universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Antioxidant properties of marine dissolved organic matter produced by marine phytoplankton"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfillment of the requirements for the degree of

Master of Science (MSc)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 833
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Ecology and Ecosystems
Betreut von / Supervisor:	Univ.-Prof. Dr. Gerhard J. Herndl

Acknowledgements

I want to thank the entire MOVie working group for their great support, help and advice during my project work. I am very thankful for having the chance to work in such a nice and helpful environment. Thanks to the captain and his crew on RV Thuwal, and also thanks to all the scientists on board. It was a pleasure for me to join this research cruise in the Red Sea organized by KAUST with such a pleasant atmosphere on board. Furthermore, I want to thank Elisa Berdalet and Mara Abad of the Marine Science Institute (CSIC) in Barcelona for the DOC analyses and Michael Schagerl from the Division Limnology for providing space in the algal culture facilities for my experiments. For introducing me to this research topic and guiding me during my project, I want to thank my Co-Supervisor Cristina Romera-Castillo. Special thanks go to Barbara Mähnert for the cell counting and her excellent support and knowledge on phytoplankton cultivation. Many thanks also to my friends and the other Master students of the working group (Christian, Julie, Meriel, Paula and Teresa) for their mental support, for their encouragement and of course, for all the funny moments in the office and parties we had. I am especially grateful to my family for supporting me emotionally and financially, for their patience and always believing in me.

Last but not least my dearest thanks go to my supervisor Gerhard J. Herndl. My thesis would not have been possible without him and his expertise. I am very thankful for his trust and advice, the great opportunity to start this research and for providing the opportunity to join the research cruise in the Red Sea, which gave me memorable and fascinating impressions on Biological Oceanography. I am very grateful for an always open door and for his continuous support and guidance throughout my entire working period at the Division Bio-Oceanography.

This research was supported by the Austrian Science Fund (FWF; P28781-B21) project ARTEMIS.

Abstract

Environmental stress affects the biochemical composition of microalgae frequently resulting in the generation of reactive oxygen species (ROS). Free radical scavengers (antioxidants) can protect biological systems against ROS. Phytoplankton can produce antioxidants as a defence against the harmful free radicals and thus are a potential source of antioxidants. Antioxidants are also part of the dissolved organic matter (DOM) in the ocean. Little is known, however, about the antioxidant production and release by marine phytoplankton under different nutrient regimes. In this study, three non-axenic marine phytoplankton species (*Chaetoceros affinis*, *Cylindrotheca fusiformis*, *Picochlorum* sp.) were grown under different N:P ratios. Phytoplankton-derived DOM was extracted from the media using solid phase extraction (SPE) and antioxidant activity of the DOM was measured using the colorimetric DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Additionally, DOM was sampled at different water column depth in the Red Sea to determine its antioxidant activity. DOM collected in the Red Sea as well as DOM produced by phytoplankton cultures exhibited antioxidant activity. Overall, highest antioxidant activity was detected in phytoplankton cultures grown under P-limitation and lowest under N-limitation. The total antioxidant activity in the DOM was similar in the two diatom species (*Chaetoceros affinis*, *Cylindrotheca fusiformis*). In conclusion, the results indicate that antioxidant properties are present in phytoplankton-derived DOM, but nutrient stress does not necessarily lead to enhanced release of antioxidant-DOM in all phytoplankton species.

Introduction

Antioxidants are specific molecules that can protect biological systems against reactive oxygen species (ROS) and free radicals (King et al. 2016; Schlesier et al. 2002). Free radicals are any molecule or atoms containing one or more unpaired electrons, which make them highly reactive (Birben et al. 2012). ROS are produced by living organisms from molecular oxygen as a result of normal cellular metabolism (endogenous source) and in response to environmental stressors such as air pollutants, ultraviolet radiation or heat exposure (exogenous source) (Birben et al. 2012). Once they are formed, they are highly reactive and can start harmful chain reactions of radical production (Assunção et al. 2017; Birben et al. 2012). Antioxidants interact with free radicals by donating one electron to the unpaired free radical molecule, stabilizing it and interrupting the radical chain reaction. They can even prevent the formation of reactive oxidants (Huang et al. 2005).

Thus, antioxidants are produced by living cells as a defence against the harmful free radicals. Some plants are a source of antioxidants, which have been also isolated and commercialized as additives in food and cosmetic (Payum et al. 2015). However, antioxidants have also been found as part of the dissolved organic matter (DOM) in seawater (Romera-Castillo and Jaffé 2015).

DOM constitutes a large organic carbon pool in the ocean and most of the DOM originates either directly from phytoplankton extracellular release or indirectly from grazing on phytoplankton (Biddanda and Benner 1997; Dittmar et al. 2008). In coastal waters, however, the contribution of terrestrial DOM can also be important. A substantial fraction of DOM is composed of polyphenols such as tannins, lignins and flavonoids (Romera-Castillo and Jaffé 2015), which exhibit antioxidant activity (Amarowicz 2007; Dizhbite et al. 2004; Rice-Evans et al. 1996). Although the antioxidant activity of the aquatic DOM has been previously reported (Romera-Castillo and Jaffé 2015), little is known about its sources. Karthikeyan et al. (2013) investigated seven diatom species regarding their antioxidant capacities and found that *Odontella mobiliensis* and *Pleurosigma angulatum* exhibited the highest capacities and can be potentially used as sources of natural antioxidants. Living phytoplankton release DOM into the medium, however, they also contribute as dead organic material to the DOM pool (Thornton 2014). Thus, we hypothesized that the phytoplankton-derived DOM has antioxidant properties.

Phytoplankton growth requires inorganic nitrogen and phosphorus (El-Kassas 2013). While nitrogen is an important element for all proteins in the cells of algae, phosphorus is essential in the metabolic energy pathway in living phytoplankton cells (El-Kassas 2013). The elemental composition of phytoplankton in terms of carbon : nitrogen : phosphorus (C:N:P) centers around 106:16:1 (Redfield 1934), hence essentially the same as the inorganic N:P ratio of 16 in the deep ocean. This Redfield ratio is now broadly used as reference to determine inorganic nutrient limitation of phytoplankton production (Ptacnik et al. 2010). In coastal waters, however, this N:P ratio can differ due to terrestrial nutrient input and the composition of different phytoplankton species (Ptacnik et al. 2010). Thus, the availability of nutrients can affect the growth of phytoplankton and environmental stress conditions. Nutrient limitation will cause a decrease in the reproduction of cells (El-Kassas 2013; Thompson 1996). It has already been shown that nutrient stress exhibits a strong effect on the biochemical composition of microalgae (Goiris et al. 2015) and generates reactive oxygen species (Apel and Hirt 2004), which may change the antioxidant content of the DOM (Goiris et al. 2015). Microalgae can produce carotenoids, ascorbic acid (vitamin C), tocopherols (vitamin E), or phenolic compounds that are considered to be valuable antioxidants (Goiris et al. 2015). Accumulation of carotenoids due to nutrient stress has been observed in *Dunaliella bardawil* (Ben-Amotz and Avron 1983; Goiris et al. 2015). Another microalga, *Dunaliella salina,* shows an increase in ascorbic acid (El-Baky et al. 2004; Goiris et al. 2015) and vitamin E (Durmaz 2007; Goiris et al. 2015) content if limited in nitrogen availability.

The percentage of phytoplankton extracellular release is highest during stationary to senescent phase (Obernosterer and Herndl 1995; Sharp 1977) thus, it is expected that antioxidants are detectable in this phytoplankton growth phase. Another aim of this work was to explore whether phytoplankton release more antioxidants if stressed under different nutrient limitation conditions. We used three phytoplankton species that are abundant in the marine coastal ecosystems: two diatoms (*Chaetoceros affinis* and *Cylindrotheca fusiformis*) and one green alga (*Picochlorum* sp.). Generally, diatoms are ubiquitous in the euphotic layers of the oceans constituting the largest portion of new production (Wetz and Wheeler 2007). Green algae are also known to have high growth rates (El-Kassas 2013). *Chaetoceros affinis* and *Cylindrotheca fusiformis* were found to be limited in their growth under nutrient depletion (Magaletti et al. 2004; Obernosterer and Herndl 1995). El-Kassas (2013) found that *Picochlorum* sp. (Chlorophyta) tolerate high salinity concentrations (halotolerant) and is

responding to nutrient limitation with decreased cell abundance. Generally, in natural environments, nutrient-limited conditions (N- and P-limitations) are common (El-Kassas 2013). Recent studies have shown that phytoplankton contribute to the production of DOM to a large extent (Puddu et al. 2003) and this DOM represents a major marine carbon pool (Thornton 2014). Through exudation, cell lysis due to viruses, grazing and zooplankton sloppy feeding, phytoplankton-derived organic matter is released into the oceanic DOM pool (Myklestad 2000). This DOM provides substrate for heterotrophic bacteria (Azam et al. 1983).

In the present study, antioxidant properties of DOM in the stationary growth phase of three non-axenic marine phytoplankton species (*Chaetoceros affinis*, *Cylindrotheca fusiformis*, *Picochlorum* sp.) grown under different N:P ratios were examined. Additionally, four water column profiles were collected during a research cruise in the Red Sea. The Red Sea harbors one of the warmest deep waters and most saline waters in the world's ocean (Kheireddine et al. 2017). Temperature in the Red Sea varies between 21°C and 28°C in the north and between 26°C and 32°C in the south (Kheireddine et al. 2017; Nandkeolyar et al. 2013). Low precipitation rates, minor input by rivers (Kheireddine et al. 2017; Patzert 1974) and high evaporation rates (Sofianos and Johns 2003) are characteristic for the Red Sea. In the upper layers, nutrients are depleted and thus oligotrophic conditions dominate (Kheireddine et al. 2017). Significant physical variability characterises the northern Red Sea. During the winter, the deep layers are well mixed, while a distinct stratification can be observed during the summer. Pereira et al. (2015) showed that the microalga *Picochlorum* sp., isolated from the Red Sea, produces antioxidants, but thus far antioxidant properties of DOM have never been measured in the Red Sea.

