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„Anaplerotic dissolved inorganic carbon fixation by marine *Alteromonas* – culture and natural community analysis"

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List of abbreviations

Formula symbols

Symbol	*Unit*	*Description*
A	mol/total incubation time	Leucine/Bicarbonate incorporation
g	h	Generation time
k	mol/cell/h	Cell-specific carbon uptake rates
N	number of cells	Abundance

Abbreviations

ASW	Artificial Sea Water
C	Carbon
DIC	Dissolved inorganic carbon
DPM	Disintegration per minute
MICRO-CARD-FISH	Microautoradiography combined with catalyzed reporter deposition fluorescence in situ hybridization

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1 Introduction

The marine carbon cycle is of fundamental importance, serving as long-term sink for atmospheric carbon (C) and providing organic C to the marine food web. One of the most important carbon fluxes is primary production, which is the transformation of CO_2 into organic carbon compounds. Photosynthesis by phytoplankton produces the majority of particulate and dissolved organic carbon in the sunlit ocean accounting for half of the global primary production (Field et al., 1998). On average 30 % of the photosynthetically produced organic carbon is exported out of the euphotic layer, exponentially attenuating with depth since the majority of the sinking organic matter is remineralized in the mesopelagic realm (Baltar et al., 2010; Herndl & Reinthaler, 2013). Despite limited nutrients in the deep sea, the prokaryotic carbon demand exceeds the supply (Reinthaler et al., 2006). Research agreed on that, besides photoautotrophic phytoplankton, chemoautotrophs provide a significant source of organic carbon in the dark ocean (Reinthaler et al., 2010). However, it is known, that also heterotrophs are able to fix dissolved inorganic carbon (DIC) representing another important player, which provides a non-considered source of newly synthesized organic carbon in the dark ocean.

Heterotrophs are defined as organisms that respire CO_2 after taking up organic substrates to gain energy and produce biomass. Via a wide range of carboxylation reactions, like the synthesis of amino acids, fatty acids and anaplerosis, which form an integral part in the heterotrophic metabolism, heterotrophic organisms do fix DIC (Braun et al., 2021; Erb, 2011). The anaplerotic pathway is a mechanism to refill tricarboxylic acid cycle' intermediates that have been released for biosynthesis (Erb, 2011). Especially in oligotrophic conditions being the ocean's most nature, anaplerotic reactions are found to be important. This is because the oxidation of DIC provides an additional C1 to existing organic carbon substrates, thus compensating metabolic imbalances (Braun et al., 2021; Erb, 2011; Palovaara et al., 2014). Recent studies agree that heterotrophic bacteria fixing CO_2 via anaplerosis could be relevant in the dark ocean DIC fixation (Alonso-Sáez et al., 2010; Baltar et al., 2016; Baltar & Herndl, 2019; Teira et al., 2012).

In this study, we expand prior work by quantifying the anaplerotic dark DIC fixation of heterotrophic microbes in the marine environment and providing cell-specific rates to test our hypothesis that *anaplerotic pathways are key contributors to oceanic dark DIC fixation*.

As representatives of the heterotrophic marine prokaryotes, we focus on Alteromonadales which are positive for DIC fixation in previous Stable Isotope Probing experiments. Additionally they are putative heterotrophic marine bacteria, like *Alteromonas mediterranea DE*, whose genomes do not carry any genes for photo- or chemoautotrophic DIC fixation (DeLorenzo et al., 2012). Lacking those genes in their genomes ensures that the incorporation of DIC must occur via anaplerosis.

To test our hypothesis we performed both, lab and field studies. In the lab culture studies, we calculated how much anaplerotic DIC fixation contributes to the *Alteromonas* metabolic rates such as biomass production and respiration. Therefore, we cultured *Alteromonas mediterranea DE* under different temperature and nutrient conditions incorporating radioactive-labelled ^{14}C-bicarbonate and ^{14}C-leucine. In the environmental experiments, we determined the contribution of anaplerotic dark DIC fixation by *Alteromonas* to the whole prokaryotic DIC assimilation in the marine environment. We performed MICRO-CARD-FISH (MICROautoradiography combined with CAtalyzed Reporter Deposition Fluorescence In Situ Hybridization) using a probe targeting on *Alteromonadales* addressing their activity of incorporating ^{14}C-bicarbonate and ^{3}H-leucine.

The objective of this study was to determine to what extend *Alteromonas*' DIC fixation via anaplerosis contributes to the total prokaryotic dark inorganic carbon assimilation in the ocean, and what might influence (temperature and nutrient concentration) their anaplerotic DIC fixation. Specifically, the present work provides answers to following research questions:

i. How is anaplerotic dark DIC fixation influenced by temperature and nutrient supply?
ii. Do *Alteromonas* reach their potential cell-specific DIC fixation rate observed in culture in the marine environment?
iii. How does anaplerotic dark DIC fixation by *Alteromonas* varies within depth and region?
iv. Hoch much do anaplerotic reactions by *Alteromonas* contribute to the total dark oceanic inorganic carbon fixation?

2 Material and methods

2.1 Anaplerotic DIC fixation rates using *Alteromonas* culture

2.1.1 Preparation of Artificial Sea Water

To prepare 1 L of Artificial Sea Water (ASW) anhydrous salts were dissolved in 600 mL dH_2O (MilliQ) and hydrous salts in 300 mL MilliQ individually and were combined. One mL of major nutrient stocks, 100 µL of trace metal stock solutions and vitamin B12 was added (Table 1). To avoid contamination, the bottles were sterilized by autoclaving and stock solutions sterile filtered and manipulation were performed under a clean bench. The C:N:P of the ASW with glucose as carbon source was either 50:10:1 µM (Goldman et al., 1987) or 500:100:10 µM depending on the assay. We decided to use these glucose concentrations because 50 µM C in glucose is similar to the dissolved organic carbon concentration in the ocean and 500 µM is the highest possible concentration of organic carbon during phytoplankton bloom in coastal regions (Fisher et al., 1998; Hansell, 2013; Simjouw et al., 2004).

Table 1: Component list for preparing artificial sea water.

Component	Molar concentration in final medium
Anhydrous salts	
NaCl	4.2×10^{-1} M
Na_2SO_4	2.88×10^{-2} M
KCl	9.39×10^{-3} M
$NaHCO_3$	2.38×10^{-3} M
KBr	8.40×10^{-4} M
H_3BO_3	4.85×10^{-5} M
NAF	7.15×10^{-5} M
Hydrous Salts	
$MgCl_2.6H_20$	5.46×10^{-2} M
$CaCl_2.6H_20$	1.05×10^{-2} M
$SrCl_2.6H_20$	6.38×10^{-5} M
Vitamins	
Cyanocobalamin Vit.B12	3.8×10^{-11} M
Nutrients	
NH_2PO_4 H_2O*	1 µM
NH_4Cl	10 µM
Glucose ($C_6H_{12}O_6$)	8.3 µM
Trace metals	
$FeCl_3$ $6H_2O$	0.5 nM
$ZnSO_4$ $7H_2O$	5 nM
$MnCl_2$ $4H_2O$	0.3 nM
$CoCl_2$ $6H_2O$	0.02 nM
Na_2MoO_4 $2H_2O$	105 nM
$CuSO_4$ $5H_2O$	3 nM
Na_2SeO_3	1.7 nM
Ni	8 nM

2.1.2 Culture setup

Laboratory experiments were conducted with cultures of *Alteromonas mediterranea DE* previously grown in ASW with ×10 nutrient conditions (C:N:P of 500:100:10 µM) and stored in glycerol stock at -80°C. One mL of the thawed stock was added into 100 mL ×10 ASW. This culture was incubated at 20°C for several days. When the cell abundance reached approximately 10^6 cells per mL, the culture was inoculated to a new ×10 ASW and repeated twice to obtain freshly grown cells. Subsequently, the culture was stored in 50 mL Greiner tubes at 4°C as initial culture for the following pre-culture steps. The cell abundances were measured by flow cytometry (BD Accuri C6). The samples were fixed with glutaraldehyde (0.5 % final concentration and stained with SYBR Green I.

All cultures were incubated in HCl washed and autoclaved glass flasks closed with an air-permeable silicone plug covered with aluminium foil. During the incubation at 13°C or 23°C, the flasks were shaken gently at 60 rpm. The initial cell abundance of all cultures was approximately 10^4 cells per mL. For all experiments a pre-culture was performed with 100 mL of ASW and *Alteromonas mediterranea DE* kept at 4°C above mentioned. From the pre-culture, the culture experiments were conducted in 150 mL ASW in triplicates except kinetics without replicates.

To determine the growth curves, incubations were conducted under different temperature (13°C and 23°C) and nutrient conditions (×1 and ×10 of C:N:P in ASW) by subsampling at each 5 – 8 h until stationary phase of cell growth was reached. Based on the growth curves, the incubation times of the pre-culture and the cultures were 72 h and 24 h for the 13°C experiments, respectively, and 24 h and 6 h for the 23°C experiments, respectively.

We decided for 13°C and 23°C incubation temperature because 13°C is the temperature of Mediterranean deep where *Alteromonas mediterranea DE* originally come from, and 10°C+ being the classical ways for this kind of assay (e.g. Q10).

2.1.3 Leucine concentration kinetics

To determine the saturation concentration of leucine, kinetics experiment for substrate concentration was conducted with *Alteromonas* in the mid-exponential phase grown in ASW with ×1 nutrients. In total eight concentrations in duplicate samples and one killed control were incubated with ^{3}H-leucine working solution (one part radioactive, nine parts non-radioactive leucine) into 5 mL of culture. Controls were killed with 0.2 µm filtered formaldehyde (2 % final concentration) at 4°C keeping it for 15 min. After ^{3}H-leucine was added (Table 2), the samples were incubated at 23°C for 2 h, and then terminated the incubation of the live-samples with 0.2 µm filtered formaldehyde (2 % final concentration). The samples were kept overnight at 4°C before filtering through 0.2 µm polycarbonate filters (GTTP, 25 mm filter diameter; Millipore) supported by cellulose acetate filter below (HWAP, 0.45 µm pore size) using minimal vacuum (200 mmHg). The tubes were rinsed twice with approximately 5 mL of 5 % TCA (kept at 4°C) and incubated for 5 min before vaccumed and rinsed again with MilliQ. The filters were transferred to 20 mL scintillation vials and 8 mL of scintillation cocktail (Filter Count, Perkin Elmer) was added. The samples were kept over night and counted with scinitillation counter

Tri-Carb 4910 TR (Perkin Elmer). Consequently, we decided to use 140 nM of leucine as final concentration of for the further experiments (Figure 1).

Table 2: Volumes of ^{3}H-leucine working solution added during the concentration kinetics experiment.

