA extracts in replicate using well-established primers described in Zehr & McReynolds (1989) and Zani *et al.* (2000). Reagent blanks were also amplified to screen for contamination. Amplicons were pooled and gel purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), cloned using an Invitrogen TOPO TA kit for sequencing (Carlsbad, CA, USA) according to the manufacturer's guidelines, and plasmids were isolated and purified from the resulting clone libraries using a Montage Plasmid $Miniprep_{96}$ Kit (Millipore, Billarica, MA, USA). Recombinant plasmids were sequenced using Sanger technology at the University of California Berkeley DNA Sequencing Center.

Raw sequences were trimmed of vector contamination and low quality reads using Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI, USA). The partial *nifH* fragments remaining were imported into a *nifH* database maintained and curated at UCSC (Heller *et al.*, 2014), translated into amino acid sequences, and aligned to the existing hidden markov model-aligned sequences. In order to evaluate whether sequences closely related to PCR contaminants were recovered, amino acid sequences were clustered at 92% identity (a conservative threshold used in other studies such as Farnelid *et al.* (2010) using CD-HIT (Huang *et al.*, 2010), and

neighbor joining trees were built using both amino acids and nucleotides in the ARB software environment (Ludwig *et al.*, 2004). In order to determine representative sequences, amino acid sequences were clustered at 97% identity using the CD-HIT suite (Huang *et al.*, 2010). Maximum likelihood trees of partial *nifH* amino acid sequences were built in MEGA 5.2 using the JTT matrix based model to calculate branch lengths and a bootstrap test with 500 replicates. *nifH* cluster designations for each phylotype follow the convention of Zehr *et al*. (2003a).

2.4. Nutrient Analysis

Methods for the determination of NO_3^- and PO_4^{3-} are provided in full by Louis et al., (subm. This issue). Briefly, seawater samples were filtered at 0.2 µm (Nuclepore, Whatman) into clean polethylene bottles in a laminar flow cabinet and acidified for storage to pH < 2 using HCl suprapur. Samples were stored and returned to the LOV laboratory for analysis using a liquid waveguide capillary cell (LWCC) coupled to a spectrophotometer for absorbance analysis at 710nm for PO_4^{3-} and 540nm for NO_3^-. PO_4^{3-} and NO_3^- were determined colorimetrically according to Chen *et al.* (2008) and Murphy & Riley (1962) respectively. Detection limits were determined as 3 nmol L^{-1} for PO_4^{3-} and 10 nmol L^{-1} for NO_3^-.

2. 5. Data analysis and statistics

All data collected during this experiment are freely available on Pangaea: http://doi.pangaea.de/10.1594/PANGAEA.810331.

Principal component analyses (PCA) were conducted using Primer v6 (Clarke & Gorley, 2006). Variables, which included representative diazotroph phylotypes, N_2

fixation rate, pCO_2 and pH_T, were normalised by subtracting the mean value across all samples and dividing by the standard deviation prior to analysis.

3. Results

3.1. Environmental conditions

The temperature in each of the individual mesocosms followed very closely the conditions experienced in surrounding waters (Fig. 2a). Mean water column temperature on day 0 was 22.1°C, this increased steadily to a maximum of 24.7°C on day 18 and then decreased to 24.3°C on day 20. There was a transient period of thermal stratification in the near surface (<5m) within the mesocosms between days 5 and 8, which was thoroughly disrupted on Day 9 (Gazeau et al, this issue a). Surface irradiance was relatively constant during the entire experiment with minimal and maximal daily (sunrise to sunset) average values of 531and 735 μmol photons m-2 s-1. Maximum irradiance levels (~1300-1400 μmol photons m-2 s-1 were reached at around 12:00 pm and the Light:Darkness (L:D) cycle was 16.5:7.5 and 16:8, respectively at the start and at the end of the experiment. Chl *a* concentrations were similar between all mesocosms (Fig. 2b) and remained low throughout the experiment and varied between 0.05 and 0.09 μgL^{-1} (mean 0.07 ± 0.01 μgL^{-1}, n=161). There was some shading caused by the mesocosm walls which reduced values of PAR inside the enclosures relative to outside. This might have been responsible for these values being approximately half the concentration measuredoutside of the mesocosms which ranged between 0.10 and 0.19 μgL^{-1}(n=19). Phytoplankton biomass decreased during the acidification phase in all mesocosms, independently of pCO_2 conditions, as shown by fluorometric data acquired using daily CTD profiles (data not shown; see Gazeau et al., sbm, this issue). This corresponded to organic matter sedimentation at the start

of the experiment (first few days) that further stabilised at low rates until the end of the experiment (Gazeau et al., in prep, this issue).

Nutrient concentrations are described in full by Louis et al. (subm this issue) and summarised for day 0 and day 20 in Table 1. The initial conditions reflected the oligotrophic nature of the summertime Mediterranean with mean observed concentrations for all mesocosms on day 0 of 47 ± 14 $nmolL^{-1}$ NO_3^- and 23 ± 4 $nmolL^{-1}$ PO_4^{3-}. There was a rapid change in nutrient conditions both within and outside of mesocosms (Louis et al., this issue) so that by Day 1, the mean concentration for all mesocosms was 97.5 ± 21.9 $nmolL^{-1}$ and 9.5 ± 0.8 $nmolL^{-1}$ for NO_3^- and PO_4^{3-} respectively. Whilst PO_4^{3-} then remained stable for the duration of the experiment (8.5 ± 1.8 nmol L-1), NO_3^- decreased with time to reach a minimum at Day 11 (9.9 nmol L-1). This saw a progressive change in NO_3^- : PO_4^{3-} between Day 1 to Day 11 of 10.4 to 1.4, with a minimum on Day 8 of 1.3. Whilst there was some variability between the individual nutrients which led this heterogeneity in the nutrient stoichiometry, there was no evidence of any sensitivity in nutrient dynamics relative to increases in seawater pCO_2.