Hence, the goals of this study were: 1) to test whether the DOM released by phytoplankton exhibits antioxidant activity; 2) to determine the extent of species-specific differences in the antioxidant production among dominant phytoplankton species; 3) to examine whether nutrient limitation affects antioxidant production.

Material and Methods

Phytoplankton culture conditions

Two different non-axenic marine diatoms, *Chaetoceros affinis* (CCAP 1010/27, Culture Collection of Algae and Protozoa, Scottish Marine Institute), *Cylindrotheca fusiformis* (CCAP 1017/2, Culture Collection of Algae and Protozoa, Scottish Marine Institute), and one non-

axenic marine green alga *Picochlorum* sp. (Institut Ruđer Bošković, Martin A. Pfannkuchen), were grown under different N:P ratios. Stock cultures were maintained in an f/2 medium (f/2 + Si Guillard's medium) and provided with new medium every other week. For the experiment, media with varying N:P ratios were prepared and inoculated with the above mentioned phytoplankton species. They were grown in f/2 medium with nutrient conditions under an N:P = 24:1, under nitrogen limited conditions (N:P = 9:1) and under phosphorus limited conditions (N:P = 184:1) (Table 1). All treatments were established in triplicate per species and nutrient condition. The experimental culture bottles were inoculated with 20 ml phytoplankton culture taken from the respective stock cultures at the end of the exponential phase. All media were prepared with sterile artificial seawater. The cultures were grown at 18°C placed on a shaker to simulate constant turbulence. For the cultures, 1 L glass flasks containing 1 L of medium were used and kept at a light:dark cycle of 16:8 h. For illumination, fluorescence lamps with a photosynthetic active radiation (PAR) of 80 µmol m^{-2} s^{-1} were used. The growth phases of phytoplankton were determined via optical density (OD) of the cultures measured spectrophotometrically (Shimadzu UV-VIS Spectrophotometer UV-1800) at 550 nm wavelength every 24 h. An exponential growth phase was assumed when the measured optical density doubled within 24 h, while the stationary growth phase was reached when the optical density remained constant.

Phytoplankton-derived DOM was sampled from the cultures at the end of the experiment by filtering the cultures in their stationary phase. One litre was filtered through pre-combusted (450°C for 6 h) Whatman GF/F glass fibre filters using a glass filtration system and a peristaltic pump. Filtration was conducted after five (N:P = 9:1, N:P = 24:1) and six days (N:P = 184:1) for *Chaetoceros affinis*, after seven (N:P = 9:1, N:P = 24:1) and nine days (N:P = 184:1) for *Cylindrotheca fusiformis* and after seven (N:P = 9:1, N:P = 24:1) and eleven days (N:P = 184:1) for *Picochlorum* sp.. An aliquot of ~20 ml for DOC was collected into 20 ml pre-combusted glass vials, acidified with 2 drops of conc. HCl (37%) and stored at 4°C until analysis.

Table 1: *Initial nutrient concentrations in the different phytoplankton cultures used for the experiments*

NO_3^- (µM)	PO_4^{3-} (µM)	N:P (atomic ratio)
332	36	9
882	36	24
882	4.8	184

Phytoplankton abundance

Samples for cell abundance were taken every 24 h to determine the growth of each algal species. For that, 250 µl of each sample were fixed with Lugol's solution (iodine-potassium iodide solution). Each sample was placed in a cell counting chamber (Fuchs-Rosenthal, volume 3.2 µl, total area of 16 mm^2 and a chamber depth of 0.2 mm). At least 100 phytoplankton cells per sample were counted under the microscope (Zeiss Axio Imager.A1) and the cell number per ml was calculated using the following equation:

$$Z_U = (Z_a * V_f * 1000)/(F_a * \text{chamber depth [mm]})$$

Where Z_U = cells per ml sample; Z_a = counted cells; F_a = counted area; V_f = dilution factor of the sample.

For each species, cells of the N:P = 24:1 treatment were counted and correlated with the optical density (OD) measured at the same time in the culture. Thus, the cell abundance for each phytoplankton species and nutrient condition was calculated and growth curves for each species were compiled.

Bacterial abundance

Bacterial abundance in the phytoplankton cultures was sampled (1 ml) at 24 h intervals. Samples were immediately fixed with glutaraldehyde (0.5% final concentration) at 4°C for 15 min and subsequently frozen at -80°C. Prior to the flow cytometric analyses, samples were brought to room temperature and 0.5 ml were stained with SYBR Green (1 x final concentration) in the dark for 10 min. Bacteria were counted on an Accuri C6 flow cytometer (Becton Dickinson).

Solid phase extraction

Water samples were filtered through Whatman GF/F glass fibre filters and acidified to pH 2 with HCl (25%) (Dittmar et al. 2008). The PPL cartridges (styrene divinyl benzene polymer type sorbents) were rinsed following Dittmar et al. (2008) with four cartridge volumes of methanol and four cartridge volumes of 0.01 M HCl immediately before use (Fig. 1, step 1 and 2). Seawater samples were passed through the cartridges by gravity (Red Sea samples: 5 L per cartridge; culture samples: 1 L per cartridge) and rinsed afterwards with seven cartridge volumes of 0.01 M HCl to remove the salts (Fig. 1, step 3 and 4). The sorbents were dried in a speed vac for 4 h. Solid phase extraction-DOM was eluted with 8 ml methanol into combusted glass vials and stored at -20°C (Fig. 1, step 6).

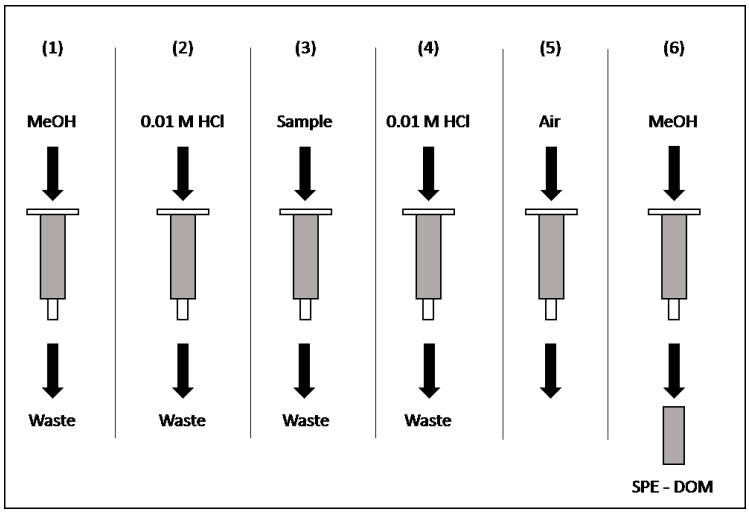

Figure 1: *Scheme for the isolation of solid phase extraction (SPE) – DOM of phytoplankton cultures (from Dangl, 2017)*

An aliquot of the PPL extract (100 µl) was collected for dissolved organic carbon (DOC) analysis. Each aliquot was dried in the speed vac for 2 h and re-dissolved in 10 ml Milli-Q water. Samples for DOC analyses were acidified with conc. HCl (37%) and analysed using a Shimadzu TOC VCSH analyser and standard methods (Dickson et al. 2007).

Determining antioxidant activity

The antioxidant activity of the PPL extracts obtained from the three different phytoplankton species was determined using the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) (DPPH$^{\bullet}$) method following Romera-Castillo and Jaffé (2015). Briefly, a volume of 0.8 ml of a methanol solution DPPH$^{\bullet}$ (7.09 x 10^{-5} M) with an absorbance of 0.8 ± 0.03

measured at 515 nm, was mixed with 0.3 ml of each methanol PPL extract (eluate collected from the cartridge). After the addition of the DPPH• reagent, the samples were shaken and left reacting in the dark and at room temperature (21°C) for 15 min. The decrease in absorbance after the reaction time was measured in a Shimadzu UV-VIS Spectrophotometer UV-1800. Each absorbance measurement was performed in triplicate on five dilutions (Table 2). Absorbance is given as the average of these triplicate measurements. A blank of 0.4 ml of methanol mixed with 0.8 ml of the corresponding free radical solution was also measured at time 0. The absorbance of the methanol extracts was measured as well (0.4 ml of each solution mixed with 0.8 ml of methanol) at time zero. The antioxidant activity (AxA) or quenching percentage is expressed as:

$AxA = [1-(A_A-A_C)/A_B] \times 100$

Where A_A is the absorbance of the antioxidant sample at the end of the reaction time; A_C is the absorbance of the sample blank at t = 0; and A_B is the absorbance of the methanol blank (0.4 ml MeOH + 0.8 ml DPPH• solution) at t = 0.

The results of the antioxidant activity measurements of the DPPH assay were expressed in two ways: 1) as AxA_{total}, the total antioxidant activity of the methanol extract (100 µL aliquot); 2) AxA_{100}, the DOC-normalized antioxidant activity for 100 mg L^{-1} of DOC of the methanol extract determined using a calibration curve.

Table 2: *Dilutions of the sample extracts with methanol to obtain a volume of 0.4 ml plus 0.8 ml DPPH• reagent*

Sample extract [µl]	Methanol [µl]	DPPH• [µl]
300	100	800
200	200	800
150	250	800
100	300	800
75	325	800

Sampling of the water column of the Red Sea

In the Red Sea, sampling was carried out during the *Deep DOC* research cruise aboard the RV Thuwal in April 2017 in collaboration with the Red Sea Research Center, King Abdullah University of Science and Technology (KAUST) in Saudi Arabia (Fig. 2). In total, four stations were sampled: while Station 1 was located near a brine pool, Station 2 was near an oil seep.