Leucine concentration [nM]	1	2	5	20	40	80	150	320
Volume of 20 µM Leucine [µL] (HOT+COLD = 1+9)	0.3	0.5	1.3	5	10	20	40	80

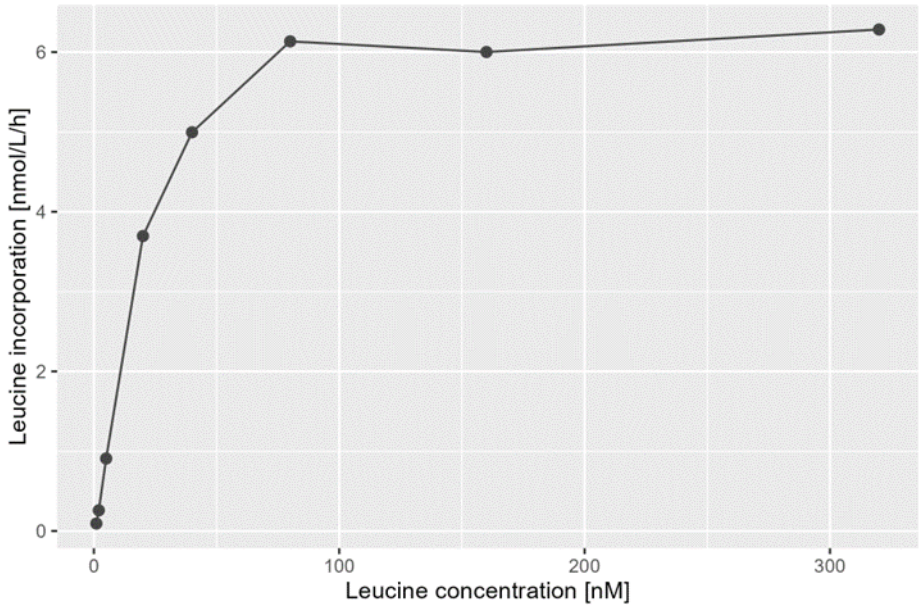

Figure 1: Leucine concentration kinetics. The points represent the leucine incorporation at different leucine concentrations. As final concentration of ^{14}C-leucine which is added in the samples to measure biomass production 140 nM were defined since incorporation is already saturated at this point.

2.1.4 Leucine and bicarbonate time kinetics

To determine the duration of incubation, 5 mL *Alteromonas* culture were prepared in duplicate samples and killed control (2 % final concentration of 0.2 µm filtered formaldehyde). Final concentration of ^{14}C-leucine and ^{14}C-bicarbonate was 140 nM and 8 µCi/sample, respectively. The samples were incubated at 23°C for 1 h, 2 h or 3 h for leucine and 2 h, 3 h, 4 h, and 6 h for DIC by terminating with 0.2 µm filtered formaldehyde (2 % final concentration) at the above points. The samples were kept overnight at 4°C before filtering through 0.2 µm polycarbonate filters (GTTP, 25 mm filter diameter; Millipore) supported by cellulose acetate filter below (HWAP, 0.45 µm pore size) using minimal vacuum (200 mmHg). The tubes of the leucine samples were rinsed twice with approximately 5 mL of 5 % TCA (kept at 4°C) and incubated for 5 min before vaccumed and rinsed again with MilliQ, while the tubes of the DIC samples were rinsed only with MilliQ twice. The filters of the DIC samples were exposed to 37 % HCl fume for 12 h, before transferred into 20 mL scintillation vials and adding 8 mL scintillation cocktail (Filter Count, Perkin Elmer). The samples were kept at room temperature for overnight and counted in the scinitillation counter Tri-Carb 4910 TR (Perkin Elmer). The appropriated duration of incubation was defined by the highest incorporation which were 2 h for ^{14}C-leucine and 4 h for ^{14}C-bicarbonate (Figure 2).

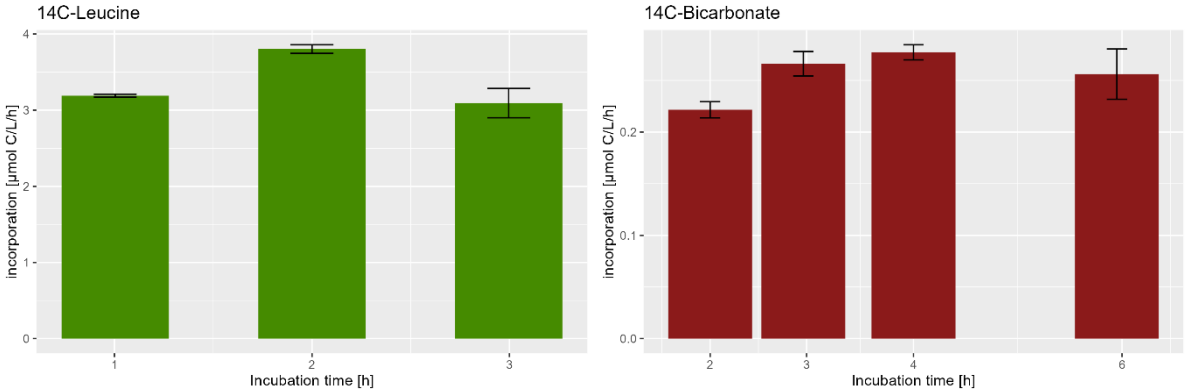

Figure 2: Time kinetics. The bar plots indicate the incorporation of ^{14}C-leucine (left) and ^{14}C-bicarbonate (right) after different incubation times. The appropriated duration of incubation was defined by the highest incorporation which were 2 h for ^{14}C-leucine and 4 h for ^{14}C-bicarbonate.

2.1.5 Heterotrophic and anaplerotic activity detection

To determine the heterotrophic and anaplerotic activity of *Alteromonas mediterranea DE*, DIC fixation and leucine incorporation rates under the different culture conditions were measured. For each culture condition, the rates were determined with three biological replicates. From each replicate, 2 samples and 1 killed control were taken to measure the bulk activity with the scintillation counter. Samples for MICRO-CARD-FISH were also taken to access cell-specific activities.

For the bulk leucine incorporation, 20 mL of *Alteromonas* culture were poured into three BOD bottles and 10 mL into two of 15 mL-Greiner tubes. One of each was immidiately fixed with 0.2 µm-filtered formaldehyde (2 % final concentration) as control. After the samples were inoculated with ^{14}C-leucine (final concentration 140 nM determined in leucine kinetics), the BOD bottles were closed with rubber lids including a bucket with a filter paper wick in. Samples were incubated at either 13°C or 23°C for 2 h. For the MICRO-CARD-FISH samples, the incubation was terminated by adding 37 % formaldehyde (2 % final concentration) into the Greiner tubes keeping it at 4°C for >20 min. Whereas 400 µL of 2N H_2SO_4 and subsequently 200 µL of phenethylamine were injected onto the filter paper wick in the BOD bottles before placing it on a shaker at room temperature for 1 – 2 h. The fixed samples were filtered through 0.2 µm polycarbonate filters (GTTP, 25 mm filter diameter, Millipore) supported by cellulose acetate filter below 0.45 µm pore size, twice rinsed with MilliQ. While filters for Micro-CARD-FISH were stored at -20°C until analysis, the filters for bulk leucine uptake measurement and the filter paper wicks from the previous step were placed into 20 mL scintillation vials and 8 mL of scintillation cocktail was added (Filter Count, Perkin Elmer). The samples were kept over night and measured with the scintillation counter (Tri-Carb 4910 TR, Perkin Elmer).

For DIC fixation measurement, 10 mL of *Alteromonas* culture were poured into in total 5 of 15 mL Greiner tubes (2 samples and 1 killed-control for the bulk DIC fixation rate measurements, 1 sample and 1 killed-control for MICRO-CARD-FISH). The samples were inoculated with 8 µL of 1 mCi/mL DI^{14}C (8 µCi/sample) and incubated at either 13°C or 23 °C for 4 h. To terminate the incubation 37 % formaldehyde (2 % final concentration) were added and the samples kept at 4°C for >20 min. The fixed samples were filtered through 0.2 µm polycarbonate filters (GTTP, 25 mm filter diameter, Millipore) supported by cellulose acetate filter below (0.45 µm pore size). The Bulk-DIC samples were rinsed twice with MilliQ, vaccumed and the filters

placed in 37 % HCl fume for 12 – 18 h. The filters for MICRO-CARD-FISH were rinsed twice with MilliQ and stored at -20°C until analysis. The Bulk-DIC filters were placed into 20 mL scintillation vials and 8 mL of scintillation cocktail (Filter Count, Perkin Elmer) were added. The samples were kept at room temperature for overnight, before measuring with the scintillation counter (Tri-Carb 4910 TR, Perkin Elmer).

2.1.6 Statistical analysis

Disintegrations per minute (DPM) of the leucine samples were converted into carbon production over time with the conversion factor 1.55 kg C/mol leucine (Giering & Evans, 2022). Inorganic carbon fixed over the time course was calculated by 1 µCi corresponding to 2.2×10^6 DPM and the DIC concentration in the media of 0.002380754 g C/M. Cell-specific carbon uptake rates were calculated by $k = \frac{ln^2 \times A}{g \times (N2 - N1)}$, the integral of the cell growth assuming k as specific activity [mol C/cell/h] to be constant.

The statistical analysis was conducted in R (Version 4.2.2) using two-way ANOVA as significance test. Errors were calculated by error propagation.

2.2 Heterotrophic and anaplerotic activity of *Alteromonas* in environmental studies

2.2.1 Sampling location

Seawater samples of meso- and bathypelagic depths were collected during two cruises in the Atlantic and Pacific Ocean to determine cell-specific heterotrophic and anaplerotic activity of *Alteromonas* with MICRO-CARD-FISH. The Atlantic samples were collected at three closely located stations off Iberian margin in the North Atlantic during the RadProf cruise in July/August 2015. Pacific samples analysed in this study were taken during the SONNE248 cruise in May 2016 (Figure 3 and Table 3).

Table 3: Station list. It gives an overview of the details of the sampling positions.

Cruise	Ocean	Station	Month/Year	Latitude	Longitude	Depth [m]	Substrate
SONNE	Pacific	6	05/2016	0 °N	180 °E	200	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	6	05/2016	0 °N	180 °E	400	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	6	05/2016	0 °N	180 °E	500	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	6	05/2016	0 °N	180 °E	1000	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	6	05/2016	0 °N	180 °E	2000	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	10	05/2016	21°58'N	178°19'E	200	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	10	05/2016	21°58'N	178°19'E	400	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	10	05/2016	21°58'N	178°19'E	500	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	10	05/2016	21°58'N	178°19'E	1000	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	10	05/2016	21°58'N	178°19'E	2000	^{3}H-Leu, ^{14}C-Bicarb
SONNE	Pacific	14	05/2016	45°N	178°45'E	200	^{14}C-Bicarb
SONNE	Pacific	14	05/2016	45°N	178°45'E	300	^{14}C-Bicarb
SONNE	Pacific	14	05/2016	45°N	178°45'E	500	^{14}C-Bicarb
SONNE	Pacific	14	05/2016	45°N	178°45'E	1000	^{14}C-Bicarb
SONNE	Pacific	14	05/2016	45°N	178°45'E	2000	^{14}C-Bicarb
SONNE	Pacific	16	05/2016	50 °N	179°33'E	200	^{3}H-Leu

SONNE	Pacific	16	05/2016	50 °N	179°33'E	500	^{3}H-Leu
SONNE	Pacific	16	05/2016	50 °N	179°33'E	1000	^{3}H-Leu
SONNE	Pacific	16	05/2016	50 °N	179°33'E	2000	^{3}H-Leu
RadProf	Atlantic	8	08/2015	43°N	9°W	1000	^{3}H-Leu, ^{14}C-Bicarb
RadProf	Atlantic	11	08/2015	43 °N	10°W	1800	^{3}H-Leu, ^{14}C-Bicarb
RadProf	Atlantic	16	08/2015	43°N	11°W	2000	^{3}H-Leu, ^{14}C-Bicarb