3.2. Carbonate Chemistry

Measured A_T and C_T, were determined and used to compute daily values of pCO_2 and pH_T (Fig. 3a and b). Starting conditions of pCO_2 on day 0 were very close to the targeted levels and achieved levels of 609, 731, 790, 920, 1198 and, 1353 µatm (for P1, P2, P3, P4, P5 and P6 respectively) relative to controls of 474, 465 and 456 µatm for C1, C2 and C3 respectively (Table 1). These produced a range of pH_T values of between 8.02 to 7.61 for controls and P6 respectively. A_T values gradually increased during the experiment as a consequence of evaporation and followed the

variability in salinity (Gazeau et al., subm this issue - a). pCO_2 values remained more or less constant throughout the experiment for control and perturbed mesocosms P1 to P4. P5 and P6 showed a decrease of pCO_2 with time as a response mostly to exchange with the atmosphere. pH profiles acquired using the CTD and transformed to the total scale using integrated samples of A_T and C_T reflected homogeneous distribution throughout the contained water columns for the duration of the experiment (Gazeau et al, subm this issue - a).

3.3. Nitrogen Fixation

Over the whole period of the experiment N_2 fixation rates in amended mesocosms P1 to P4 (mean ± 1 s.d.) of 2.05 ± 1.67 nmol $L^{-1}d^{-1}$ were comparable to those determined in control mesocosms of 2.00 ± 1.21 nmol $L^{-1}d^{-1}$ (Fig. 4). There was a general decrease in rates over the 20 day period from a mean value for the controls on days 0 and 2 of 3.47 ± 1.64 nmol $L^{-1}d^{-1}$ to 1.28 ± 1.06 nmol $L^{-1}d^{-1}$ on days 16 and 20. Variability in rates was largely associated with the heterogeneous distribution of suspended material throughout the mesocosms, which over days 0 and 2 was reflected in particulate nitrogen concentrations of 480.7 ± 123.7 nmol L^{-1}. In contrast to the observations of control and P1 to P4 treatments, a large increase in N_2 fixation rate was observed during the first 8 days in mesocosms P5 (day 2) and P6 (days 0, 4, 6, 8), which were originally amended to 1198 and 1353 μatm CO_2, respectively (Fig. 4). The N_2 fixation rate reached a maximum of 23.3 nmol $L^{-1}d^{-1}$ in P6, 6 days after the start of the experiment, which was approximately 10 times the background rate determined in control mesocosms. The daily change in carbonate chemistry observed in Fig. 3, as CO_2 in the mesocosms equilibrates with the atmosphere allows an interrogation of daily N_2 fixation rate against a high resolution of CO_2 conditions (Fig.

5). At pCO_2 values > ~1000µatm N_2 fixation rates were generally enhanced. The maximum recorded rate was associated with 1121 µatm CO_2 (pH_T = 7.69) and seven out of nine measured rates were equal to or greater than three standard deviations of the mean control value (Fig. 5).

3.4. Diazotroph community shifts

Samples from mesocosms C1, P3, P5, and P6 on days 1, 5, 9, 13, 17, and 20, as well as samples from outside the mesocosms at the initiation of the experiment (day -3) were screened using a suite of qPCR assays targeting cyanobacterial and proteobacterial diazotrophs that have been described in oligotrophic oceans, including the Mediterranean Sea. In samples from outside the mesocosms (day -3), the only phyloytpes detected were γ-ETSP1 and γ-ETSP3, and abundances ranged from DNQ to $2x10^2$ *nifH* copies L^{-1}. Although described in previous studies in the Mediterranean (Le Moal & Biegala, 2009, Le Moal *et al.*, 2011, Man-Aharonovich *et al.*, 2007) there was no detection of the unicellular cyanobacterial symbionts of *Braarudosphaera bigelowii*, UCYN-A1 or UCYN-A2, throughout this study. *Crocosphaerea* sp. (UCYN-B) was detected, but at abundances too low to quantify (DNQ; < 10 *nifH* copies L^{-1}) in a majority of the samples screened. Het-2 and γ-ETSP1 were also detected at low abundances (DNQ) starting on day 13 and day 17, respectively. There was no detection of *Trichodesmium*, Het-1, γ-24774A11, or γ-ETSP2 outside the mesocosms at day -3, thus no further samples were screened for these phylotypes (qPCR results detailed in Supplemental Table 1). From these analyses, we concluded that none of the diazotrophs targeted with the selected qPCR assays had any measureable response to the experimental manipulations (if present at all), nor were

they plausible candidates for the diazotrophs responsible for peaks in N_2 fixation rates observed in P5 and P6.