Seawater samples from different depths for determining the antioxidant capacity of the DOM were collected with a rosette system (CTD - Conductivity-Temperature-Depth) consisting of 24 Niskin bottles each holding 10 L. Seawater was collected into acid pre-rinsed 5 L carboys and immediately subjected to SPE (solid phase extraction) using PPL cartridges as described above. Seawater was sampled from the surface down to mesopelagic layers at six depths per station. Samples from the epipelagic layers were filtered through pre-combusted (450°C for 6 h) Whatman GF/F glass fibre filters before SPE, whereas deeper layers were processed unfiltered.

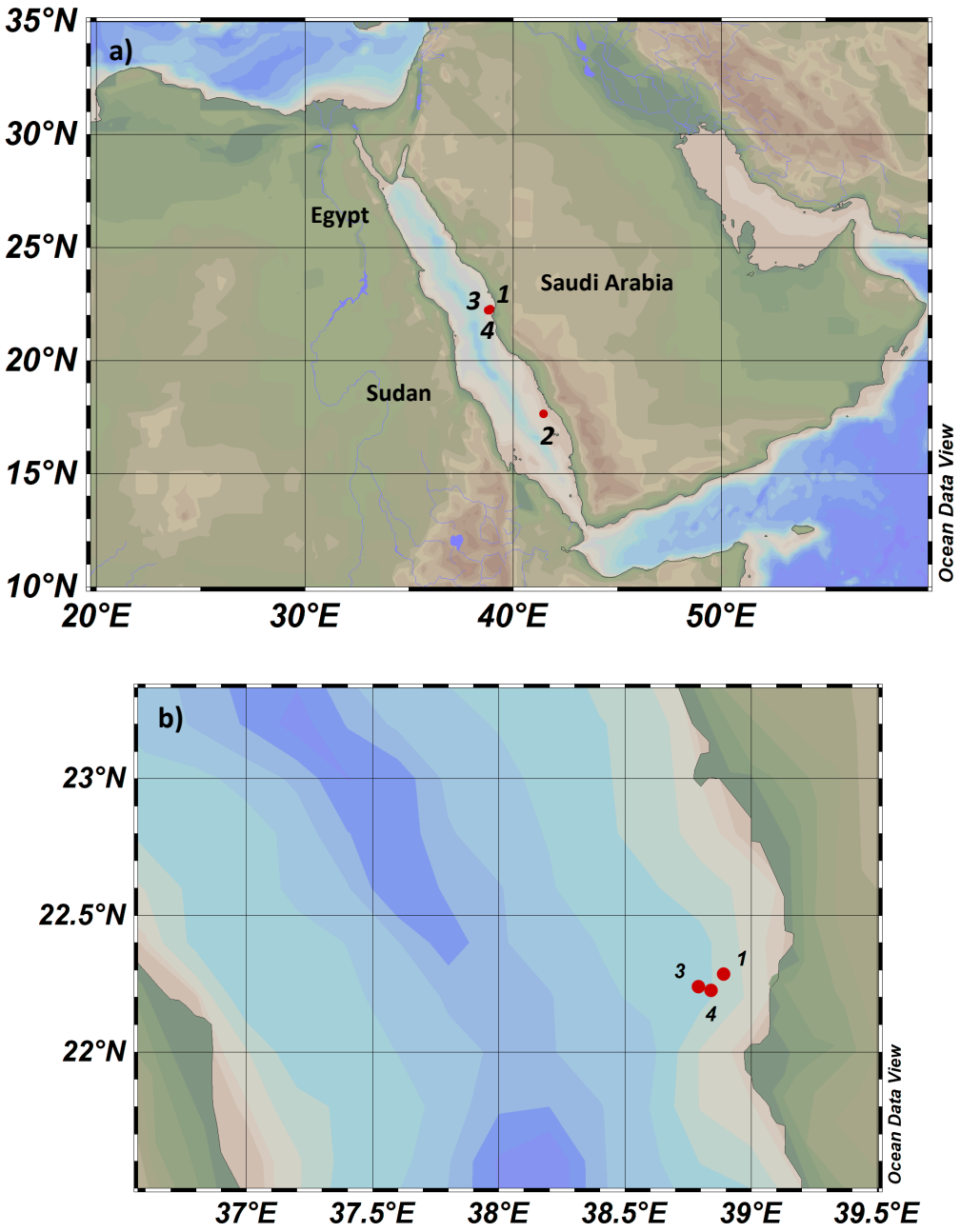

Figure 2: *Map of sampling stations in the Red Sea conducted with RV Thuwal. Overview of the four sampling stations (a). Location of the sampling stations 1, 3 and 4 in greater detail (b)*

Statistical analysis

All experiments were carried out in triplicate and the data are reported as mean ± SD (standard deviation). Two-way analysis of variance (ANOVA) was performed using RStudio (version 0.99.491). *Post hoc* analyses were explained by Tukey's multiple comparisons of means with 95% family-wise confidence level to identify significant differences ($p < 0.05$).

Results

Phytoplankton growth in the culture experiments

The maximum cell abundance was different among the three phytoplankton species (Fig. 3). The lowest abundance was obtained in the *Chaetoceros affinis* cultures (~ 0.3 x 10^4 cells ml^{-1}, Fig. 3a) while the highest abundance was found in *Picochlorum* sp. (~ 50 x 10^4 cells ml^{-1}, Fig. 3c).

In the initial phase, phytoplankton abundance of each species was similar under the different nutrient ratios. At the end of the exponential growth phase, however, differences in phytoplankton abundance became apparent under different nutrient ratios. Cultures with N:P = 9:1 and N:P = 24:1 ratios exhibited shorter growth periods than the cultures grown in N:P = 184:1 independent of the species (Fig. 3).

For all the species studied here, phytoplankton cell abundance at the stationary phase was always higher in P-limited than in N-limited and N:P = 24:1 treatments (Fig. 3). The cell abundance of *Chaetoceros affinis* grown under P-limitation (N:P = 184:1; 2,960 ± 158 cells ml^{-1}) was 1.2 and 1.3 times higher than cultures grown under N-limitation (N:P = 9:1; 2,530 ± 72 cells ml^{-1}) and under balanced nutrient conditions (N:P = 24:1; 2,363 ± 191 cells ml^{-1}), respectively. Furthermore, cell abundance of *Cylindrotheca fusiformis* grown in N:P = 184:1 (10,812 ± 442 cells ml^{-1}) media was about 1.2 times higher than in N:P = 9:1 cultures (9,338 ± 180 cells ml^{-1}) and about 1.1 times higher than in N:P = 24:1 cultures (10,178 ± 536 cells ml^{-1}). For *Picochlorum* sp., a longer growth period was observed for N:P = 184:1 cultures than for the other two nutrient conditions (Fig. 3c). In the exponential growth phase, it already started to differ from the cultures grown in N:P = 9:1 and N:P = 24:1 media, exhibiting lower cell abundance. However, at the end of the exponential growth, cell abundance in the P-limited cultures (N:P = 184:1; 540,314 ± 43,313 cells ml^{-1}) was only about 10% higher than in the N-limited cultures (N:P = 9:1; 491,647 ± 35,346 cells ml^{-1}) and under balanced nutrient conditions (N:P = 24:1; 497,647 ± 10,066 cells ml^{-1}).

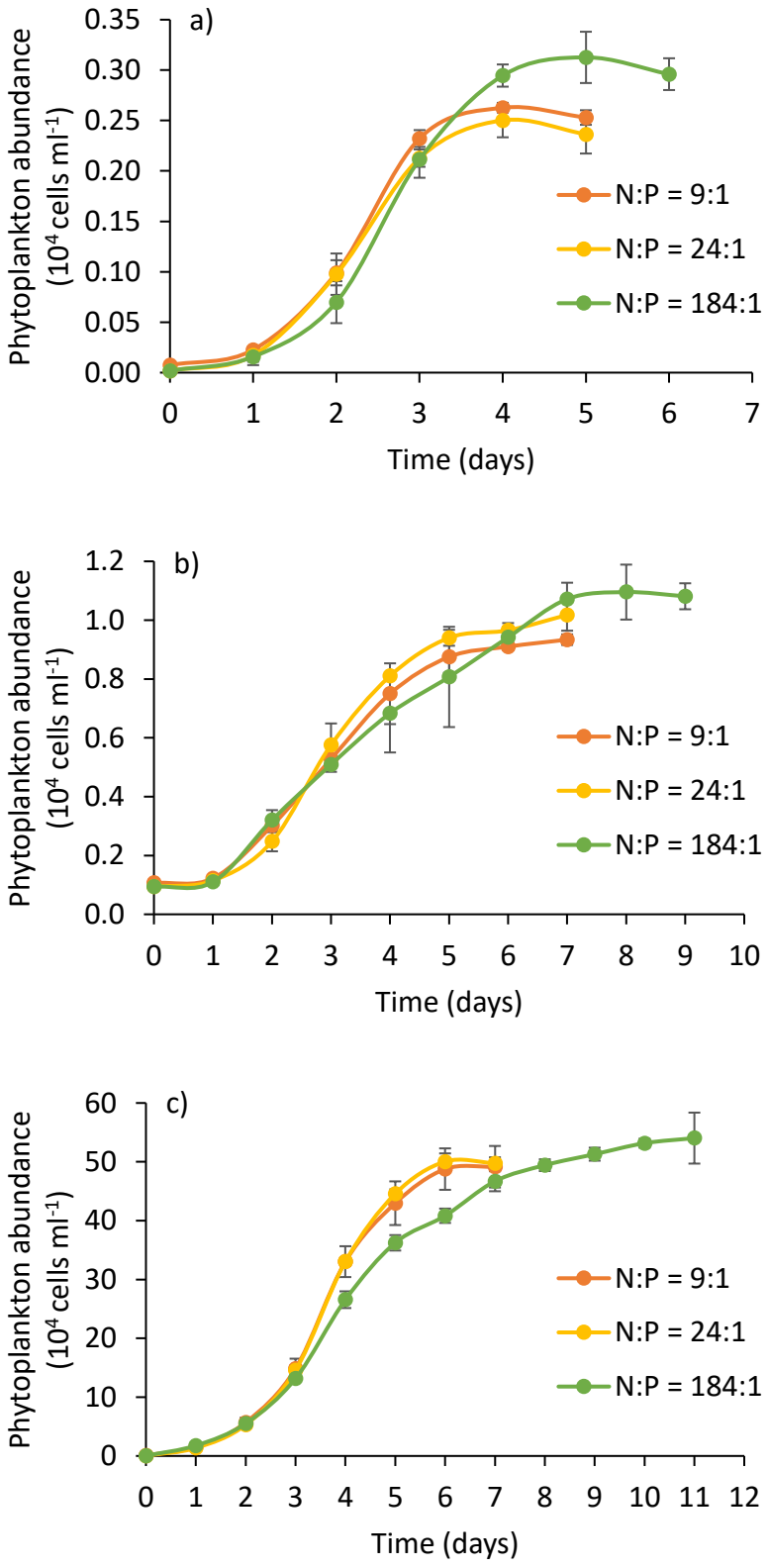

Figure 3: *Growth curves of* Chaetoceros affinis *(a),* Cylindrotheca fusiformis *(b) and* Picochlorum *sp. (c) cultures under different N:P ratios. Means ± standard deviation of three replicate are shown*