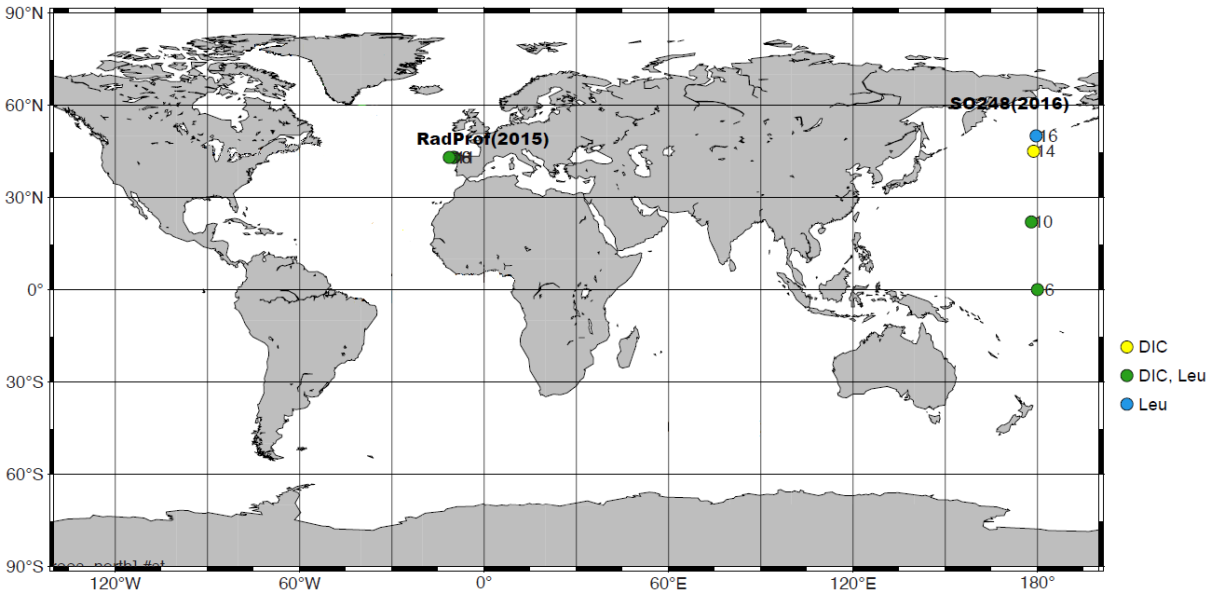

Figure 3: Map of the sampling station. The colours represent the substrate the samples were incubated with. Green shows the samples incubated with ^{14}C-bicarbonate (DIC) as well as ^{3}H-leucine (Leu), yellow only ^{14}C-bicarbonate and blue only ^{3}H-leucine.

Samples were taken from Niskin bottles. Tritium labelled leucine (5 nM final concentration) was added to volumes between 10 – 40 mL (depending on the depth) and incubated at in situ temperature for 4 – 29 h in the dark. For bicarbonate uptake 100 µCi of ^{14}C-sodium bicarbonate was added to 80 mL of seawater and incubated at 4°C and 10°C for 48 – 72 h. After fixing the samples with formaldehyde (2 % final concentration) at 4°C for up to 18 h, the samples were filtered through 0.2 µm polycarbonate filters (25 mm diameter) and frozen at -80°C.

Bulk DIC fixation rate was measured by assimilation of ^{14}C-bicarbonate into the cells. Forty to eighty mL samples and formaldehyde-killed-blanks (2 % final concentration) were incubated at in situ temperature for 48 – 72 h in the dark. The incubations were terminated by adding either formaldehyde or glutaraldehyde (2 % final concentration) depending on the cruise and filtered through 0.2 µm polycarbonate filters (25 mm diameter). After exposing the filters to HCl fume for 12 – 24 h, they were placed into 8 mL scintillation cocktail (FilterCount, Canberra-Packard). After 18 h, the samples were counted in a liquid scintillation counter (Perkin Elmer Tricarb).

2.2.2 MICRO-CARD-FISH

To determine the leucine and bicarbonate uptake by *Alteromonas*, the filter samples were processed with MICRO-CARD-FISH. The whole filters were embedded in 0.1 % agarose, while in the following steps only a twelfth part was used. The cells were permeabilized in lysozyme

mix (final concentrations of 10 mg/mL lysozyme in the buffer containing 0.1 M Tris-HCl, 0.05 M EDTA) at 37°C for 1 h. Subsequently, the filters were placed in 0.01 M HCl at room temperature for 25 min to inactivate endogenous peroxidases. The filter sections were hybridized at 35°C for 15 h in the hybridization buffer with 55 % formamide and the appropriate HRP labelled probe, either NON338 (3'-ACT CCT ACG GGA GGC AGC-5') as a negative control, EUB338 I (3'-GCT GCC TCC CGT AGG AGT-5') as a positive control or Alt1413 (3'-TTT GCA TCC CAC TCC CAT-5') for *Alteromonas* detection (Eilers et al., 2000). Afterwards, the filter sections were washed in washing buffer (final concentrations of 13 mM NaCl, 20 mM Tris-HCl, 5 mM EDTA, 0.01 % SDS). For the amplification, the filter sections were incubated in PBS-T-Mix (PBS with a final concentration of 0.05 % Triton X100) at room temperature for 15 min. Afterwards, they were incubated in amplification buffer (0.0015 % H_2O_2, 0.016 mg/mL Alexa488-Tyramide, 10 % Dextran Sulfate, 2 M NaCl, 0.1 % Blocking reagent in PBS) at 46°C for 15 min and again washed in PBS-T-Mix at room temperature for 15 min in the dark. The filter sections of the negative and positive control were mounted on microscopy slides in DAPI mix (final concentration of 2 µg/mL DAPI), while *Alteromonas* targeted filter sections were prepared for the microautoradiography process.

For the autoradiography previously hybridized filter sections were transferred onto slides covered with photographic emulsion (Ilford Nuclear Emulsion Type K 5, 0.2 µm crystal size), which was melted in a water bath at 43°C for 1 h, in complete darkness. The slides were stored in a light-tight box and exposed either 14 days for leucine or 30 days for bicarbonate uptake at 4°C. Afterwards, the samples were developed and fixed in the dark by putting them 4 min into developer (Ilford Phenisol developer), 10 s into MilliQ, 6 min into fixer (Ilford Hypam Fixer) followed by >5 min rinse in MilliQ. Before the cells were stained in DAPI mix (final concentration of 2 µg/mL DAPI), the filter sections were gently peeled off.

2.2.3 Image analysis to detect cell abundance and activity

The absolute cell abundance was calculated from DAPI stained cell counts acquired by *ACMEtool3 Version 2020-07-27*. Silver grain area surrounding active cells was analysed from overlaid images, two images taken in the epifluorescence mode of the microscope *Zeiss Axio Imager M*; one of DAPI stained prokaryotes and one of probe Alt1413 targeted bacteria; and the other image of the silver grain areas taken in the transmission mode. More than 1000 DAPI-stained cells per sample in at least 10 images per sample were analysed in the software *AxioVs40x64 Version 4.9.1.0*. By this process it was possible to count DAPI stained cells, Alt1413 CARD-FISH positive cells, silver grain area surrounded DAPI stained cells and silver grain area surrounded probe-positive cells. By the correlation of average silver grain area to the bulk leucine incorporation and DIC fixation measured on board, the conversion factor of silver grain area sizes into incorporation rates were determined (Figure 4) (Sintes & Herndl, 2006). The conversion factor for incorporation of leucine was 15,100,000 × silver grain area and of DIC 1390 × silver grain area. The ratio of probe-positive cells to the absolute abundance was used to calculate the absolute abundance of *Alteromonas* in the samples.

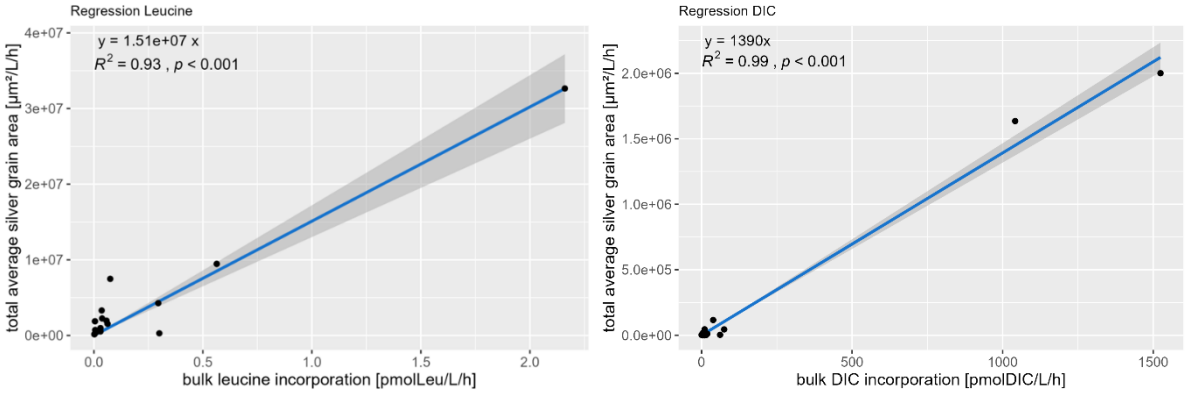

Figure 4: Relationship between total silver grain area and bulk incorporation of ^{3}H-leucine and ^{14}C-bicarbonate. Correlation of silver grain area [µm³/L/h] and bulk incorporation [pmol/L/h] of leucine (left) and DIC (right) provides the conversion factor of silver grain area into substrate uptake rates.

2.2.4 Outlier exclusion

Since we have seen an increase in the abundance of *Alteromonas* only in long time incubation samples as compared to the abundance before incubation, we did an outlier exclusion for the final estimation of the contribution of *Alteromonas* assimilating carbon by heterotrophic and anaplerotic processes (see chapter 3.2.7).

Therefore, the relative contribution of total bicarbonate and leucine incorporation, respectively, by *Alteromonas* to total prokaryotic incorporation was calculated as well as the relative contribution of substrate positive *Alteromonas* to substrate positive prokaryotes for each station. Three samples (SONNE station 10 at 200 m, RadProf station 8 at 1000 m, and RadProf station 16 at 2000 m) were identified as outliers and excluded from the final assessment. This was done by dividing the difference of total leucine positive prokaryotes and corresponding DIC positive prokaryotes by the total leucine positive prokaryotes of each station. The samples were defined as outliers if the relative contribution of total leucine positive prokaryotes to the difference of total leucine positive prokaryotes and corresponding DIC positive prokaryotes varied more than 10 % out of 100 %.

3 Results

3.1 Lab culture studies

3.1.1 *Alteromonas* growth curve at different incubation conditions

Figure 5 displays the growth curve of the *Alteromonas* culture, indicating that temperature and nutrient conditions influenced their growth. At the increased temperature of 23°C, *Alteromonas* started to grow more rapidly compared to an incubation temperature of 13°C. Additionally, with an increased glucose concentration of 500 µM C, *Alteromonas* reached a higher maximum abundance of around 5×10^7 cells/mL, than at a lower glucose concentration of 50 µM C, where they reached one order of magnitude lower abundance of 5×10^6 cells/mL.