Therefore, in order to characterise the diazotrophs present in P5 and P6 during the period where N_2 fixation rates were stimulated as a result of pCO_2 and pH changes, we amplified a partial fragment of the *nifH* gene using well-established universal PCR primers. Sequences were also recovered from the Bay of Calvi (day -3), C1 and P3 mesocosms for comparison. Characterization of community composition based on clone libraries are qualitative in nature, and due to biases inherent in this approach, including the preferential amplification of proteobacterial diazotrophs with this assay (Turk et al., 2011), the number of times each sequence type is recovered is not necessarily indicative of starting abundances and must be interpreted with caution. The diazotrophic community present in the water column prior to the onset of the experiment (day -3) was comprised mainly of three cyanobacterial phylotypes (cluster 1B), several putative γ-proteobacterial (cluster 1G) phylotypes, as well as multiple cluster III phylotypes, which are likely to be anaerobic diazotrophs (Figure 6b,c). Among the cyanobacterial phylotypes, two appear to cluster with the *Chroococcales*: 59013A11 and 59013A17 are 99% and 98% similar to the endolithic cyanobacteria *Hyella* sp. LEGE 07179 (AGG68340.1), respectively. The third, 59013A30 has 100% amino acid similarity in the amplified region to *Leptolyngbya saxicola* LEGE 0713 (AGG68333.1). Cluster 1G and III phylotypes recovered from day -3 samples were not closely related to cultivated organisms. Of the 20 different phylotypes represented at the onset of the experiment, a single cluster III phylotype (59030A23) was the only one recovered at any other time point (P5, day 1).

By day 1, the diazotrophic community present in the control mesocosm appeared to shift to several cluster 1G phylotypes (dominated by 59031A5, 59024A32, 59024A21), a single phylotype (59036A19) closely related to *Burkholderia* spp. that clusters with other 1J/1K (represented by α-proteobacterial genera including *Rhizobium*, *Azospirillum*, *Rhodobacter*, etc.), and a single cluster III phylotype (59024A3). After 5 days of incubation, entirely different cluster 1G phylotypes were present in the control mesocosms as well as two *Burkholderia*-like cluster 1K phylotypes. The 1K phylotype, 59036A19, was the most abundant sequence type recovered, and was found in a majority of the samples analyzed. Although *Burkholderia*-like *nifH* sequences have been described as contaminants of PCR reagents in several studies (Farnelid *et al.*, 2009, Moisander *et al.*, 2014, Zehr *et al.*, 2003)), a majority of the phylotypes recovered from this study shared <92% nucleotide identity to described contaminants. There were 5 sequences that clustered with a previously reported contaminant and were removed from our analysis (See Supplemental Figure 1). It is nearly impossible to rule out any uncultivated proteobacterial *nifH* sequences as a potential contaminant; however, given the lack of similarity to known PCR contaminants and previous reports of *Burkholderia*-like organisms in the Mediterranean Sea (Man-Aharonovich *et al.*, 2007 and Le Moal *et al.*, 2011), it is reasonable to assume the *Burkholderia*-like phylotypes recovered in this study were from an organism in the environment.

In the P5 and P6 mesocosms, the general diazotrophic community succession is characterised by a reduction in the number of phylotypes recovered, with respect to both the day -3 sample and the C1 control mesocosm. In P5, where elevated N_2 fixation rates peaked early (day 2) during the experiment, a cluster III phylotype (59030A23) was the most abundant sequence recovered. By day 5, the cyanobacterial

phylotype 59030A23 which was 98% similar (amino acid) to *Leptolyngbya saxicola* LEGE 0713 (AGG68333.1) was the most abundant sequence type recovered. In contrast, in the P6 mesocosm, where elevated N_2 fixation rates peaked by day 6, the diazotrophic community shifted from being primarily one cluster III phylotype (59031A7; day 1) to being primarily a cluster 1K phylotype (59039A16; day 5).

With the exception of the cluster III phylotype 59030A23, which was present in the day -3 sample, but no control mesocosms, none of the phylotypes that were the dominant sequence types recovered in P5 and P6 mesocosms at days 1 and 5 were found in any other treatment or the controls. In contrast, in mesocosm P3, where pCO_2 did not stimulate peaks in N_2 fixation rates, the cluster 1K phylotype 59036A19, which is also present in the control, was the most abundant sequence recovered from day 1 and day 5 samples. Thus diazotrophic community succession was not as evident in this treatment.

4. Discussion

Although there a relatively few studies on the spatial and temporal distribution of N_2 fixation in the Mediterranean Sea, and there is a very limited amount of information about its diazotrophic community composition, understanding how this process may be impacted by projected changes in OA over the next century is a critical undertaking. Together, the susceptibility of the Mediterranean to anthropogenic influences (The MerMex Group, 2011) and the indications that decreases in pH of 0.05 to 0.14 units are already evident (Touratier & Goyet, 2011) infer that microbial communities and biogeochemical cycles will become increasingly pressured on a decadal time scale.