Bacterial abundance

Since phytoplankton cultures used in this study were non-axenic, bacterial abundance was additionally enumerated for each phytoplankton culture and nutrient condition (Fig. 4). Generally, phytoplankton cultures grown under P-limitation (N:P = 184:1) showed lower abundance in bacterial cells than cultures grown under N-limitation (N:P = 9:1) or under balanced nutrient conditions (N:P = 24:1).

In the stationary growth phase of the phytoplankton, the highest abundance was found in cultures of *Picochlorum* sp. (7 x 10^6 cells ml^{-1}) followed by the cultures of *Chaetoceros affinis* (4 x 10^6 cells ml^{-1} for N:P = 9:1 and N:P = 24:1 and 2 x 10^6 for N:P = 184:1) and *Cylindrotheca fusiformis* (5 x 10^6 cells ml^{-1} for N:P = 9:1, 6 x 10^6 cells ml^{-1} for N:P = 24:1 and 2 x 10^6 cells ml^{-1} for N:P = 184:1). In the stationary phase of phytoplankton, bacterial growth in *Chaetoceros affinis* and *Picochlorum* sp. cultures was in the exponential phase. *Cylindrotheca fusiformis* peaked at day 3 and decreased thereafter.

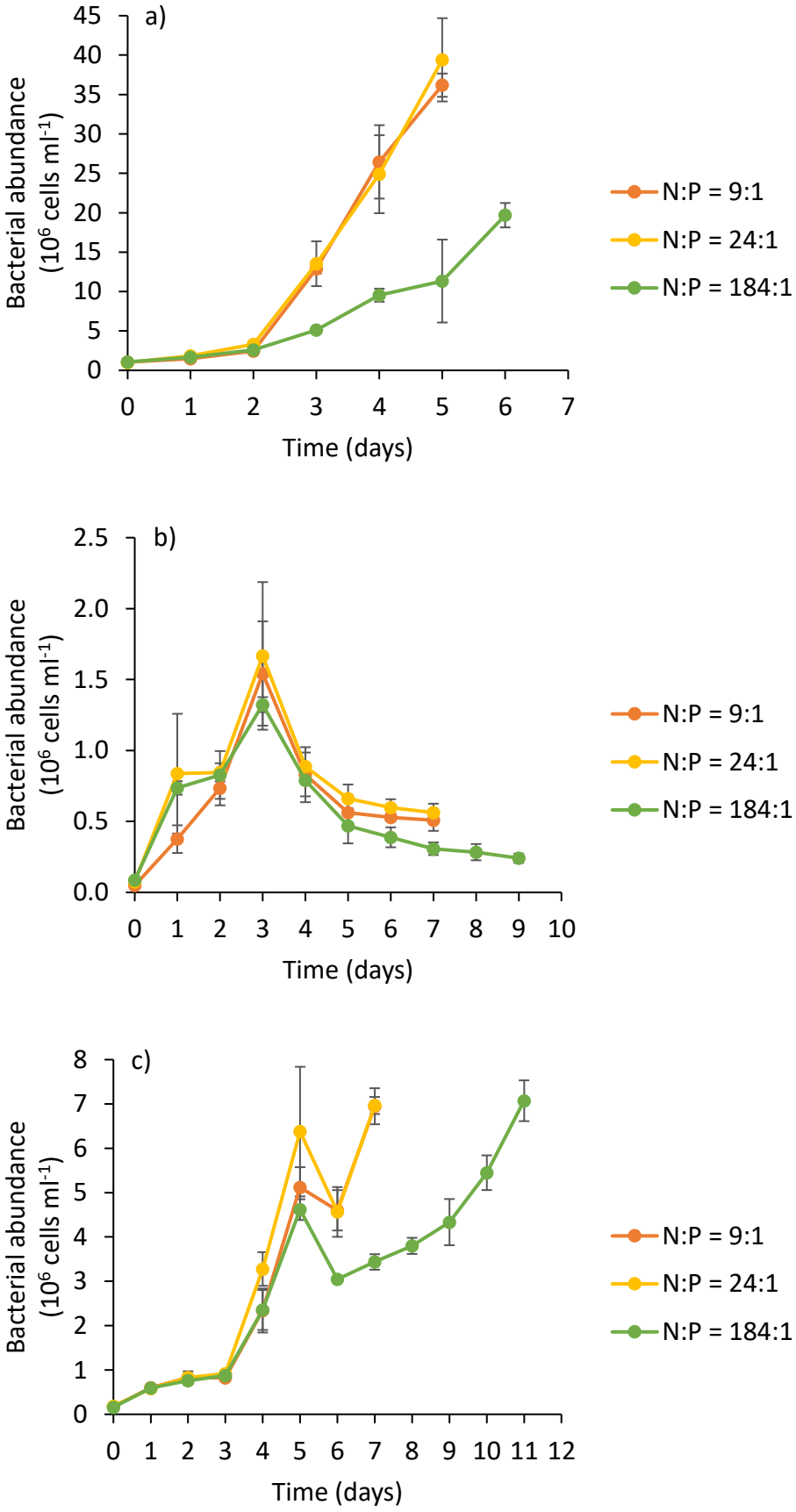

Figure 4: *Bacterial abundance of* Chaetoceros affinis *(a),* Cylindrotheca fusiformis *(b) and* Picochlorum *sp. (c) cultures under different nutrient conditions. Means ± standard deviations of three replicate cultures are shown.*

Dissolved organic carbon

In the stationary phase of phytoplankton, DOC concentration was highest in *Cylindrotheca fusiformis* and lowest in *Picochlorum* sp. cultures. The differences in DOC production were significant between the three species (ANOVA, $p < 0.001$). DOC concentrations produced by phytoplankton oscillated between 2.02 ± 0.02 mg L^{-1} and 2.28 ± 0.62 mg L^{-1} for *Chaetoceros affinis* cultures, between 2.92 ± 0.13 mg L^{-1} and 3.32 ± 0.34 mg L^{-1} for *Cylindrotheca fusiformis* cultures and between 1.65 ± 0.05 mg L^{-1} and 1.70 ± 0.06 mg L^{-1} for *Picochlorum* sp. cultures (Table 3). Comparing the values between the individual treatments, *Chaetoceros affinis* and *Picochlorum* sp. cultures with an N:P = 184:1 ratio exhibited the highest DOC concentrations and those with an N:P = 9:1 and N:P = 24:1 showed the lowest ones. *Cylindrotheca fusiformis* showed a different pattern in cultures with an N:P = 184:1 exhibiting the lowest DOC concentrations and those with an N:P = 24:1 the highest ones.

Table 3: *Values of AxA_{100} (%), DOC bulk (mg L^{-1}), and DOC recovery (%) of each nutrient condition (N:P = 9:1, N:P = 24:1, N:P = 184:1)* ± SD for Cylindrotheca fusiformis*,* Chaetoceros affinis *and* Picochlorum *sp.*

	AxA_{100} (%)	**DOC bulk (mg L^{-1})**	**DOC recovery (%)**
Chaetoceros affinis			
N:P = 9:1	35.11 ± 1.73	2.14 ± 0.03	17.93 ± 0.83
N:P = 24:1	39.30 ± 1.31	2.02 ± 0.02	20.21 ± 4.22
N:P = 184:1	28.05 ± 5.15	2.28 ± 0.62	21.61 ± 6.50
Cylindrotheca fusiformis			
N:P = 9:1	21.68 ± 4.06	3.12 ± 0.29	21.55 ± 10.26
N:P = 24:1	23.30 ± 4.43	3.32 ± 0.34	19.57 ± 2.10
N:P = 184:1	53.73 ± 4.11	2.92 ± 0.13	13.37 ± 1.85
***Picochlorum* sp.**			
N:P = 9:1	19.87 ± 2.87	1.65 ± 0.05	37.29 ± 21.23
N:P = 24:1	36.27 ± 2.31	1.66 ± 0.06	35.85 ± 23.73
N:P = 184:1	47.83 ± 5.83	1.70 ± 0.06	26.70 ± 10.06

The recovery of the PPL extracts (calculated as the fraction of DOC eluted from the originally present DOC of the sample) varied between 17.93 ± 0.83% and 21.61 ± 6.50% for *Chaetoceros affinis*, between 13.37 ± 1.85% and 21.55 ± 10.26% for *Cylindrotheca fusiformis* and between 26.70 ± 10.06% and 37.29 ± 21.23% for *Picochlorum* sp. (Table 3). In general, cultures with an N:P = 184:1 ratio showed the lowest DOC recovery while cultures treated with N:P = 9:1 exhibited the highest recovery. *Chaetoceros affinis*, however, exhibited an opposite

trend with N:P = 9:1 exhibiting the lowest (17.93 ± 0.83%) and N:P = 184:1 the highest (21.61 ± 6.50%) DOC recovery.