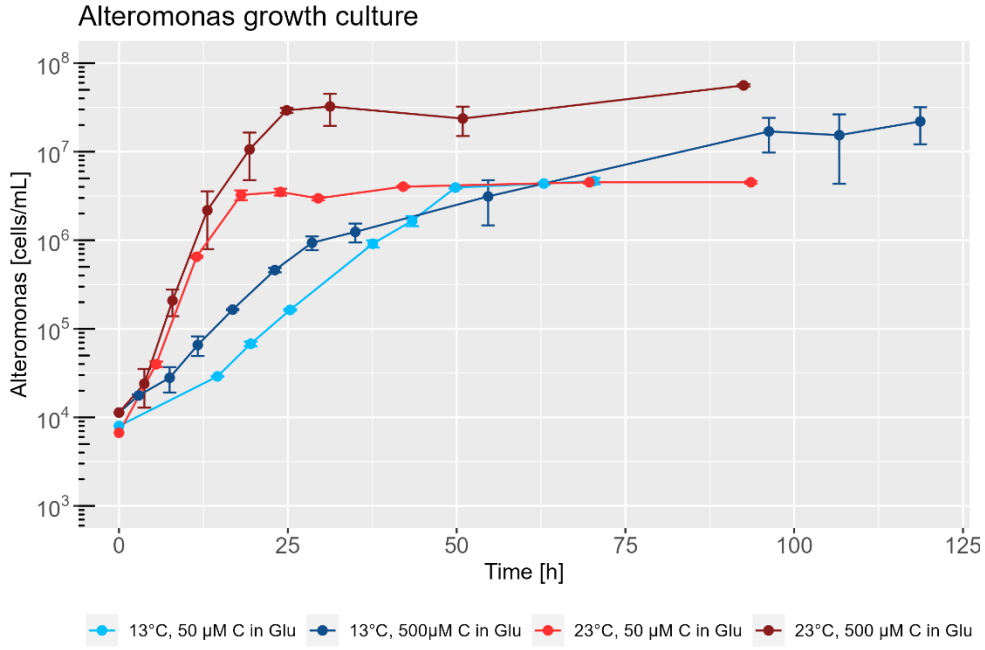

Figure 5: *Alteromonas* growth curve. The plot displays the growth of *Alteromonas* [cells/mL] in culture at 13°C incubation temperature and 50 µM C in glucose concentration (light blue), 13°C and 500 µM C in glucose concentration (dark blue), 23°C and 50 µM C in glucose concentration (light red), 23°C and 500 µM C in glucose concentration (dark red) to time in hours.

3.1.2 Temperature and nutrient effects on *Alteromonas'* biomass production, respiration and DIC fixation

Figure 6 represents biomass production, respiration and DIC fixation of *Alteromonas* under the four different incubation conditions. Biomass production constituted the major part of the metabolic processes (2.93 to 7.90 fmol C/cell/h), while DIC fixation showed the lowest cell-specific C uptake (0.12 to 0.30 fmol C/cell/h) (Appendix A). The assumption that temperature affects *Alteromonas*' metabolism more than changes in nutrients was tested with a two-way ANOVA (Figure 7). The obtained p-values are shown in Appendix D. The effect of temperature under constant nutrient conditions was highly significant for DIC fixation and biomass production ($p < 0.001$), and moderately significant for respiration ($p < 0.01$). While there was no significant effect of increased nutrient concentration at 13°C incubation temperature on all three metabolic processes ($p > 0.05$), DIC fixation and biomass production were significantly influenced by increased nutrient supply at 23°C ($p < 0.001$).

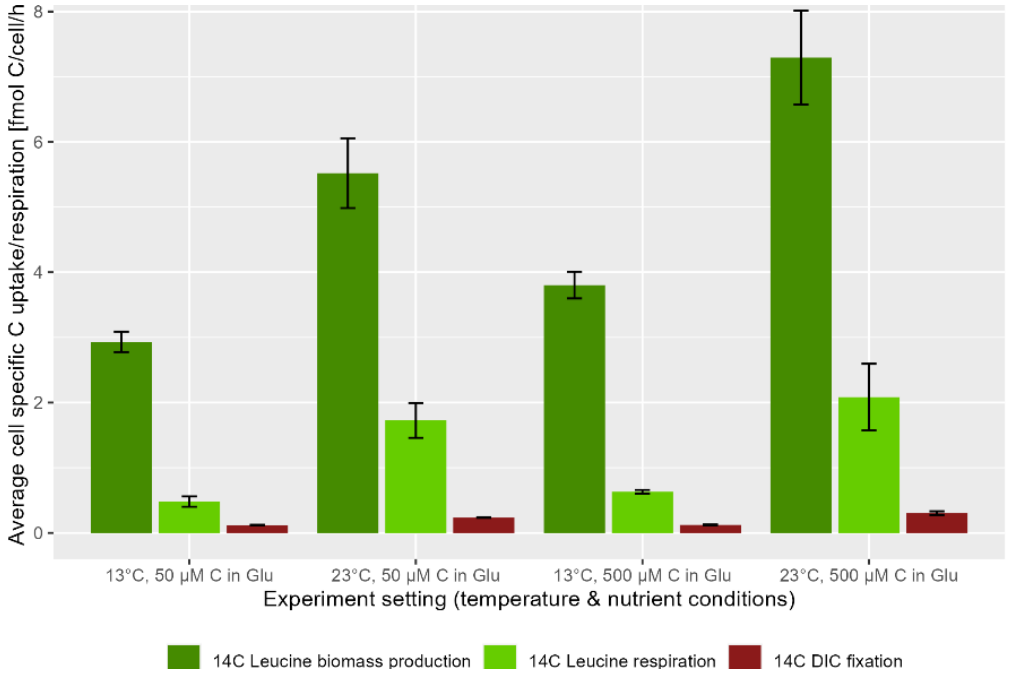

Figure 6: Average cell-specific C uptake and respiration per assay. The graph provides an overview of the average cell-specific carbon uptake and release in fmol C/cell/h for biomass production (dark green), respiration (light green) and DIC fixation (dark red) for each assay.

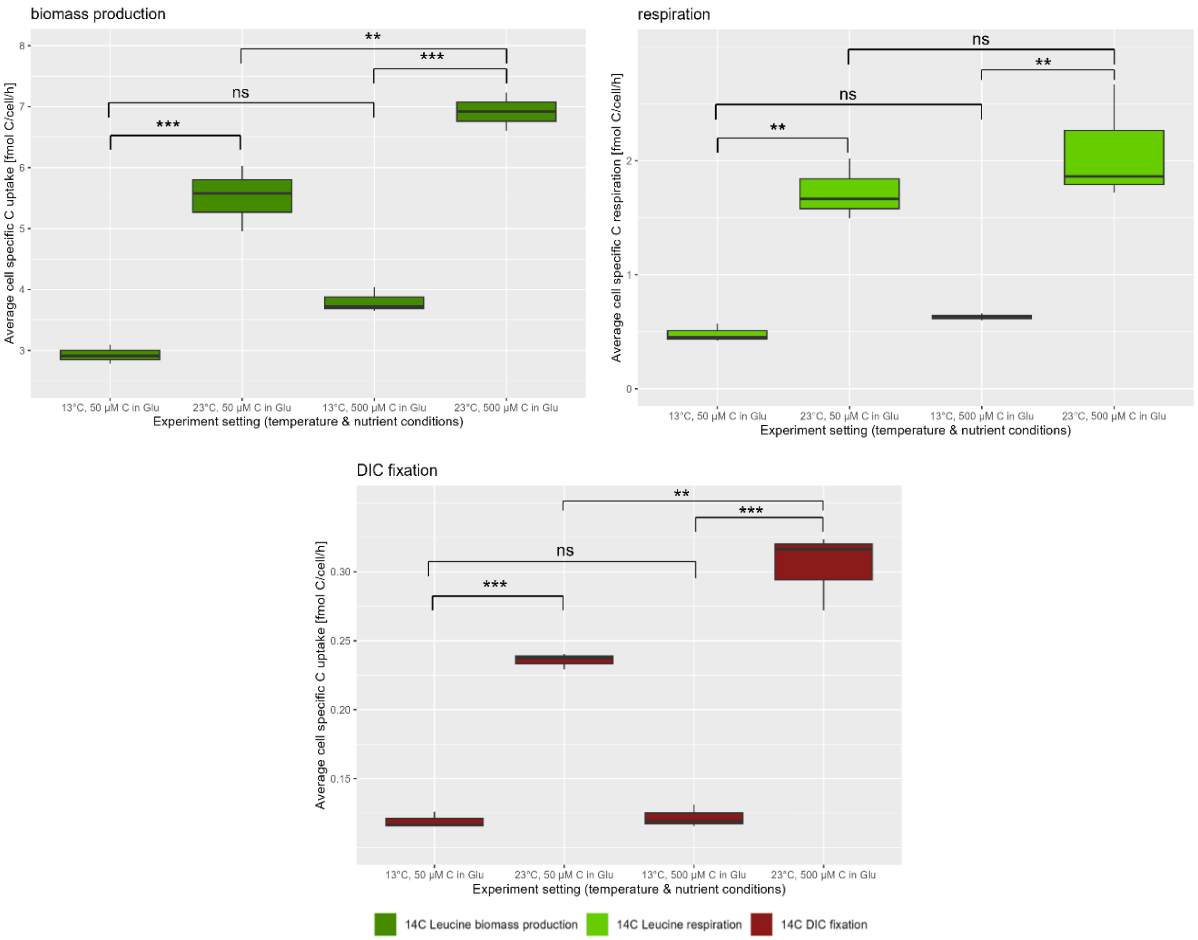

Figure 7: Boxplots with significance for average cell-specific C uptake and respiration per assay. The error bars indicate the standard deviation. To analyse the differences between the means of the metabolic processes and the interactions of the assays, it was tested for significance with a two-way ANOVA. The results are presented for biomass production, respiration and DIC fixation. The meaning of the symbols are: ns – not significant p > 0.05, * – less significant p <= 0.05, ** – moderately significant p <= 0.01, *** – highly significant p <= 0.001.

While there was an increase in cell-specific level biomass production and respiration with increasing temperature and nutrient concentration (Figure 6a), the proportions of the metabolic processes remain relatively constant (Table 4). The proportion of biomass production to the overall metabolism (sum of biomass production, respiration and DIC fixation) was higher (83 ±6 %) at 50 µM C in glucose concentration. Under low nutrient supply, respiration decreased to 14 ±1 % and 14 ±2 %, relative to contributions of respiration at 500 µM C in glucose concentration (22 ±6 % and 23 ±4 %). However, the relative contribution of DIC fixation remained stable at approximately 3 ±0.3 % under all the incubation conditions (Table 4).

Table 4: Relative contribution of metabolic processes in each assay. The table presents the relative contribution of biomass production, respiration and DIC fixation to the sum of all three metabolic process ± error in %.

setting	Biomass production [%]	Respiration [%]	DIC fixation [%]
13°C 50 µM C in Glu	83 ± 6	14 ± 2	3.4 ± 0.3
13°C, 500 µM C in Glu	74 ± 9	23 ± 4	3.2 ± 0.3
23°C, 50 µM C in Glu	83 ± 6	14 ± 1	2.7 ± 0.2
23°C, 500 µM C in Glu	75 ± 10	22 ± 6	3.1 ± 0.4

3.2 Environmental studies

3.2.1 Physicochemical property of seawater and *Alteromonas'* abundances at the stations

An overview of the environmental parameters is shown in Figure 8. The figures exhibit profiles of temperature, salinity and fluorescence (i.e., a proxy for chlorophyll-a concentrations). In the Pacific Ocean, Stations 6 and 10, which were located at lower latitudes, had warmer water temperatures ranging from 15°C to 7°C in the mesopelagic zone, while Stations 14 and 16 at higher latitudes had temperatures at around 7°C to 3°C. All stations showed decreased temperatures in the bathypelagic zone ranging from 4°C to 2°C. Salinity did not vary significantly across the stations, but maximum chlorophyll-a concentrations slightly increased with latitude, reaching a maximum of 0.065 mg/m³ at Station 16. The Atlantic stations exhibited generally higher temperature, salinity and chlorophyll-a concentrations compared to the Pacific stations, even in the bathypelagic zone (Figure 8).