The current study provides evidence of an increase in N_2 fixation rates for these waters when *p*CO2 was elevated above ~1000 µatm and pH_T decreased below 7.74 and according to Fig. 5 a maximum rate is reached at conditions in the order of 1134 µatm *p*CO2 (pH ~ 7.69). There is though some complexity to this situation as the elevation of rates was not universally observed. N_2 fixation rates determined at pCO_2 levels of 1064 and 1082 µatm on days 10 and 16 were lower than mean control rates. Whilst there is some variance in the impact of OA on N_2 fixation the sensitivity of this relationship at pCO_2 >1000 µatm is supported by a comparison of F test results. The similarity in variance between P3 versus controls ($F = 0.903$) contrasts hugely with those between P5 and P6 with controls, of $F = 6.7e^{-05}$ and $8.8e^{-07}$ respectively. Projections afforded by the current generation (CMIP5) models (Moss *et al.*, 2010) indicate decreasing ocean pH of between 0.07 and 0.33 units for the 2090s relative to the 1990s for "high mitigation" RCP2.6 and the "business as usual" RCP8.5 scenarios respectively (Bopp *et al.*, 2013). The change indicated by RCP8.5 would see pH values of Mediterranean waters in the late 21st century at ~7.70, well within the affected range identified by this experiment. Our findings suggest that no change in N_2 fixation rates are likely to be seen as a result of increasing acidification for several decades. However, based on the "business as usual" scenario and indications of the sensitivity of the Mediterranean to OA (The MerMex Group, 2011; Touratier & Goyet, 2011), there is the potential for a 10 fold change in activity by the end of the 21st century.

The mean rates in control and amended (P1, P2, P3, P4) mesocosms of 2.00 ± 1.21 nmol $L^{-1}d^{-1}$ and 2.05 ± 1.67 nmol $L^{-1}d^{-1}$ respectively are comparable to those published elsewhere for coastal waters of the western Mediterranean: 4 – 8 nmol $L^{-1}d^{-}$

[1] (Garcia *et al.*, 2006, during summer at DYFAMED); between 2 and 7.5 nmol $L^{-1}d^{-1}$ (Sandroni *et al.*, 2007 at DYFAMED); 1.8 ± 0.19 nmol $L^{-1}d^{-1}$ (Bonnet *et al.*, 2011, in the Rhone plume); Maximum of 1.3 ± 1.0 nmol $L^{-1}d^{-1}$ (Ridame *et al.*, 2013, DUNE experiment, Corsica). The rates of Ridame *et al.* (2013) were geographically closer and might be expected to be similar in magnitude. Control rates reported during that study were though of the order of 10 times smaller, at approximately 0.2 nmol $L^{-1}d^{-1}$. That said, initial nutrient (N and P) conditions between this study and those described during DUNE were quite different (Ridame *et al.*, 2014; Luis et al, this issue). Ridame described N_2 fixation limited by phosphate concentrations of 2 – 5 nmol L^{-1}. During the current study DIP on Day 0 was in the order of 20 to 30 nmol L^{-1} 4 to 15 times higher and considered unlikely to be limiting diazotroph activity. DIP decreased rapidly after Day 0 to an average of 8.5 ± 0.5 nmol L^{-1}, though the total P availability was likely supported by mean dissolved organic phosphorus (DOP) concentrations of 11 ± 2 nmol L^{-1}.

Ridame *et al.* (2013) indicate that the activity of the diazotroph community in the Mediterranean Sea is affected by resource availability. During mesocosm experiments, they (Ridame *et al.*, 2013) observed increases of up to 5.3 fold in N_2 fixation rates following the addition of dust to seawater in three separate mesocosm experiments performed off the coast of Corsica. During those studies, initial DFe concentrations of 2.3 to 3.3 nmolL^{-1}, were not thought to be limiting N_2 fixation and that DFe concentrations of the order of 1.5 nM and higher did not limit N_2 fixation in the western Mediterranean Sea. During the current study DFe was not determined, though previous measurements of DFe in this region indicate fairly stable concentrations in the order of 2.5 nmol L^{-1} (Ridame *et al.*, 2013; Wagener *et al.*, 2010; Louis et al., this issue). The iron demand from the diazotrophic community was

estimated by converting N-fixation rates to a carbon equivalent assuming Redfield stoichiometry (Redfield, 1934) and an assumed cellular Fe:C ratio for cyanobacterial diazotrophs of between50 µmol:mol (Laroche & Breitbarth, 2005) and 16µmol:mol (Tuit *et al.*, 2004). The Redfield C:N value of 6.6 might be considered to be towards the upper extreme for cyanobacterial diazotrophs (Knapp et al, 2012), though is in the mid-range for marine bacterioplankton (Vrede et al., 2002). The iron requirements for N_2 fixation increased from between 0.2 and 0.7 pmol $L^{-1}d^{-1}$ in control mesocosms to a maximum of 2.5 to 7.7 pmol $L^{-1}d^{-1}$ in P6 on day 8. Even if maintained for the duration of the experiment, this would not reduce DFe to limiting concentrations identified by Ridame *et al.*, (2013), thus it is likely that the diazotrophic community was not Fe limited at the time of this experiment.

During both a west to east transect of the Mediterranean and a dust addition experiment in Corsica, Ridame *et al.* (2011) and Ridame *et al.* (2013) found clear evidence that N_2 fixation was limited, or co-limited by the availability of P. During the current study, starting concentrations of 23 ± 4 nmolL^{-1} PO_4^{3-} in all mesocosms were within the range of concentrations reported for the wider western Mediterranean (e.g. (Pujo-Pay *et al.*, 2011) and Table 3 of Louis et al. (subm this issue)), although they were between ~ 4 and ~15 times greater than those reported by Ridame *et al.* (2013). According to Redfield stoichiometry, the starting ratio of NO_3^- : PO_4^{3-} (hereafter N:P) of 1.9 (Table 1) might indicate N rather than P limitation, a condition recognised to favour N_2 fixation (e.g. Bonnet *et al.*, 2011). Following rapid decreases in DIP between Day 0 and Day 1, mean N:P ratios of 10.4 ± 3.3 still indicate N rather than P to be limiting. Taking the same approach as was used for Fe above, the P demand required to support observed rates of N_2 fixation during this experiment was estimated (from Redfield stoichiometry) to range from 0.14 nmol $L^{-1}d^{-1}$ for controls to