Antioxidant activity

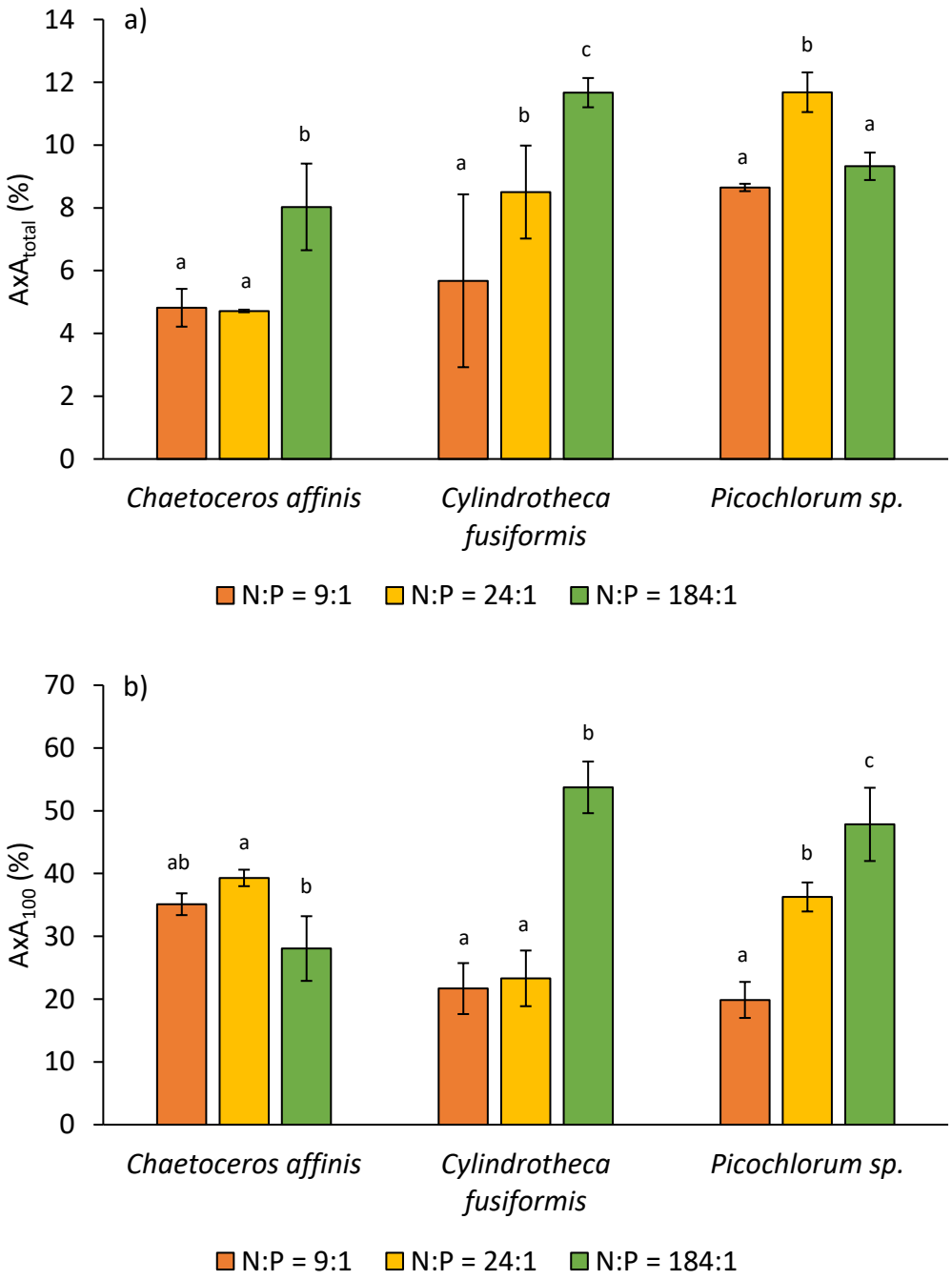

Figure 5: *a) Total antioxidant activity (AxA$_{total}$ ± SD) and b) antioxidant activity (AxA$_{100}$ ± SD) normalised to 100 mg L^{-1} of DOC given as percentage observed in the DOM of* Chaetoceros affinis*,* Cylindrotheca fusiformis *and* Picochlorum *sp. grown under varying nutrient ratios (N:P = 9:1, N:P = 24:1, N:P = 184:1). A different letter for each treatment within each species indicates statistically differences (*p < *0.05, two-way ANOVA, with post-hoc Tukey's test).*

The total antioxidant activity derived from the extracts of DOM obtained from phytoplankton grown under N:P = 24:1 was significant different among the phytoplankton species (ANOVA, $p < 0.001$) tested with extracts of *Picochlorum* sp. showing the highest antioxidant activity, followed by *Cylindrotheca fusiformis* and *Chaetoceros affinis* (Fig. 5a).

The antioxidant activity was compared between the different nutrient ratios for each phytoplankton species. Extracts from *Chaetoceros affinis* showed the highest antioxidant activity for cultures grown under P-limitation (ANOVA, $p < 0.01$) at 8.03 ± 1.38%, while cultures with an N:P ratio of 9:1 and 24:1 showed the lowest antioxidant activity with 4.82 ± 0.60% and 4.72 ± 0.04%, respectively. Moreover, extracts of *Cylindrotheca fusiformis* exhibited significant differences between the three treatments (N:P = 9:1, N:P = 24:1 and N:P = 184:1) (ANOVA, $p < 0.01$). The total antioxidant activity of the *Cylindrotheca fusiformis*-derived DOM extracts decreased with decreasing N availability with the highest activity obtained in P-limited cultures (11.67 ± 0.47%) followed by cultures grown under balanced nutrient ratios (8.50 ± 1.48%) and N-limited cultures (5.68 ± 2.75%). The extracts of *Picochlorum* sp. exhibited the highest antioxidant activity (11.68 ± 0.63%) for cultures grown under balanced nutrient conditions (ANOVA, $p < 0.05$), while extracts of N-limited and P-limited cultures showed similar values of 8.65 ± 0.12% and 9.32 ± 0.44%, respectively.

Normalising the antioxidant activity to 100 mg L^{-1} of DOC (AxA_{100}) allows comparing the free radical scavenging capacity (antioxidant capacity) among samples of varying DOC concentrations and thus indicates the quality of the DOM produced by each species. AxA_{100} in the DOM released by the species studied here ranged between 19.87 ± 2.87% and 53.73 ± 4.11% (Fig. 5b). Comparing the AxA_{100} between the treatments across all phytoplankton species under P-limitation, extracts of *Cylindrotheca fusiformis* and *Picochlorum* sp. showed the highest and *Chaetoceros affinis* the lowest AxA_{100} (ANOVA, $p < 0.001$). Under N-limitation, extracts of *Chaetoceros affinis* presented significantly higher values (ANOVA, $p < 0.001$) than those of *Cylindrotheca fusiformis* and *Picochlorum* sp., while under balanced nutrient conditions the highest values were observed in *Chaetoceros affinis* and *Picochlorum* sp. cultures (ANOVA, $p < 0.001$).

In particular, extracts of *Chaetoceros affinis* exhibited significantly higher AxA_{100} when cultures were grown under balanced nutrient conditions (39.30 ± 1.31%) than when grown under P-limitation (28.05 ± 5.15%) (ANOVA, $p < 0.001$). Extracts of *Cylindrotheca fusiformis* had the highest AxA_{100} (53.73 ± 4.11%) when cultures were grown under P-limitation (ANOVA,

$p < 0.001$), while cultures with an N:P ratio of 9:1 and 24:1 showed the lowest AxA_{100} at 21.68 ± 4.06% and 23.30 ± 4.43%, respectively. Furthermore, the extracts of *Picochlorum* sp. indicated significant differences in their AxA_{100} between the three nutrient conditions (ANOVA, $p < 0.001$) (N:P = 9:1, N:P = 24:1, N:P = 184:1). The highest AxA_{100} was found in the extracts of P-limited cultures (47.83 ± 5.83%), decreasing continuously with decreasing N availability, thus followed by the activity of N:P = 24:1 cultures (36.27 ± 2.31%) while the lowest AxA_{100} at 19.87 ± 2.87% was found in the extracts of N-limited cultures.

In general, a positive linear relationship between the AxA_{total} and phytoplankton abundance was found. For *Chaetoceros affinis* and *Cylindrotheca fusiformis* the relationship was significant ($R^2 = 0.92$; $p < 0.05$ and $R^2 = 0.67$; $p < 0.05$, respectively) (Fig. 6a, 6c) while for *Picochlorum* sp., it was not ($R^2 = 0.33$, Fig. 6e). In contrast, a significant inverse relationship was found between AxA_{total} and bacterial abundance for *Chaetoceros affinis* ($R^2 = 0.92$; $p < 0.05$) and *Cylindrotheca fusiformis* ($R^2 = 0.74$; $p < 0.05$) (Fig. 6b, 6d). However, the AxA_{total} of *Picochlorum* sp. extracts showed a significant positive correlation ($R^2 = 0.63$; $p < 0.05$) with bacterial abundance in their cultures (Fig. 6f).