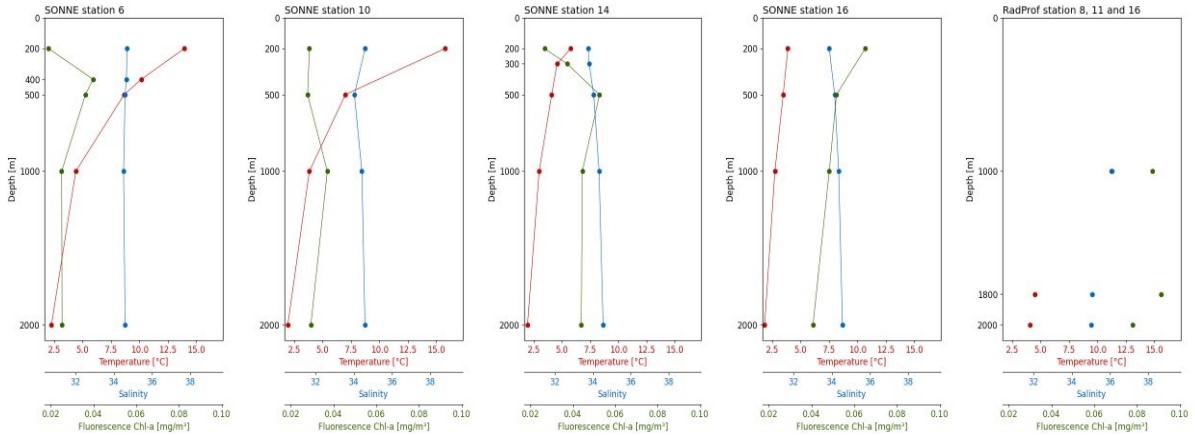

Figure 8: Overview of the sampling stations. The graphs display the distribution of environmental parameters such as temperature [°C] (red), salinity (blue) and chlorophyll-a [mg/m³] (green) for each station.

3.2.2 Bulk DIC fixation

The bulk DIC fixation of the Pacific samples measured on board is presented in Figure 9. Station 10 exhibited high DIC fixation rates of 5 µmol/m³/d in the epi- and mesopelagic realm decreasing in the bathypelagic to 4 – 0 µmol/m³/d. Stations 6, 14 and 16 showed a decreasing bulk DIC fixation profile starting from 1 µmol/m³/d in the epi- and mesopelagic to 0 µmol/m³/d in the bathypelagic realm.

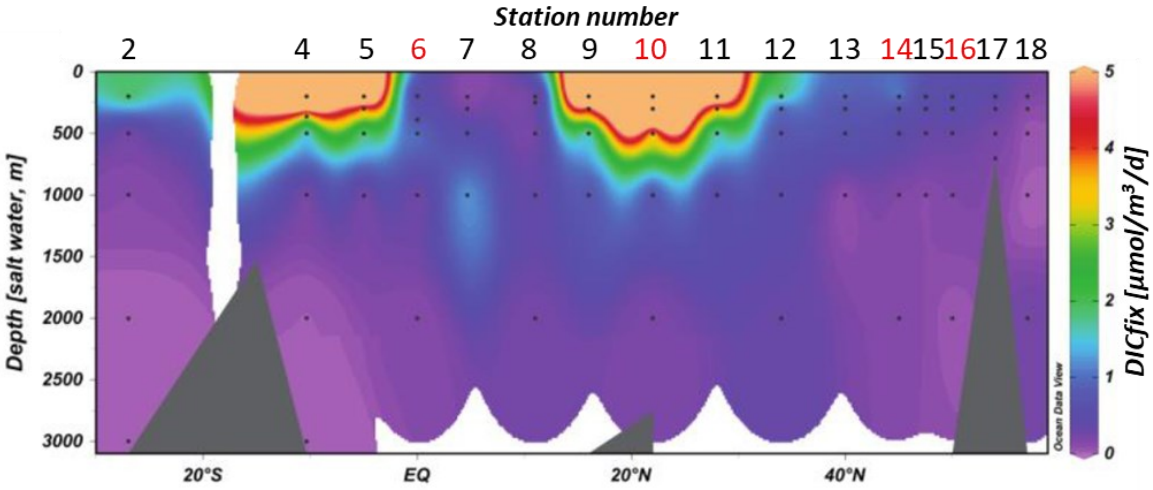

Figure 9: Bulk DIC fixation rate in the Pacific Ocean during SONNE 248 cruise. Contour plot of DIC fixation rates shows the DIC fixation rate in µmol/m³/d (Simon, 2016). The red marked station numbers are the stations analysed in this work.

3.2.3 Abundance of total Prokaryotes and *Alteromonas*

The abundances were determined at the end of the assay with either ^{14}C-bicarbonate or ^{3}H-leucine. The prokaryotic abundance at all stations decreased with depth, ranging from 10^5 cells/mL in the mesopelagic to approximately 2×10^4 cells/mL in the bathypelagic zone, as observed in both DIC and leucine samples. *Alteromonas* abundance is again similar in DIC and leucine samples decreasing with depth from 5×10^2 cell/mL to 10^2 cells/mL except for DIC samples at 200 m in Station 10 and RadProf samples at 1000 m depth, where *Alteromonas* abundance unexpectedly increased to over 10^4 cells/mL (Figure 10).

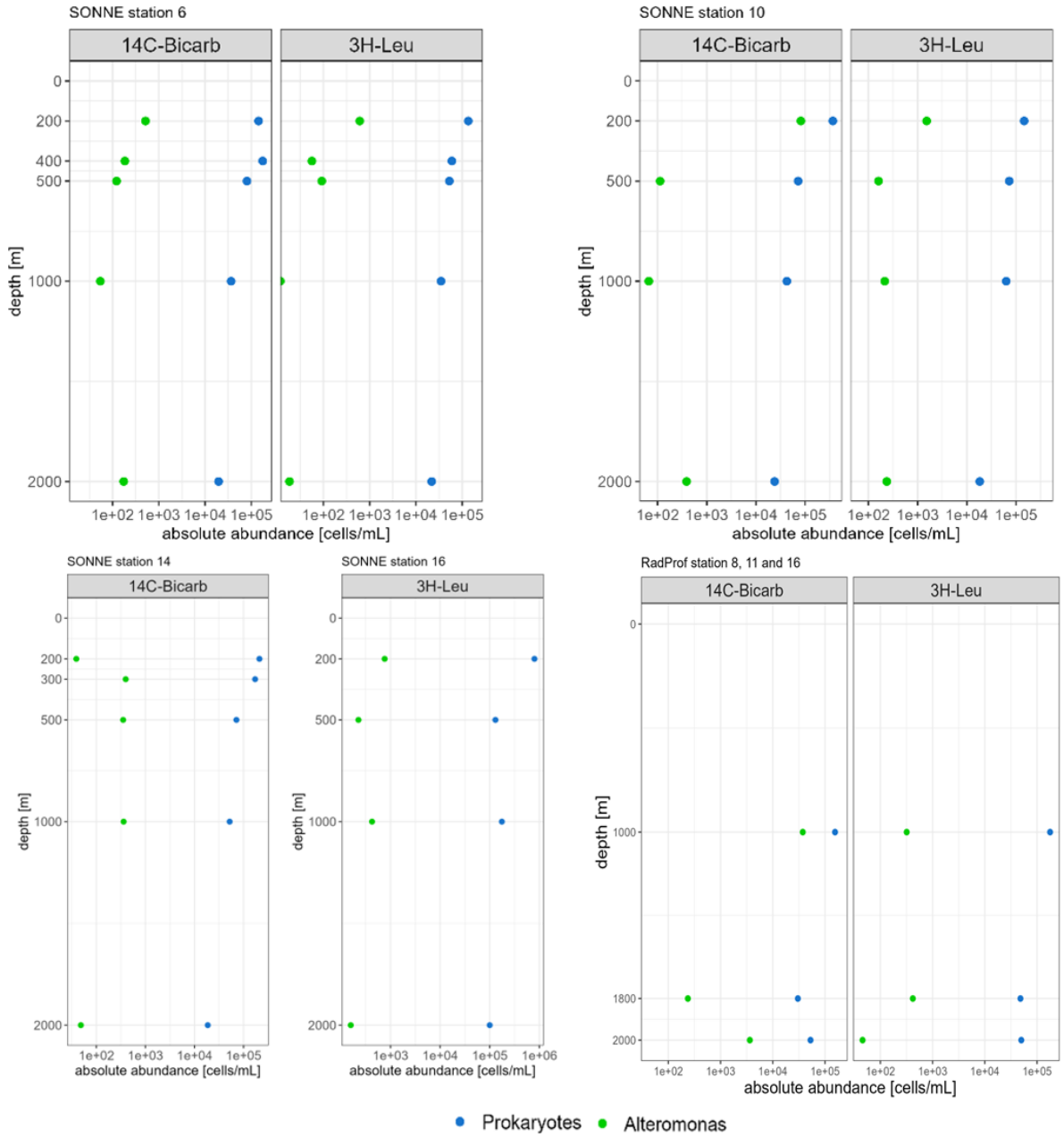

Figure 10: Overview of total prokaryotic and *Alteromonas*' abundance. The plots show the abundance of prokaryotes (blue) and *Alteromonas* (green) for either ^{14}C-bicarbonate or ^{3}H-leucine assay for each station.

3.2.4 Relative abundance of *Alteromonas*

Generally, the relative abundance of *Alteromonas* to the total prokaryotic community was typically below 1 % for both leucine and DIC samples. However, there are two exceptions in the DIC samples where *Alteromonas* compromise more than 20 % of prokaryotic community (Figure 11, Appendix B).

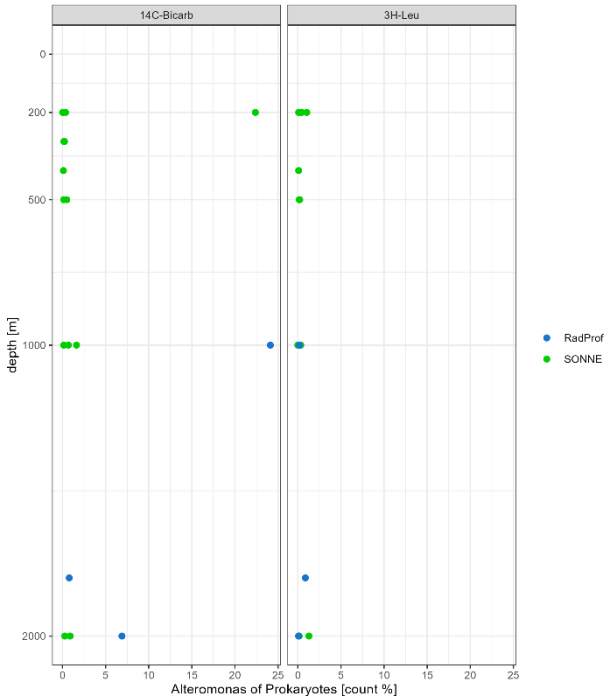

Figure 11: Proportion of *Alteromonas* in the prokaryotic community. The plot displays the contribution of *Alteromonas* in the prokaryotic community in count % of ^{14}C-bicarbonate inoculated samples (left) and ^{3}H-leucine inoculated samples (right) from the RadProf cruise (blue) and SONNE cruise (green).