a maximum of 1.45 nmol $L^{-1}d^{-1}$ where peak N_2 fixation rates were measured. Whilst concentrations of PO_4^{3-} remained low throughout the experiment, alkaline phosphatase activity (APA) was found to be positively correlated to pCO_2 and remineralised PO_4^{3-} from the dissolved organic pool at rates of ~ 180 nmol $L^{-1}h^{-1}$ for the first 12 days and at ~60 nmol $L^{-1}h^{-1}$ for the remaining 8 days (Celussi et al, in press, this issue), thus it is considered unlikely that the diazotrophic community was experiencing either Fe or P limitation. Environmental variables such as seawater temperature, stratification and incident irradiation may all play a part in controlling diazotrophic activity but are considered not important to this discussion as they were comparable throughout treatments and variability was equal between mesocosms (Fig 2 and Gazeau et al. subm this issue - a).

None of the diazotrophs typically recognized as important N_2-fixers in the ocean (e.g. *Trichodesmium*, UCYN-A, diatom-associated diazotrophs, etc.) were found in the Bay of Calvi, or in the experimental treatments, instead, a diverse diazotrophic community comprised of possible anaerobes (cluster III), α (1J/1K) and γ (1G) proteobacteria and filamentous (or pseudo-filamentous) cyanobacteria (1B; Figure 6a,b) was recovered. Cluster III phylotypes were the most abundant sequence type recovered at the beginning of the experiment (Figure 6c), representing ~58 % of the recovered sequences in day -3 samples, and 60% and 63% in P5 and P6, respectively, at day 1. Although cluster III sequences have been characterized from oligotrophic marine waters (Church *et al.*, 2008, Farnelid *et al.*, 2009, Langlois *et al.*, 2008, Turk-Kubo *et al.*, 2014), the occurrence of these possibly anaerobic phylotypes was unexpected. This was a shallow (~25 m) though well oxygenated environment, mean O_2 concentrations were 226 ± 1 µmolL^{-1} (103% saturation). The mesocosm

bags were 12 m deep and were deployed in a manner not thought to disturb benthic sediments. They were also allowed to flush with seawater for 2 days before the enclosures were sealed (see Gazeau et al. sbm, this issue, for more details). Despite calm conditions prior to the experiment and the low tidal conditions experienced in the Mediterranean, coastal processes must be sufficient to mobilise and maintain these bacteria within the pelagic environment. It is possible that they were associated with suspended particulate material, as observed for other diazotrophs by Le Moal *et al.* (2011) and Benavides *et al.* (2013), which settled fairly quickly following containment of the mesocosm enclosures. Daily export fluxes determined from sediment traps at the base of each mesocosm decreased from a mean rate of 7.8 mg C $m^{-2}d^{-1}$ for days -1 to +1 to 3.0 mg C $m^{-2}d^{-1}$ for days 4 to 6 (Gazeau et al., in preparation, this issue - b). These decreased further to 2.1 mg C m^{-2} d^{-1} by the end of the experiment. As there was no evidence of increased primary production or phytoplankton biomass throughout this period (Maugendre et al., in press; Gazeau et al., in preparation, this issue - c), it would appear that this settling fraction was detrital material suspended in the water column through mixing processes associated with the coastal circulation. Over the first 5 days of the experiment, there was a transition in the relative abundance of recovered phylotypes from cluster III to both α and γ proteobacterial sequences (Fig. 6c) in both control and treatment mesocosms. The disappearance of cluster III phylotypes by day 5 suggests that they may have been associated with settling detrital material, the fluxes of which were maximal over this period. By day 5, mesocosms C1, P3 and P6 contained populations of α and γ proteobacteria only, and in P6, the phylotype 59039A16 represented 82% of the community. In contrast, the diazotroph community in P5 on day 5 was dominated by a *Leptolyngbya*-like cyanobacterial phylotype 59038A29 (70%) and the α

proteobacterial phylotype 59036A19, which was the most frequently occurring of the diazotrophs observed. Only four of the diazotroph phylotypes recovered in this experiment are similar (>92% nucleotide identity) to previously reported diazotrophs from the Mediterranean Sea, and they all belong to cluster 1J/1K (Man-Aharonovich *et al.*, 2007; Le Moal *et al.*, 2011; Yogev *et al.*, 2011).

There is no clear association between diazotrophic community structure and changes in pCO_2 or N_2 fixation rates, as shown by PCA (Fig. 7), where 74.6% of the variance is explained by components 1 and 2. The molecular data does indicate a shift in the diazotroph communities present in the control mesocosm (C1) during the first 5 days of the experiment from the community present in the day -3 water column. Waters outside the mesocosms were not sampled throughout the experiment, so insufficient evidence exists to determine whether the diazotroph community composition changes in C1 were reflected in the natural environment or were a response to containment. The molecular data also implies that the diazotrophic community composition can rapidly change in response to environmental conditions. Although a more in-depth sequencing effort, or the development of qPCR assays targeting the most abundant phylotypes recovered, might provide additional valuable information, these approaches were not pursued, as the sampling resolution would have been insufficient to correlate patterns of successional change with observed changes in N_2 fixation with any confidence. Furthermore, samples for molecular work were not taken on days where peaks in N_2 fixation rates occurred in P5 and P6 mesocosms.