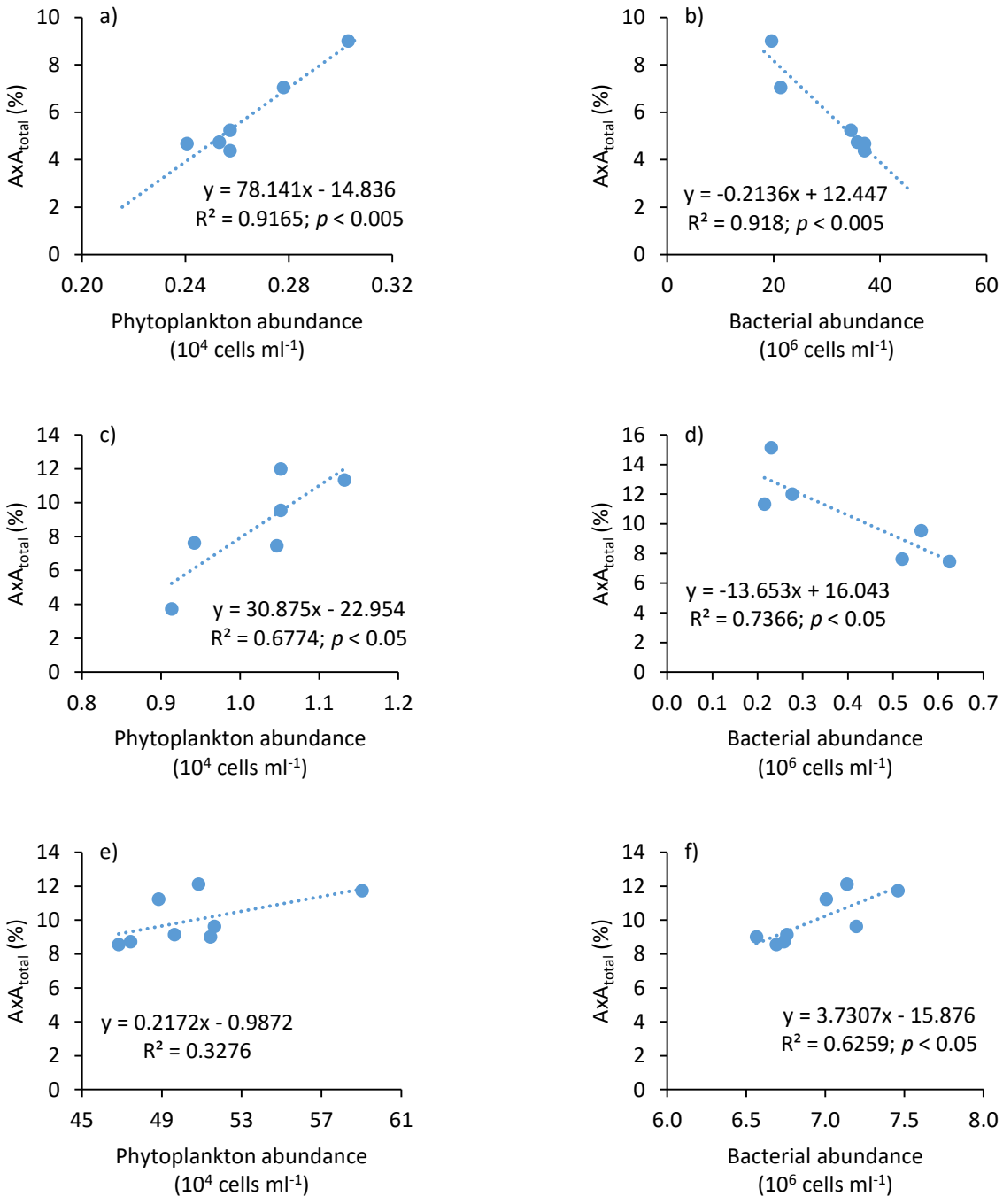

Figure 6: *Relationship between total antioxidant activity (AxA_{total}) and (left) phytoplankton abundance (x 10^4 cells ml^{-1}); and (right) bacterial abundance (x 10^6 cells ml^{-1}) for* Chaetoceros affinis *(a, b),* Cylindrotheca fusiformis *(c, d) and* Picochlorum *sp. (e, f)*

Water column antioxidant activity in the Red Sea

Antioxidant activity (AxA_{100}) measured in the DOM extracts from the Red Sea ranged between 13.61 ± 0.94% and 25.11 ± 0.86% for Stations 2 - 4, but it was much lower for Station 1 with 5.14 ± 0.97% - 7.24 ± 0.40% (Fig. 8). Station 1 was near a brine pool characterized by higher salinity and methane concentrations than in the surrounding waters. Also the recovery

of the PPL extractions was lower at Station 1 (39.18% - 48.53%), while at Stations 2 - 4, DOC recovery varied between 37.32% and 63.03%. These values are close to the average PPL extraction efficiency of 62% reported by Dittmar et al. (2008).

For all the stations, the total antioxidant activity (AxA_{total}) was similar along the sampled profiles and did not show any clear pattern (Fig. 7). Unfortunately, surface samples were not collected, where the largest differences were observed based on previous work (Romera-Castillo and Jaffé 2015). Only samples from Station 1 indicated an increase in the AxA_{total} with increasing Chlorophyll *a* concentrations (Fig. 9).

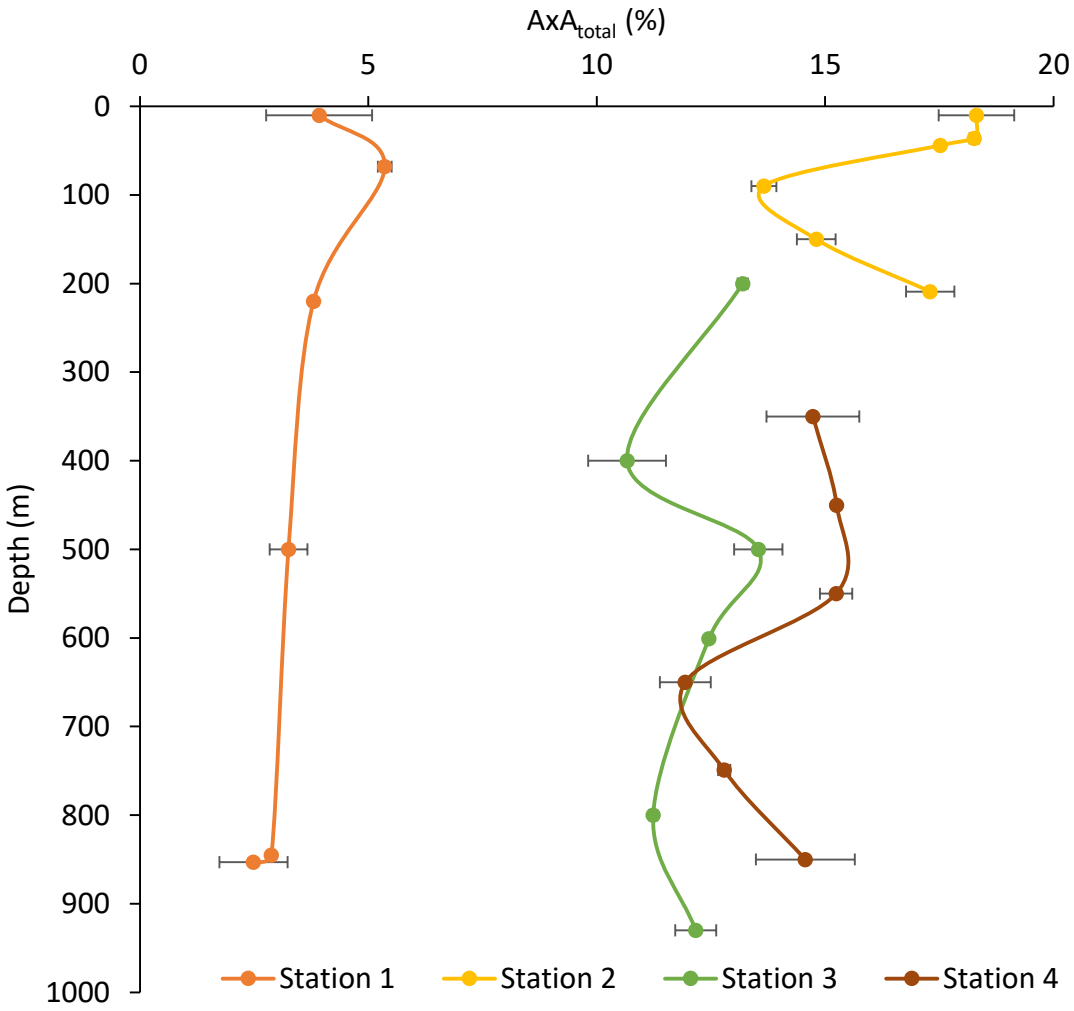

Figure 7: *Distribution of the total antioxidant activity (AxA_{total}) along the water column at Stations 1, 2, 3 and 4*