3.2.5 Abundance of *Alteromonas* taking up DIC and leucine

While *Alteromonas* were present in almost all samples, DIC positive *Alteromonas* were not found in more than half of the samples. However, when we found DIC positive *Alteromonas*, they accounted for a significant proportion of DIC positive prokaryotes, ranging from 5 % to 38 % in the Pacific samples, and to a maximum of 75 % found in the RadProf sample at 2000 m depth (Figure 12a). Around 10 – 20 % of the *Alteromonas* were DIC fixing positive in the Pacific samples, and 10 – >80 % in the Atlantic (except in the samples where no DIC positive *Alteromonas* were found). *Alteromonas* taking up leucine contributed approximately 1 % of the prokaryotic cells incorporating leucine (Figure 12(a), Appendix B). The proportion of leucine positive *Alteromonas* varied from 20 % to 100 % if leucine positive *Alteromonas* were present in the samples (Figure 12 (b), Appendix B).

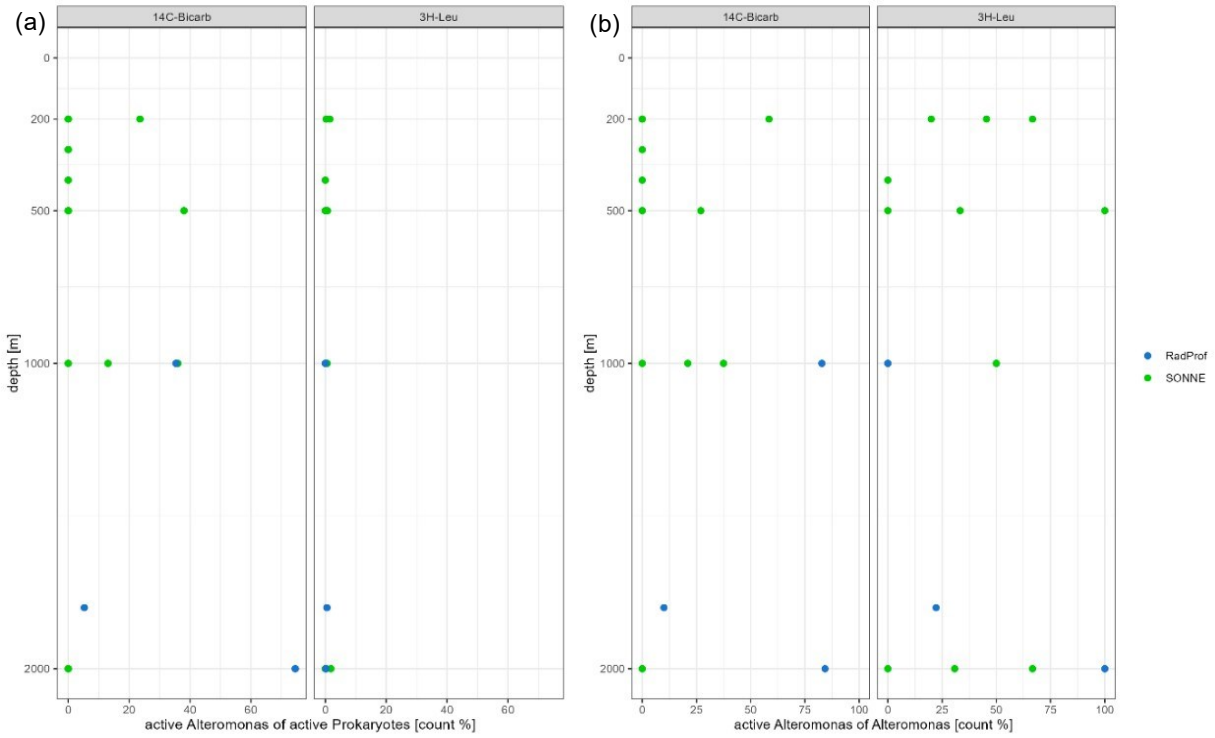

Figure 12: Proportion of active *Alteromonas*. The proportion of active *Alteromonas* in active prokaryotes is given in (a) for ^{14}C-bicarbonate inoculated samples (left) and ^{3}H-leucine inoculated samples (right) of RadProf cruise (blue) and SONNE cruise (green). (b) shows the proportion of active *Alteromonas* in *Alteromonas* found for ^{14}C-bicarbonate inoculated samples (left) and ^{3}H-leucine inoculated samples (right) of RadProf cruise (blue) and SONNE cruise (green)

3.2.6 Cell-specific activity of *Alteromonas*

Figure 13 shows cell-specific activities of prokaryotes and *Alteromonas* in the different samples. The distribution of the DIC positive cells represented mostly a bell-shaped pattern. The majority of cells exhibited medium activity levels (>0.05 to 0.148 fmolC/cell/h [>-2.7 to -1.37 log fmolC/cell/h]), while less cells exhibited relatively low (0.004 to 0.05 fmolC/cell/h [-3.36 to -2.7 log fmolC/cell/h]) and high (>0.148 to 0.198 fmolC/cell/h [>-1.37 to -0.07 log fmolC/cell/h]) activity levels. *Alteromonas* incorporating DIC showed a similar pattern to the total prokaryotes, exhibiting the majority of DIC positive *Alteromonas* a medium activity range of >0.1 to 0.115 fmolC/cell/h [>-2.03 to -1.81 log fmol C/cell/h]. DIC positive *Alteromonas* reached maximum proportions of 18.18 % and 9.45 % of prokaryotic DIC fixation upper medium activity classes of >0.1 to 0.132 fmolC/cell/h [>-2.03 to -1.59 log fmolC/cell/h] (Figure 13 (a), (b), (c), (d)). In comparison to DIC fixation, *Alteromonas* contributed far less to the total leucine incorporation (Figure 12 (a)). This trend was also observed in the single cell activity distributions. In each activity class, *Alteromonas* contribute a maximum of 0.52 % to the active prokaryotes (Figure 13 (e), (f), (g), (h)).

Altogether, *Alteromonas* played a notable role in DIC fixation, particularly in the bathypelagic zone of the coastal Atlantic waters west of the Iberian Peninsula, where DIC positive *Alteromonas* represented a substantial percentage of total DIC positive prokaryotes across all single cell activity classes (Figure 13). In contrast, they generally played a minor role in leucine uptake; however, when *Alteromonas* incorporated leucine, they exhibited medium to high single cell activity in both the mesopelagic and bathypelagic zones, with a concentration of activity in the upper half of the activity classes (Figure 13 (e), (f), (g), (h)). The median of the cell-specific activity distributions is provided in Appendix C.

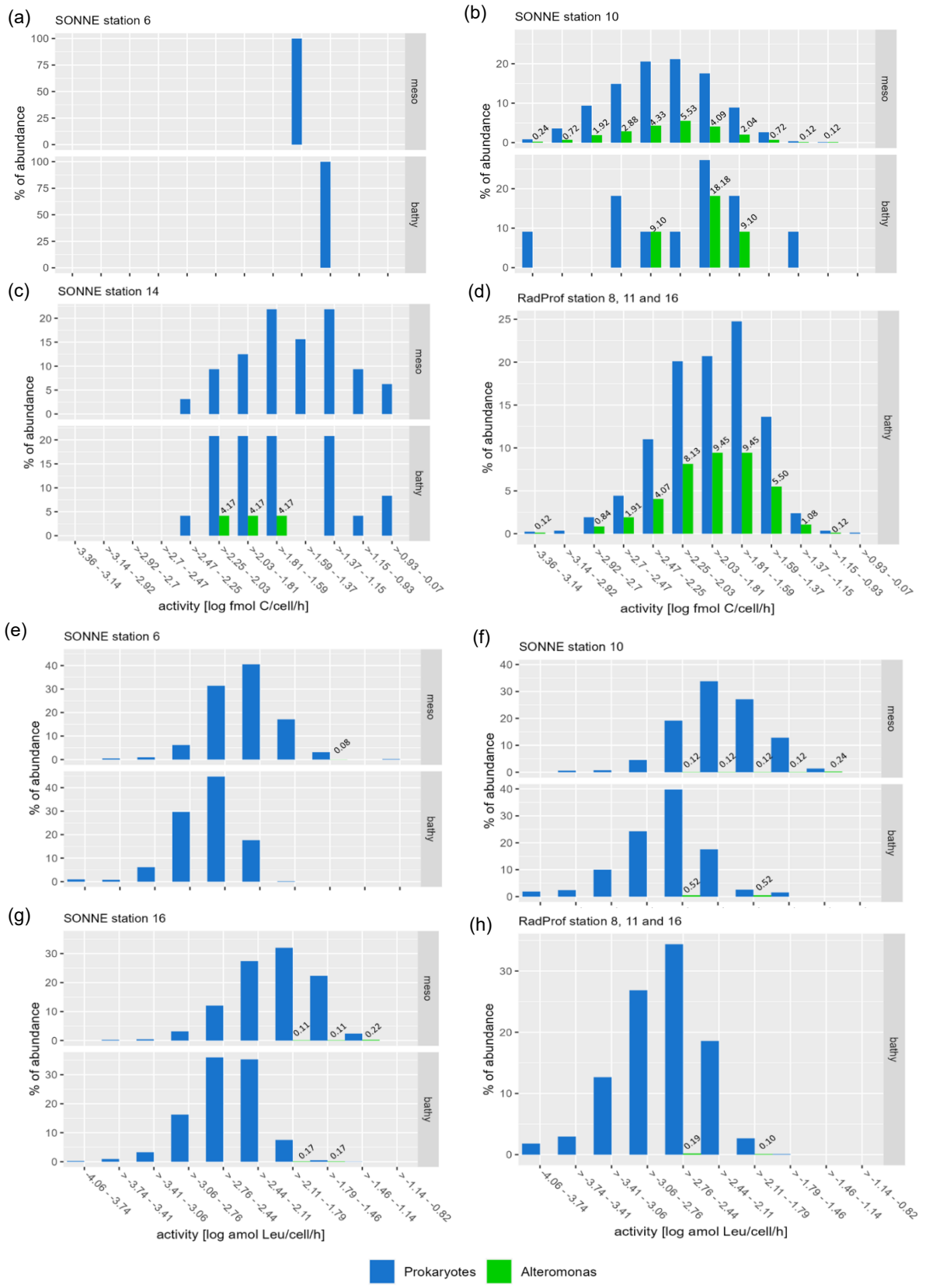

Figure 13: Histograms of the bacterial abundance in single cell activities classes for ^{14}C-bicarbonate (a-d) and ^{3}H-leucine (e-h). The plots show the distribution of prokaryotic (blue) and *Alteromonas* (green) abundance in % in single cell activity classes in log fmol C/cell/h for (a) SONNE station 6, (b) SONNE station 10, (c) SONNE station 14 and (d) RadProf station 8, 11 and 16 as well as in log amol Leu/cell/h for (e) SONNE station 6, (f) SONNE station 10, (g) SONNE station 16 and (h) RadProf station 8, 11 and 16. Relative abundance of *Alteromonas* is highlighted by the values on top of the corresponding bar in %.

3.2.7 Contribution of *Alteromonas* taking up DIC to the total DIC uptake

An overall estimation of the oceanic dark DIC fixation by *Alteromonas* can be formulated based on the analysed environmental data. Our results indicate that *Alteromonas* contributed 6.2 ±14 % (mean ± SD, n = 14) to the oceanic dark DIC fixation by prokaryotes, whereas leucine incorporation by *Alteromonas* contributed only 1.2 % ±1 % (mean ± SD, n = 13) to that by total prokaryotes. Bicarbonate positive *Alteromonas* contributed on average more to total DIC assimilating prokaryotes (6.7 ±13 %) than leucine incorporating *Alteromonas* to total prokaryotes taking up leucine (0.4 ±0.5 %) (Table 5).