These findings underscore the challenges of relating changes in N_2 fixation rates with successional changes in diazotroph community structure or changes in the abundances of specific groups. This is particularly true in an environment that has not

been well characterised, and where clone-library based approaches recover non-cyanobacterial phylotypes (Turk-Kubo et al., 2014). Although these results provide important insight into the diazotroph diversity in the Bay of Calvi, this qualitative data must be interpreted with caution. Non-cyanobacterial diazotrophs have been implicated in many studies as putative marine N_2-fixers (e.g. Farnelid *et al.*, 2011, Halm *et al.*, 2012, Le Moal, *et al.*, 2011), yet their importance in marine N_2 fixation has yet to be proven. In rare cases where quantitative abundance data for non-cyanobacterial diazotrophs exists in parallel with N_2 fixation rates, it is evident that these organisms would have to sustain extremely high cellular specific rates of N_2 fixation to account for bulk rates, which presents a paradox in highly oxygenated photic zone waters (Turk-Kubo et al., 2014).

In previous studies where N_2 fixation rates increased with elevated conditions of OA, the overall consensus was that nitrogen fixation increased due to a decreased energy demand by carbon concentrating mechanisms during cyanobacterial photosynthesis, which increased the energy available for N_2 fixation (e.g. Barcelos E Ramos *et al.*, 2007; Hutchins *et al.*, 2007; Kranz *et al.*, 2010; Kranz *et al.*, 2009; Levitan *et al.*, 2007). In a recent study (Hutchins *et al.*, 2013) described taxon-specific variability in the sensitivity of N_2 fixation to CO_2, whilst others have shown no response under natural conditions to OA (Law *et al.*, 2012; Böttjer *et al.*, 2014; Gradoville *et al.*, 2014). During this experiment there were no observed changes in primary production (Maugendre et al., in press, this issue), chlorophyll-a or phytoplankton biomass (Gazeau et al., submitted this issue), neither were there any observed changes in heterotrophic prokaryote abundance, whilst bacterial production decreased with increasing CO_2 (Celussi et al., in press, this issue). It is apparent therefore that increases in N_2 fixation rates observed here were de-coupled from

inorganic and organic C acquisition and were limited by some factor after day 8, when all amended and control mesocosms showed similar rates.

We speculate that this may be a result of either some direct effect on the diazotroph population or indirectly through the availability of a limiting nutrient. Bacterial enzymatic activity has been observed to increase under lower seawater pH in coastal waters (Grossart et al.,2006) and from this study, Celussi et al., (in press, this issue) report on a community of CTC+ (highly active) prokaryotes whose abundance relative to total prokaryotes was positively correlated to pCO_2. Not only did the percentage CTC+ increase at elevated levels of pCO_2 but there was a decrease in this number over the time of the mesocosm period in a manner which is positively correlated to N_2 fixation rates ($r^2 = 0.85$). Ridame et al., (2011, 2013) showed that whilst Fe was not limiting to diazotrophy in Mediterranean coastal waters, N_2 fixation was limited or co-limited by the availability of DIP or an unidentified trace element. The bioavailability of trace metals may be affected by OA (Hoffmann et al., 2012). Whilst the concentrations of DIP and DOP coupled with high rates of APA during this experiment suggest that P is unlikely to be the controlling nutrient, the bioavailability of a limiting trace element which increased at elevated levels of OA might have provided the driver for the elevated rates of N_2 fixation observed.

This study is the first to investigate the impact of OA on a mixed population of coastal diazotrophs, but questions remain regarding the mechanisms and diazotroph(s) responsible for the elevated N_2 fixation rates observed. Coastal waters of the Mediterranean remain enigmatic with regards to the characterisation of their diazotrophic communities, the heirarchy of nutrients which are controlling their activity and indeed their sensitivity to OA. Whilst these observations indicate the potential for enhanced N_2 fixation in Mediterranean coastal waters under pCO_2

conditions projected for the end of this century, this study argues strongly for greater characterisation of diazotrophs and diazotrophy under fixed conditions of pCO_2 and under controlled conditions of nutrient stoichiometry.

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Figure Legends

Fig. 1 Location of the Bay of Calvi, Corsica, in the Northwestern Mediterranean Sea

Fig. 2 Temporal evolution of a) temperature (mean value for depth 0 to 10 m) and b) chlorophyll *a* in 3 control (C1 to C3) and 6 amended mesocosms (P1 to P6) relative to waters outside of the mesocosm enclosures (OUT) during the MedSeA ocean acidification experiment, Bay of Calvi (Corsica, France), June – July 2012.

Fig. 3 Temporal evolution of a) pCO_2 and b) pH_T (total scale) in 3 control (C1 to C3) and 6 amended mesocosms (P1 to P6) relative to waters outside of the mesocosm enclosures (OUT) during MedSeA ocean acidification experiment, Bay of Calvi (Corsica, France), June – July 2012.

Fig. 4 Nitrogen fixation rates in 6 amended mesocosms (P1 to P6) relative to mean control (C) ± 1 standard deviation (SD) during MedSeA ocean acidification experiment in the Bay of Calvi (Corsica, France), June – July 2012. Filled red circles indicate the days when partial *nifH* sequences were investigated in the control mesocosm C1, and the amended mesocosms P3, P5 and P6.