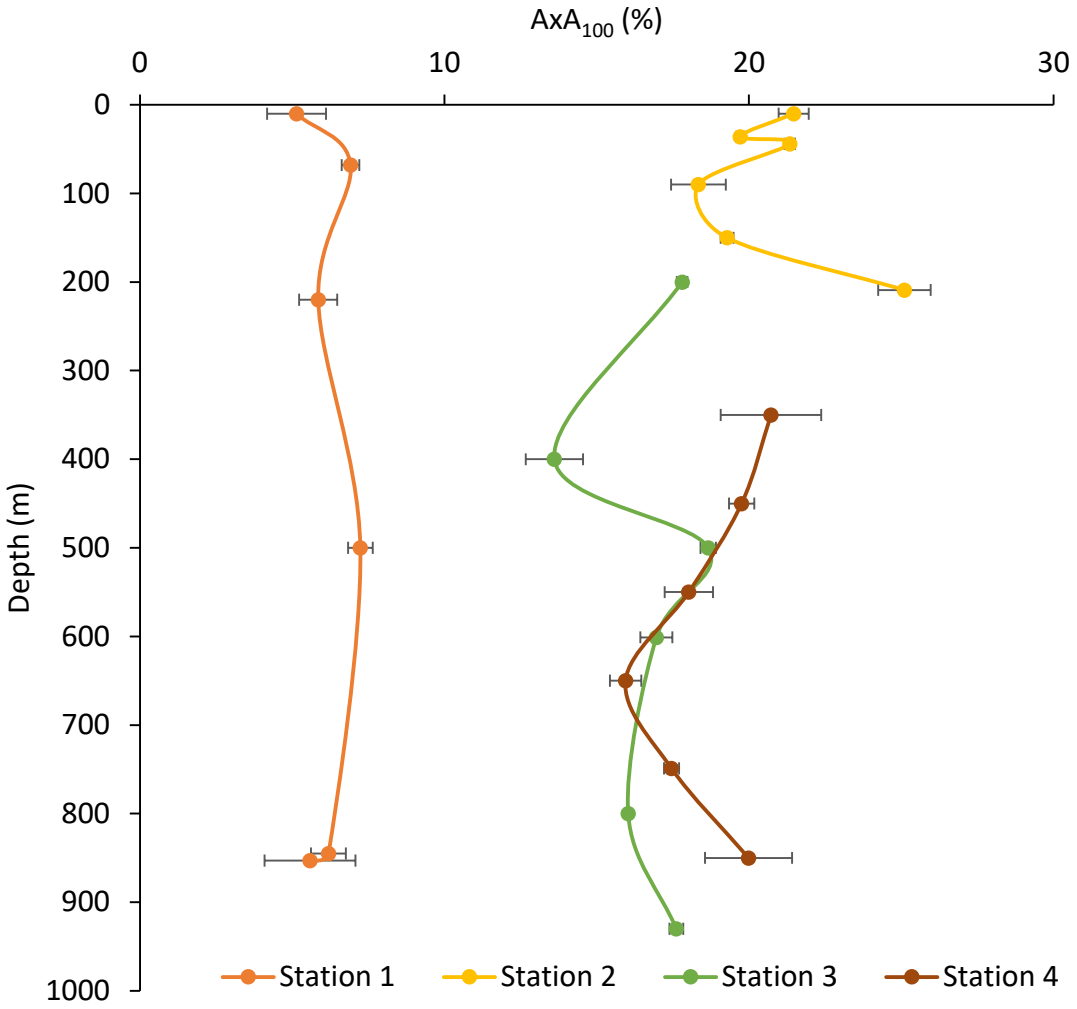

Figure 8: *Distribution of the antioxidant activity in 100 mg L^{-1} of DOC (AxA_{100}) along a depth gradient in the water column at Stations 1, 2, 3 and 4*

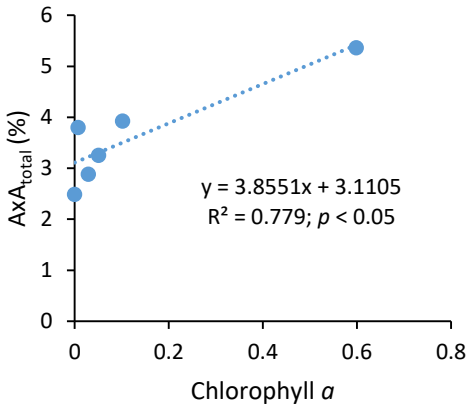

Figure 9: *Relationship between the total antioxidant activity (AxA_{total}) and the Chlorophyll* a *concentration at Station 1*

Discussion

This study was conducted to examine the response of different phytoplankton species to nutrient limitations in terms of antioxidants released as DOM and to investigate the antioxidant activity of natural DOM in the water column of the Red Sea. Our work shows that marine phytoplankton release DOM with antioxidant properties. Generally, with increasing phytoplankton abundance antioxidant activity increases (Fig 6a, 6c, 6e). Correlating the total antioxidant activity with the bacterial abundance, we observed a significant decrease in the AxA_{total} with increasing bacterial abundance in the two diatom cultures. Thus, we conclude that bacteria are able to take up phytoplankton-derived DOM with antioxidant properties. In contrast, in *Picochlorum* sp. cultures, bacterial abundance increased with increasing AxA_{total} (Fig. 6f). This indicates that bacteria in the *Picochlorum* sp. cultures either did not take up antioxidant-DOM efficiently or that the release of antioxidants exceeds uptake by bacteria. Those contrasting results might be due to differences in the DOM composition. In this study, the composition of the DOM was not analysed. Therefore, further investigations are needed to better understand the composition of the DOM produced by different phytoplankton species. Moreover, diatoms are known to release extracellular polymeric substances or mucilage, which serve as food source for other organisms such as bacteria (Puddu et al. 2003). The extracellular release of phytoplankton might have different functions including maintenance of symbiotic relationships with bacteria or release of extracellular enzymes such as alkaline phosphatase allowing the cleavage of phosphate moieties from organic compounds (Buchan et al. 2014; Dyhrman and Palenik 1999). Generally, antioxidant compounds in the DOM might affect microorganisms. They can have beneficial effects on the microbial activity, as Scully et al. (2003) reported for lakes indicating that the presence of antioxidants promoted bacterial growth. These findings correspond to our observations obtained from *Picochlorum* sp. cultures where the bacterial abundance was positively correlating with increasing AxA_{total}.

Antioxidant activities have been reported in marine phytoplankton by Karthikeyan et al. (2013) in which they investigated seven different species. Among them, *Odontella mobiliensis* and *Chaetoceros simplex* exhibited the highest DPPH radical scavenging activities ranging between 55.69 ± 2.52% and 70.04 ± 4.17%. Comparing these results with those obtained here, antioxidant activity in the DOM released by phytoplankton into the media is apparently lower than actually present inside the cells. Antioxidant activity within the cells was not investigated in our study. Moreover, studies have shown that antioxidants are also

found in terrestrial environments (Rimmer and Smith 2009). Allochthonous (terrestrial) organic material reaches the coast and ultimately might enter the ocean via river input. Thus DOM produced by phytoplankton is likely not the only source of antioxidants in coastal areas. AxA_{100} between 23.7% and 29.6% were found in estuarine sites, which are tidally influenced and dominated by mangroves (Romera-Castillo and Jaffé 2015). It has been reported that mangroves produce and release a substantial amount of tannins (Romera-Castillo and Jaffé 2015) which are well-known antioxidants (Amarowicz 2007; Romera-Castillo and Jaffé 2015).

However, antioxidants also play a role in the oceanic DOM as detected in four water column profiles of the coastal Red Sea. DOM in the Red Sea exhibits antioxidant activity. The antioxidant activities (AxA_{100}) obtained in the oligotrophic water column of the Red Sea (13.61 ± 0.94% to 25.11 ± 0.86%) are within the range obtained in the phytoplankton cultures (19.87 ± 2.87% to 53.73 ± 4.11%) and are similar to those previously reported by Romera-Castillo and Jaffé (2015) conducted in shallow Florida Bay waters (11.3 ± 0.2% to 18.7 ± 0.1%). AxA_{100} values were rather invariable throughout the water column (Fig. 8). Unfortunately, surface samples were not collected, where higher antioxidant activity might be expected than in the deeper layers of the water column. When plotting AxA_{total} versus Chlorophyll *a* concentrations, a significant positive correlation was only found at Station 1, which is an indication that most of the antioxidants measured there is derived from DOM produced by phytoplankton (Fig. 9). This positive correlation found between AxA_{total} and Chlorophyll *a*, allows us to compare the values found in the open ocean with those from the phytoplankton cultures. The AxA_{total} values at Station 1 ranging from 2.48 ± 0.74% to 5.36 ± 0.15% are similar to those of *Chaetoceros affinis* varying between 4.72 ± 0.04% and 8.03 ± 1.38%. Moreover, a recent study by Ozhan et al. (2015) showed that reactive oxygen species are activated in phytoplankton when exposed under crude oil. The fact that the highest AxA_{100} were found at Station 2 (Fig. 8) might be caused by the presence of an oil seep near the sampling station.

DOC concentrations in the phytoplankton cultures differed considerably between the phytoplankton species. A study by Romera-Castillo et al. (2010) reported similar DOC concentrations varying between 2.0 and 3.3 mg L^{-1} for phytoplankton cultures. The significant difference in the DOC production between the three species might be explained by the different characteristics of the species. For example, Biddanda and Benner (1997) reported a weak relationship between the DOC production and cell size.

The recovery of DOC using solid phase extraction with the PPL cartridges (13.4% - 37.3%) obtained in our study are similar to the recovery found in the open ocean (43%) reported by Dittmar et al. (2008) who stated that > 60% of coastal and > 40% of deep-sea DOC can be recovered using PPL cartridges. Thus, the results obtained by phytoplankton cultures are more similar to the recovery of deep-sea DOC. In the Red Sea, the recovery efficiency of the PPL extraction ranged between 37.3% and 63.0%.

Nitrogen and phosphorus are essential nutrients for phytoplankton growth and their metabolism (El-Kassas 2013). The availability of nutrients affects their growth and thus environmental stress conditions such as nutrient limitation will cause a decrease in cell division rates (El-Kassas 2013; Thompson 1996). In this study, the different phytoplankton species showed unexpected differences in their growth with respect to the varying N:P ratios. The AxA_{total} in the DOM was comparable for the two diatom species (*Chaetoceros affinis*, *Cylindrotheca fusiformis*) with highest AxA_{total} values under P-limitation (Fig. 5a). Generally, our results indicate that DOM derived from different phytoplankton genera exhibits higher AxA_{100} under P-limitation (*Picochlorum* sp. and *Cylindrotheca fusiformis*) than under N-limitation (Fig. 5b), indicating species-specific differences in the released DOM. Obernosterer and Herndl (1995) showed that the extracellular release is highest during stationary phase of marine phytoplankton when grown under P-limitation, which might result in higher AxA_{100}.

Different diatom species responded in different ways to nutrient limitation with respect to their antioxidant activity normalised to 100 mg L^{-1} of DOC. AxA_{100} of *Chaetoceros affinis* differed considerably from that of the other two species. Preliminary experiments showed that *Cylindrotheca fusiformis* as well as *Picochlorum* sp. are easy to culture reaching high cell abundances, unlike *Chaetoceros affinis*, which is very sensitive to varying light intensities only reaching moderate cell abundance. This species was probably in a physiologically poor state and stressed exhibiting therefore highest AxA_{100} under balanced nutrient conditions and additional stress by nutrient limitation slowed down biomass production and release of antioxidants into the media. It is known that in the surface ocean N-limitation is more common than P-limitation and most of the DOM production can be found under nutrient-poor conditions as reported elsewhere (Biddanda and Benner 1997; Moore et al. 2013; Obernosterer and Herndl 1995; Thornton 2014). Thus, phytoplankton are probably more used to N-limitation, but become stressed under P-limitation resulting in higher release of antioxidants.