Table 5: Relative contribution of *Alteromonas*' DIC fixation and leucine incorporation to total prokaryotic DIC and leucine uptake. The table provides the relative contribution of DIC fixation by *Alteromonas* and their proportion in active prokaryotic community in %, as well as the contribution of leucine incorporation by *Alteromonas* and their proportion in the active prokaryotic community in % for each station and depth analysed. The means and standard deviations (SD) are given below, calculated without the greyish outliers which were excluded by the difference of leucine active prokaryotic cells and DIC active prokaryotic cells.

		^{14}C-Bicarbonate		**^{3}H-Leucine**	
station	**Depth [m]**	**DIC fixation [%]**	**Proportion in active prokaryotes [%]**	**Leucine incorporation [%]**	**Proportion in active prokaryotes [%]**
SO st. 6	200	0.00	0.00	1.28	0.35
SO st. 6	400	0.00	0.00	0.00	0.00
SO st. 6	500	0.00	0.00	0.00	0.00
SO st. 6	1000	0.00	0.00	0.00	0.00
SO st. 6	2000	0.00	0.00	0.00	0.00
SO st. 10	200	24.91	22.91	3.31	1.44
SO st. 10	500	0.00	0.00	0.25	0.21
SO st. 10	1000	42.33	37.50	0.42	0.56
SO st. 10	2000	36.98	36.36	5.36	1.79
SO st. 14	200	0.00	0.00		
SO st. 14	300	0.00	0.00		
SO st. 14	500	0.00	0.00		
SO st. 14	1000	5.03	13.64		
SO st. 14	2000	0.00	0.00		
SO st. 16	200			1.19	0.32
SO st. 16	500			3.19	0.68
SO st. 16	1000			1.67	0.50
SO st. 16	2000			1.25	0.25
Rad st. 8	1000	31.77	35.78	0.00	0.00
Rad st. 11	1800	2.91	5.56	1.26	0.54
Rad st. 16	2000	82.79	74.38	0.24	0.22
	Mean	**6.23**	6.65	**1.22**	0.40
	SD	±14.28	±13.38	±1.55	±0.48

4 Discussion

Thus far, it is very well established that photo- and chemoautotrophs are responsible for the production of organic carbon via the six known carbon fixation pathways based on autotrophic carboxylases (Erb, 2011). However, recent studies revealed that heterotrophic bacteria fixing CO_2 via anaplerosis could not be neglected in the dark ocean DIC fixation (Alonso-Sáez et al., 2010; Baltar et al., 2016; Baltar & Herndl, 2019; Teira et al., 2012). In this study, we expand prior work by quantifying the anaplerotic dark DIC fixation of heterotrophic microbes in the marine environment and providing cell-specific rates to test our hypothesis that anaplerotic pathways are key contributors to oceanic dark DIC fixation. In the lab culture studies, we calculated how much anaplerotic DIC fixation contributes to the *Alteromonas* metabolic rates such as biomass production and respiration to better understand what might influence anaplerotic dark DIC fixation. In the environmental experiments, we determined the contribution of anaplerotic dark DIC fixation by *Alteromonas* to the whole prokaryotic DIC assimilation in the marine environment.

4.1 Effects of temperature and organic carbon concentration on anaplerotic DIC fixation

In the culture experiment we tested the effect of temperature and nutrient concentration on DIC fixation, biomass production and respiration of *Alteromonas mediterranea DE* by inoculating either ^{14}C-bicarbonate or ^{14}C-leucine.

Regarding relative contributions of metabolic rates, overall anaplerotic dark DIC fixation contributed on average 3.1 ±0.4 % to the *Alteromonas'* metabolism. The contribution of bicarbonate assimilation was very stable throughout different temperature and nutrient conditions being perspicuous since the maintenance of anaplerotic processes keeps up the citric acid cycle and therefore the bacterial metabolism (Erb, 2011). The relative contributions of biomass production and respiration to the metabolic rates were not influenced by temperature change but by the availability of organic matter e. g., biomass production increased while respiration decreased under low organic carbon supply (see Table 4).

Regarding cell-specific activities, our results showed that cell-specific anaplerotic DIC fixation was 2.5× higher after a 10°C increase in temperature. In contrast, there was no general trend in the cell-specific DIC fixation under the different nutrient conditions: we observed a significant increase of cell-specific DIC fixation rates at higher nutrient concentrations at 23°C (0.24 ±0.01 fmol C/cell/h to 0.30 ± 0.03 fmol C/cell/h), but this was not the case at 13°C. Previous studies are contradicting. They have found an increase of dark anaplerotic DIC fixation under low (Alonso-Sáez et al., 2010; Merlin et al., 2003) as well as under enriched nutrient conditions (Baltar et al., 2016; Miltner et al., 2005). Since anaplerosis replaces intermediates which were depleted for biosynthesis in the citric acid cycle, it might be plausible that anaplerotic reactions could be enhanced during prokaryotic growth and biomass production that are facilitated by the availability of organic matter (Baltar et al., 2016; Miltner et al., 2005). Another aspect is that anaplerotic dark DIC fixation increases in oligotrophic marine environments to compensate the metabolic imbalances (González et al., 2008; Palovaara et al., 2014). From our results, we

infer that (i) the effect of temperature on dark anaplerotic DIC fixation might be more influential than the quantity of organic carbon supply; but under warm conditions with available organic matter, anaplerotic dark DIC fixation might become more important. However, to disentangle these contradicting findings further work is still needed.

Besides substrate quantity, the type of substrate can also affect anaplerotic DIC fixation. Experiments with *Bacillus subtilis* showed a higher inorganic carbon assimilation for growth on glucose than on lactate or malate (Spona-Friedl et al., 2020). Indeed, extended experiments within our culture studies (not included in this thesis) confirm that DIC fixation is higher for *Alteromonas mediterranea DE* growing on glucose than on alanin (unpublished data).

4.2 Cell-specific anaplerotic dark DIC fixation rates by *Alteromonas*

The single cell DIC fixation rates observed in the culture, are higher than the ones observed in the environmental samples. The average cell-specific bicarbonate assimilation at 13°C and 50 µM C of glucose concentration (0.12 ± 0.01 fmol C/cell/h) was higher than the maximum cell-specific DIC fixation observed in the environmental samples (0.07 fmol C/cell/h [-1.14 log fmol C/cell/h] [Figure 13]). These findings prove that (ii) *Alteromonas* in the natural environment do not reach their potential anaplerotic dark DIC fixation rates as has been observed during culturing under comparable conditions. In pure culture, the organisms face no interspecific competition as in natural community and in our case were provided with all relevant trace metals which might be limited in the marine environment. These effects as well as the fact that the cell-specific DIC fixation rates of our culture studies were obtained from mid-exponential phase, led to artificially high metabolic rates relative to in situ. However, the cell-specific bicarbonate incorporation rates by *Alteromonas* observed in the environmental studies of this work (0.0005-0.074 fmol C/cell/h) are similar to DIC fixation rates of other *Alteromonas* species as well as of other microorganisms like archaea. For example, Yakimov et al. (2014) has reported DIC assimilation rates by *Alteromonas macleodii MVD1* of 1.7 - 2.1 fg C/cell/h (0.006 - 0.007 fmol C/cell/h) in the hadal zone of the Mediterranean Sea and Herndl et al. (2005) provides a mean inorganic carbon fixation rate of 0.014 fmol C/archeon/day in the deep Atlantic Ocean.

Focusing on cell-specific activity, we could observe variations with the depths of the Pacific samples as well as between Pacific and Atlantic. In general, we found few *Alteromonas* <1 % within the prokaryotic community (Figure 11) and even less *Alteromonas* fixing DIC being 0 % in half of the samples (Figure 12a). This might be because of the low (0 – 1 µmol C/m³/d) bulk DIC fixation rate for the Pacific samples except of the mesopelagic realm at Station 10 (Figure 9). On the one hand, the Pacific samples showed slightly more anaplerotic DIC fixation by *Alteromonas* in the bathypelagic than in the mesopelagic realm. In the bathypelagic at Station 10 and 14, *Alteromonas* had a median cell-specific bicarbonate assimilation of 0.009 and 0.014 fmol C/cell/h [-1.86 and -2.03 log fmol C/cell/h], respectively. At Station 10, where the bulk DIC fixation rate was one of the highest with (4 – 5 µmol C/m³/d [Figure 9]), the single cell activity of DIC fixing *Alteromonas* in the mesopelagic zone (median of 0.007 fmol C/cell/h [-2.18 log fmol C/cell/h]) was lower than in the bathypelagic. These findings are in good agreement with prior studies suggesting an increase in dark DIC fixation in nutrient depleted environments (Alonso-Sáez et al., 2010; Merlin et al., 2003). Comparing the bathypelagic waters of the

Pacific and Atlantic, on the other hand, a higher dark DIC fixation was found in the Atlantic samples under increased nutrient supply indicated by higher Chl-a concentrations (0.08-0.1 mg/m³ [Figure 8]). Typically, waters close to the coast, such as the samples taken from waters off the Iberian Peninsula, are enriched with nutrients coming from inputs from the margins (Bauer & Druffel, 1998; Hansell & Carlson, 2014). The Atlantic samples had the highest *Alteromonas* abundances with 10,000 cells/mL (Figure 10) as well as the highest proportions of DIC fixing *Alteromonas* to DIC fixing prokaryotes with 74 % (Table 5). *Alteromonas* contributed almost half to the DIC fixation in each activity class (Figure 13d) with a median cell-specific activity of 0.012 fmol C/cell/h [-1.90 log fmol C/cell/h] in the Atlantic. More *Alteromonas* did fix DIC in the Atlantic samples as compared to the bathypelagic samples in the Pacific what supports the assumption that dark DIC fixation is enhanced by increased nutrient supply (Baltar et al., 2016; Miltner et al., 2005). Recapitulating these findings, (iii) *Alteromonas* might assimilate more DIC via anaplerosis in the bathypelagic than in the mesopelagic realm and the fixation could be enhanced in coastal waters. However, more analysis would be needed to confirm this.

4.3 Contribution of anaplerotic DIC fixation by *Alteromonas* in the ocean

The contribution of anaplerotic dark DIC fixation by *Alteromonas* to the prokaryotic dark DIC fixation varied strongly across the sampling locations. More than half of the samples showed 0 % bicarbonate assimilation by *Alteromonas* while dark DIC fixation by *Alteromonas* contributed 3 – 42 % of total prokaryotic dark DIC fixation in the other samples especially in the bathypelagic zone (Table 5). Overall, (iv) anaplerotic dark DIC fixation by *Alteromonas* contributed on average 6.2 ±14.3 % to the total prokaryotic dark DIC fixation. Braun et al. (2021) assumed that anaplerotic DIC fixation accounts for at least 1 – 5 % of the heterotrophic biomass, and Baltar & Herndl (2019) suggested that oceanic primary production would increase by 5 – 22 % if dark DIC fixation is included. Collectively, it shows that anaplerotic pathways might be key contributors to oceanic dark DIC fixation providing newly synthesized organic carbon in an environment typically limited by organic matter. This could provide additional insights to resolve the imbalance between heterotrophic carbon demand and sinking particulate carbon supply in the deep sea (Baltar et al., 2009, 2010). Although our results provide single cell anaplerotic dark DIC fixation rates in culture and natural communities and assessed the contribution of anaplerotic pathways to the marine dark inorganic carbon fixation, more than half of our environmental samples showed no DIC fixation by *Alteromonas*, suggesting that further work is required to achieve more representative results.