Fig. 5 Daily nitrogen fixation rates plotted against instantaneous values of pCO_2 from all treatments during MedSeA ocean acidification experiment in the Bay of Calvi (Corsica, France), June – July 2012. The solid line represents the mean value of all control rate measurements, the dashed line represents the mean plus 3 standard deviations of all control measurements.

Fig. 6 Maximum likelihood phylogenetic trees of cluster I (a) and cluster III (b) partial *nifH* sequences obtained from day -3 outside of mesocosms as well as mesocosms C1 (control), P3, P5 and P6 (CO_2 enriched) on days 1 and 5. Bootstrap values are reported on nodes from 500 replicate trees. The number of sequences that cluster with each representative (at 97% amino acid identity) are in parenthesis, when >10 sequences were recovered. Diazotroph community structure changes throughout the experiment, and prior to the initiation of the experiment (day -3), according to *nifH* cluster designation of each phylotype (c).

Fig. 7 Principal component analysis for diazotroph clusters, CO_2, pH and N_2 fixation for mesocosms C1, (control), P3, P5 and P6 (CO_2 enriched) on days 1 and 5, and outside of the mesocosms on day -3.

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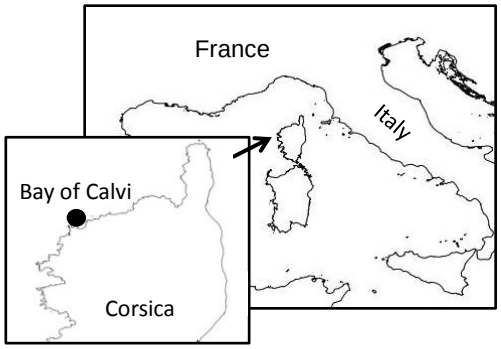
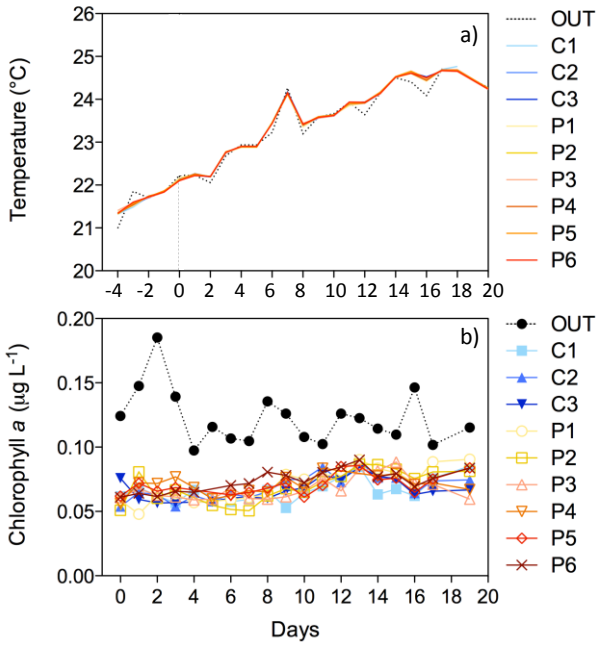


1

2 Fig. 1

3


a)
b)
Temperature (°C)
Chlorophyll a (µg L-1)
Days
OUT
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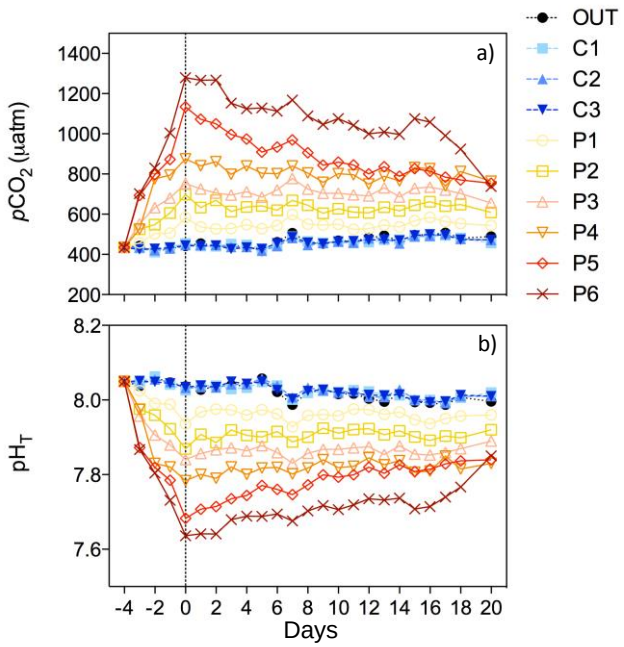
P5
P6

Fig 2


OUT
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a)
b)
pCO_2 (µatm)
pH_T
1400
1200
1000
800
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8.2
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2 Fig 3