Although the overall response to varying nutrient conditions concerning phytoplankton growth was similar, there were nevertheless obvious differences in the antioxidant activity between the individual species. The growth experiments conducted in this study showed that phytoplankton were more negatively affected under N-limitation reaching lower cell abundances than under P-limitation (Fig. 3). These results are in contrast to those reported in the study of Obernosterer and Herndl (1995). They found that *Chaetoceros affinis* grown under P-limited conditions reached significantly lower optical densities than under N-limitation or balanced nutrient conditions. In our study, light intensities of 80 µmol photons m^{-2} s^{-1} and a temperature of 18°C were used to grow phytoplankton while Obernosterer and Herndl (1995) applied light intensities of 180 µmol photons m^{-2} s^{-1} and a temperature of 22°C. Thus, these different settings and different N:P ratios might have influenced the differences in the resulting maximum cell abundances. Magaletti et al. (2004) and El-Kassas (2013) investigated *Cylindrotheca fusiformis* and *Picochlorum* sp. growth, respectively, under N- and P-limitation and found that nutrient depletion affects the cell growth resulting in decreased cell abundances. All those findings of other studies fail to explain the results shown in Fig. 3, indicating highest phytoplankton abundance under P-limitation.

A study by Grossart (1999) showed that varying environmental conditions strongly influence the interaction between marine diatoms and bacteria. When inorganic nitrogen and phosphorus are limited, bacteria growth is retarded (Grossart 1999) in agreement with this study. According to Puddu et al. (2003), DOM released by *Cylindrotheca closterium* grown under P-limitation is less available for bacterial uptake and will affect the bacteria community resulting in decreased bacterial abundance.

Conclusion

Antioxidant activity detected in the DOM of phytoplankton cultures is species specific with different responses to nutrient limitations. In general, increasing P-limitation of phytoplankton led to DOM formation with increasing antioxidant activity.

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Supplementary Information

Supplementary Table 1: *Artificial seawater recipe (salinity: 35)*

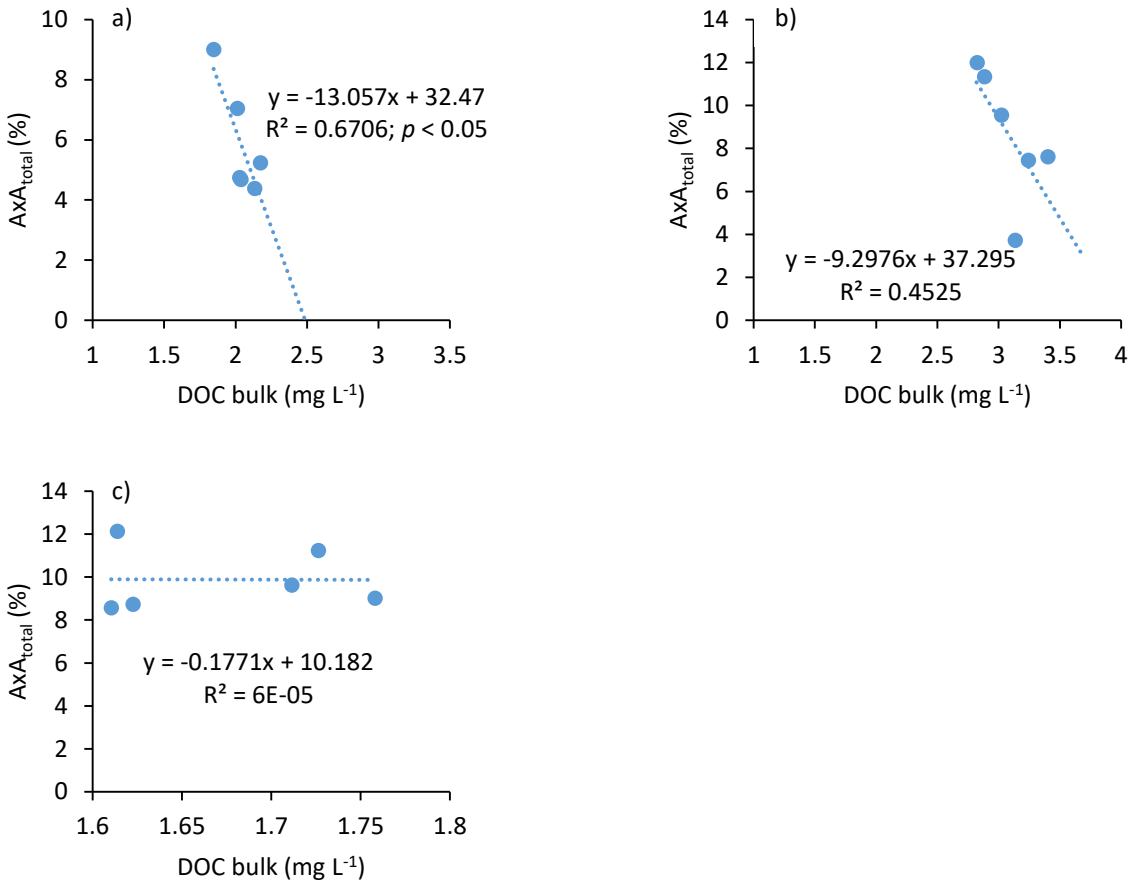
Chemical compounds	Concentration g/5l
NaCl	101.41
Na_2SO_4	16.99
KCl	2.82
$NaHCO_3$	0.831
KBr	0.413
H_3BO_3	0.110
NaF	0.0132
$MgCl_2.6H_2O$	45.90
$CaCl_2.2H_2O$	6.43
$SrCl_2.6H_2O$	0.105

Supplementary Table 2: *f/2 + Si Guillard's medium*

	$NaNO_3$	**NaH_2PO_4 $2H_2O$**	**Na_2SiO_3 $9H_2O$**	**Trace elements***	**Vitamin Complex****
Concentration	75 g/l	5.65 g/l	30 g/l	7.538 g/l	0.202 g/l
N:P ratio					
9:1	376.6 µl/l	1 ml/l	1 ml/l	1 ml/l	0.5 ml/l
24:1	1 ml/l	1 ml/l	1 ml/l	1 ml/l	0.5 ml/l
184:1	1 ml/l	132.7 µl/l	1 ml/l	1 ml/l	0.5 ml/l

Trace elements*	
Na_2 EDTA	4.16 g/l
$FeCl_3.6H_20$	3.15 g/l
$CuSO_4.5H_2O$	0.01 g/l
$ZnSO_4.7H_2O$	0.022 g/l
$CoCl_2.6H_2O$	0.01 g/l
$MnCl_2.4H_2O$	0.18 g/l
$Na_2MoO_4.2H_2O$	0.006 g/l

Vitamin Complex**	
Cyanocobalamin (Vitamin B12)	0.001 g/l
Biotin	0.001 g/l
Thiamine HCl (Vitamin B1)	0.2 g/l



Supplementary Figure 1: *Relationship between the total antioxidant activity (AxA_{total}) and DOC concentration (mg L^{-1}) produced by a)* Chaetoceros affinis*; b)* Cylindrotheca fusiformis *and c)* Picochlorum *sp.*

Zusammenfassung

Umweltstress beeinflusst die biochemische Zusammensetzung von Mikroalgen, was häufig zur Bildung reaktiver Sauerstoffspezies (ROS) führt. Radikalfänger (Antioxidantien) können biologische Systeme gegen ROS und freie Radikale schützen, indem sie diese Radikale aus den Medien entfernen. Mikroalgen können Antioxidantien zur Abwehr schädlicher freier Radikale produzieren und sind somit eine potentielle Quelle für Antioxidantien. Antioxidantien sind auch Teil des gelösten organischen Materials (dissolved organic matter, DOM) in den Meeren. Über die Produktion und Freisetzung von Antioxidantien durch marines Phytoplankton (Mikroalgen) unter Beeinflussung verschiedener Nährstoffregimen ist jedoch wenig bekannt. In dieser Studie wurden drei nicht-axenische marine Phytoplanktonarten (*Chaetoceros affinis*, *Cylindrotheca fusiformis*, *Picochlorum* sp.) unter verschiedenen Stickstoff zu Phosphor (N:P) Verhältnissen gezüchtet. Das durch Phytoplankton freigesetzte DOM wurde durch Festphasenextraktion (solid phase extraction, SPE) extrahiert und die antioxidative Aktivität des DOM wurde unter Verwendung der kolorimetrischen DPPH (2,2-diphenyl-1-picrylhydrazyl) Analyse gemessen. Darüber hinaus wurde DOM aus unterschiedlicher Tiefen der Wassersäule im Roten Meer untersucht um seine antioxidative Aktivität zu bestimmen. DOM, das im Roten Meer gesammelt wurde, sowie DOM, welches durch Phytoplankton-Kulturen produziert wurde, zeigte antioxidative Aktivität. Zusammenfassend wurde die höchste antioxidative Aktivität in jenen Phytoplankton-Kulturen nachgewiesen, welche unter Phosphor-Limitierung gezüchtet wurden, wohingegen die niedrigste antioxidative Aktivität in Stickstofflimitierten Kulturen nachgewiesen wurde. Die gesamte antioxidative Aktivität im DOM war bei beiden Kieselalgenarten ähnlich (*Chaetoceros affinis*, *Cylindrotheca fusiformis*). Zusammenfassend zeigten die Ergebnisse, dass Antioxidantien im gelösten organischen Material, welches von marinen Phytoplankton produziert wurde vorhanden sind, aber Nährstoffstress möglicherweise keine effektive Strategie für einige Phytoplanktonarten ist, um die Freisetzung von antioxidativen DOM zu verstärken.