5 Conclusion

Baltar & Herndl (2019) estimated that 1.2 – 11 Pg C/yr (2.5 – 22 % of total annual primary production) are synthesized by dark DIC fixation including chemoautotrophy and anaplerotic reactions, but it is unknown how much anaplerotic rates contribute. Our results allow the first assessment of organic carbon produced via anaplerosis in the marine dark environment. *Alteromonas* contributed 6.2 ±14.3 % to the total dark DIC fixation, indicating that 0.075 – 0.685 Pg C/yr are synthesized by anaplerotic reactions only by *Alteromonas*. This amount is in the same order to the carbon flux by chemoautotrophs below 200 m (0.11 Pg C/yr) (Baltar & Herndl, 2019). Considering that also other bacterial organisms like e.g. *Oceanospirillales* have the ability to fix a non-negligible quantity of inorganic carbon (DeLorenzo et al., 2012), anaplerotic dark DIC fixation is potentially higher than our estimation. Collectively, our results suggest that anaplerotic reactions are key contributors to the marine dark DIC fixation and should therefore be considered in the marine carbon cycle. Additionally, this so far unconsidered source of organic carbon in the dark ocean might resolve the mismatch between heterotrophic carbon demand and particulate organic carbon supply in the deep sea (Baltar et al., 2009; Baltar & Herndl, 2019). Regarding the enhancing effect of temperature on anaplerotic dark DIC fixation derived from our culture studies, it could be expected that the contribution of anaplerotic dark DIC fixation to the marine carbon flux might increase with ocean warming. Further investigations are needed to lighten the role of anaplerotic dark DIC fixation in the marine carbon cycle.

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Appendix

Appendix A: Cell-specific carbon uptake and release for biomass production, respiration and DIC fixation. The table presents the measurements of the experiment, where ^{14}C-leucine and ^{14}C-bicarbonate were inoculated in *Alteromonas* culture at different assays. The results show the biomass production, respiration and DIC fixation on cell-specific level in fmol C/cell/h for each setting. The mean and the standard deviation (sd) were calculated.

Experiment setting	**^{14}C-Leu biomass production [fmol C/cell/h]**	**^{14}C-Leu respiration [fmol C/cell/h]**	**^{14}C-Bicarb DIC fixation [fmol C/cell/h]**
13°C, 50 µM C in Gluc	3.10	0.57	0.13
	2.79	0.45	0.12
	2.91	0.42	0.12
Mean	2.93	0.48	0.12
sd	± 0.16	± 0.08	± 0.01
13°C, 500 µM C in Gluc	3.65	0.60	0.12
	4.03	0.63	0.13
	3.72	0.66	0.12
Mean	3.80	0.63	0.12
sd	± 0.20	± 0.03	± 0.01
23°C, 50 µM C in Gluc	4.96	1.49	0.23
	5.58	1.66	0.24
	6.02	2.02	0.24
Mean	5.52	1.73	0.24
sd	± 0.54	± 0.24	± 0.01
23°C, 500 µM C in Gluc	6.60	1.72	0.32
	7.23	1.86	0.27
	8.04	2.67	0.32
Mean	7.29	2.08	0.30
sd	± 0.72	± 0.51	± 0.03

Appendix B: *Alteromonas* proportions. The table provides the proportions of *Alteromonas (Alt.)* in prokaryotes (Prok.), active *Alteromonas* in active prokaryotes and active *Alteromonas* in *Alteromonas* in count % for ^{14}C-bicarbonate and ^{3}H-leucine.

station	Depth [m]	Alt. of Prok. [count %]		Active Alt. of active Prok. [count %]		Active Alt. of Alt [count %]	
		^{14}C-Bicarbonate	^{3}H-Leucine	^{14}C-Bicarbonate	^{3}H-Leucine	^{14}C-Bicarbonate	^{3}H-Leucine
SO st. 6	200	0.36	0.45	0.00	0.35	0.00	20.00
SO st. 6	400	0.10	0.09	0.00	0.00	0.00	0.00
SO st. 6	500	0.15	0.17	0.00	0.00	0.00	0.00
SO st. 6	1000	0.15	0.00	0.00	0.00	0.00	0.00
SO st. 6	2000	0.89	0.08	0.00	0.00	0.00	0.00
SO st. 10	200	22.37	1.04	23.57	1.43	58.43	45.45
SO st. 10	500	0.16	0.22	0.00	0.21	0.00	33.33
SO st. 10	1000	0.16	0.34	38.00	0.56	27.00	50.00
SO st. 10	2000	1.63	1.29	36.00	1.80	21.00	30.77
SO st. 14	200	0.02		0.00		0.00	
SO st. 14	300	0.23		0.00		0.00	
SO st. 14	500	0.49		0.00		0.00	
SO st. 14	1000	0.69		13.04		37.50	
SO st. 14	2000	0.26		0.00		0.00	
SO st. 16	200		0.10		0.32		66.67
SO st. 16	500		0.17		0.68		100.00
SO st. 16	1000		0.24		0.50		50.00
SO st. 16	2000		0.16		0.25		66.67
Rad st. 8	1000	24.11	0.18	35.40	0.00	82.83	0.00
Rad st. 11	1800	0.78	0.87	5.26	0.53	10.00	22.22
Rad st. 16	2000	6.89	0.09	74.59	0.22	84.26	100.00

Appendix C: Medians of cell-specific incorporation rates of bicarbonate and leucine per station and pelagic zone. The table provides the medians of the cell-specific ^{14}C-bicarbonate and ^{3}H-leucine activity for prokaryotes and *Alteromonas* in log fmol C/cell/h and log amol C/cell/h and n being the number of cases.

		^{14}C-Bicarbonate				**^{3}H-Leucine**			
		Prokaryotes		***Alteromonas***		**Prokaryotes**		***Alteromonas***	
Station	**realm**	**Median activity [log fmol C/cell/h]**	**n**	**Median activity [log fmol C/cell/h]**	**n**	**Median activity [log amol Leu/cell/h]**	**n**	**Median activity [log amol Leu/cell/h]**	**n**
SO st.6	meso	-1.42	3	0.00	0	-2.36	1278	-1.50	1
	bathy	-0.66	2	0.00	0	-2.68	539	0	0
SO st.10	meso	-2.24	832	-2.18	189	-2.20	820	-1.71	6
	bathy	-1.91	11	-1.86	4	-2.67	581	-2.21	6
SO st.14	meso	-1.53	32	0.00	0				
	bathy	-1.75	24	-2.03	3				
SO st.16	meso					-2.05	913	-1.46	4
	bathy					-2.48	1201	-1.80	4
Rad st.8, 11, 16	bathy	-1.90	836	-1.90	340	-2.70	1050	-2.45	3

Appendix D: P-values of the two-way ANOVA significance test. The table provides the p-values for each comparison for biomass production, respiration and DIC fixation.

comparisons	^{14}C-Leu biomass production	^{14}C-Leu respiration	^{14}C-Bicarb DIC fixation
13°C, 50 µM C in Gluc - 13°C, 500 µM C in Gluc	0.1809	0.9194	0.9965
23°C, 50 µM C in Gluc - 13°C, 50 µM C in Gluc	0.0006	0.0035	0.0001
23°C, 500 µM C in Gluc - 13°C, 500 µM C in Gluc	0.0001	0.0013	<0.0001
23°C, 50 µM C in Gluc - 23°C, 500 µM C in Gluc	0.0071	0.4772	0.0025

Abstract

While photoautotrophic phytoplankton and chemoautotrophs are known to contribute significantly to the production of organic carbon by assimilating CO_2, it was found that heterotrophic bacteria fixing dissolved inorganic carbon (DIC) via anaplerosis do contribute to the formation of organic carbon in the ocean as well. In this study, we expand prior work by quantifying the anaplerotic dark DIC fixation of *Alteromonas* in the marine environment and providing cell-specific rates to test our hypothesis that anaplerotic pathways are key contributors to oceanic dark DIC fixation. Environmental samples from the Pacific and Atlantic Ocean were analysed using MICRO-CARD-FISH. Additionally, we performed culture studies with *Alteromonas mediterranae DE* incorporating ^{14}C-bicarbonate and ^{14}C-leucine under different temperature and nutrient conditions to identify the impact on anaplerotic DIC fixation. In the culture part, it was observed that DIC fixation via anaplerosis contributed on average 3.1 ±0.4 % to the *Alteromonas* metabolism being stable under changing conditions. On cell-specific level, temperature was more influential than organic carbon supply. In the environment, an overall contribution of 6.2 ±14.3 % of anaplerotic dark DIC fixation by *Alteromonas* to the total prokaryotic dark DIC fixation in the dark ocean was observed. It shows that anaplerotic pathways might be key contributors to oceanic dark DIC fixation providing newly synthesized organic carbon in an environment typically limited by organic matter.

Zusammenfassung

Es ist bekannt, dass photoautotrophes Phytoplankton und chemoautotrophe Organismen durch die Aufnahme von CO_2 einen signifikanten Beitrag zur Bildung von organischem Kohlenstoff leisten. Es wurde jedoch festgestellt, dass heterotrophe Bakterien ebenfalls zur Bildung von organischem Kohlenstoff im Ozean beitragen, indem sie über anaplerotische Reaktionen gelösten anorganischen Kohlenstoff (DIC) binden. Mit dieser Arbeit wird bisheriges Wissen erweitert, indem wir die anaplerotische dunkle DIC-Fixierung von *Alteromonas* im Ozean quantifizieren und zellspezifische Raten liefern. Damit testen wir unsere Hypothese, dass anaplerotische Reaktionen von wesentlicher Bedeutung in der marinen dunklen DIC-Fixierung sind. Dazu wurden Umweltproben aus dem Pazifik und dem Atlantik mittels MICRO-CARD-FISH analysiert. Zusätzlich wurden Kultivierungsversuche durchgeführt, bei denen *Alteromonas mediterranae DE* in ^{14}C-Bicarbonat und ^{14}C-Leucin angereichertem Substrat unter verschiedenen Temperatur- und Nährstoffbedingungen kultiviert wurden. Dabei wurden Stoffwechselraten und der Einfluss von Temperatur und Nährstoffkonzentration auf die anaplerotische DIC-Fixierung ermittelt. Bei den Kultivierungsversuchen wurde beobachtet, dass die DIC-Fixierung durch Anaplerose durchschnittlich einen Anteil von 3,1 ±0,4 % am gesamten Stoffwechsel von Alteromonas hatte und unter wechselnden Bedingungen stabil war. Auf zellspezifischer Ebene hatte Temperatur mehr Einfluss auf die Stoffwechselvorgänge DIC-Fixierung, Biomasseproduktion und Respiration als die Nährstoffkonzentration. Die Umweltanalysen zeigten, dass die anaplerotische dunkle DIC-Fixierung von *Alteromonas* an der gesamten prokaryotischen dunklen DIC-Fixierung im dunklen Ozean einen Gesamtanteil von 6,2 ±14,3 % hat. Dies beweist, dass anaplerotische Reaktionen wesentlich zur dunklen DIC-Fixierung im Ozean beitragen könnten und somit eine wichtige Quelle für neu synthetisierten organischen Kohlenstoff sind, der in der Tiefsee typischerweise limitiert ist.