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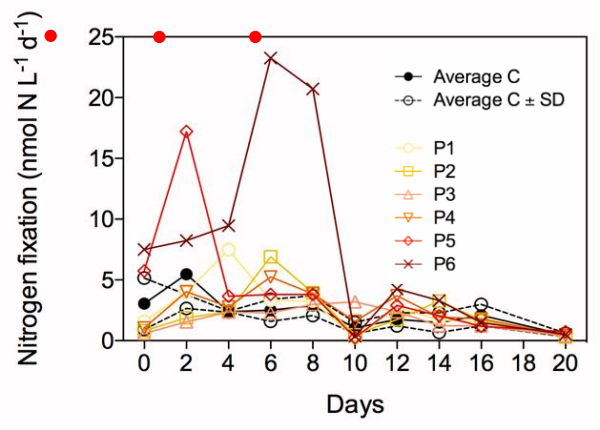
Nitrogen fixation (nmol N L-1 d-1)
25
20
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10
5
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0
2
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6
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14
16
18
20
Days
Average C
Average C ± SD
P1
P2
P3
P4
P5
P6

Fig 4

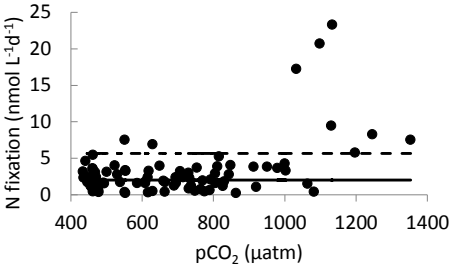

N fixation (nmol L-1d-1)
25
20
15
10
5
0
400
600
800
1000
1200
1400
pCO2 (µatm)

Fig 5

1

2

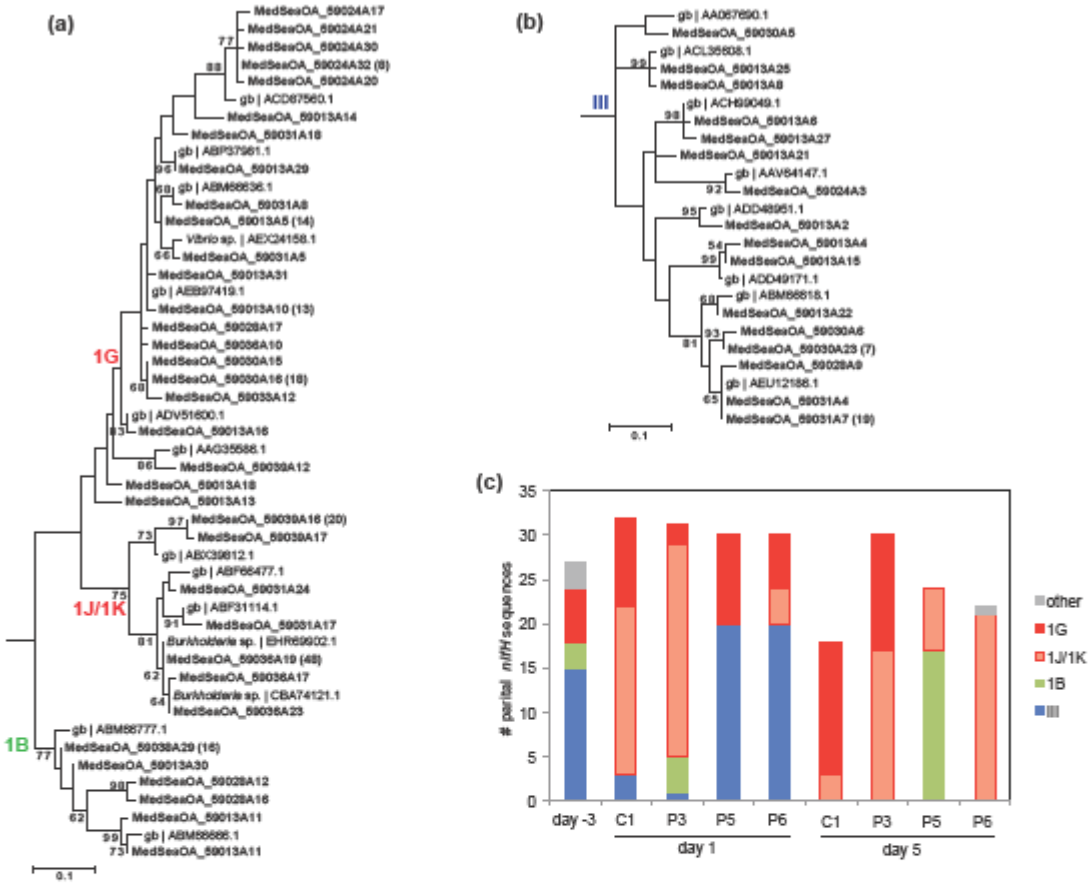
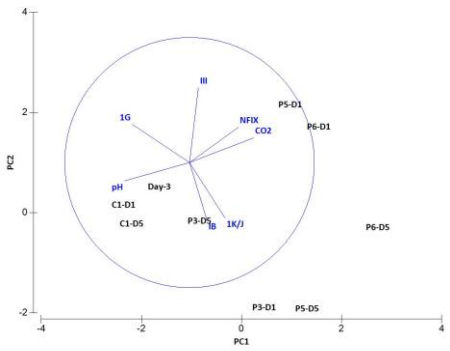

(a)
1G
1J/1K
1B
(b)
III
(c)
partial rrlrrH sequences
day -3
C1
P3
P5
P6
day 1
day 5
other
1G
1J/1K
1B
III

Fig 6


4
2
0
-2
PC2
-4
-2
0
2
4
PC1
III
1G
P5-D1
NFIX
CO2
P6-D1
pH
Day-3
C1-D1
C1-D5
P3-D5
1B
1K/J
P6-D5
P3-D1
P5-D5

1

2 Fig 7