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Picoeukaryote community ecology and functional response to trace metals disturbances in coastal environments

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Resumen

Los sistemas costeros son de vital importancia para el desarrollo humano y los procesos ecosistémicos. En estas regiones se desarrolla aproximadamente la mitad de la productividad primaria del planeta, representando así un sistema fundamental para ciclos biogeoquímicos de la tierra y una región muy relevante en términos de servicios ecosistémicos. Sin embargo, estas regiones se encuentran bajo constante presión debido a variaciones ambientales naturales o bien de origen antropogénico. Las perturbaciones de origen humano, entre ellas la polución química, someten a los organismos a constantes desafíos para su fisiología y para su contexto ecológico. En este contexto, la fracción fotosintética de los microorganismos planctónicos juegan un rol muy relevante en la estructuración, funciones y procesos biogeoquímicos de los sistemas costeros. En este grupo funcional, los organismos de menor tamaño han ganado interés debido a su alta diversidad y su gran relevancia ecológica. Así, mediante percepción remota y observaciones en terreno, sumado a aproximaciones de laboratorio al nivel de comunidades y de poblaciones, se evaluó el efecto del Cobre como un relevante agente contaminante para el medio ambiente, en la ecología del plancton eucarionte de menor tamaño. Los resultados obtenidos indican que el cobre actúa homogenizando la diversidad biológica en comunidades de picoeucariotes, mediante un desacople entre las fluctuaciones abióticas y las respuestas en el componente biótico de ambientes costeros. Estas comunidades expuestas a polución por cobre mostraron una respuesta consistente, con una aproximación de secuenciación masiva, donde el clado de Mamiellophyceae mostró una dinámica temporal de abundancias relativas diferencial. Finalmente, poniendo a prueba la hipótesis del desbalance nutricional inducido por cobre, se monitoreo los niveles de proteínas relacionadas con la homeostasis de iones y transporte relacionado con el metabolismo del nitrógeno en el organismo modelo *Ostreococcus tauri* obteniendo una respuesta concentración dependiente. En resumen, estos datos sugieren una regulación cruzada entre el metabolismo del nitrógeno y la homeostasis del cobre, con potenciales efectos en la ecología del plancton.

Abstract

Coastal environments are of special importance for human development and ecosystem processes. In these regions roughly half of total primary production take place, representing an important system for earth biogeochemistry and a fundamental region to ecosystem services. However, these regions are constantly subjects to pressure due to environmental variability of both natural or anthropogenic origin, where human-made disturbances and chemical pollution lead to constant challenges for organisms physiology and ecological processes. The photosynthetic fraction of the planktonic microorganisms plays a key role in environmental functioning and biogeochemical processes. Within this functional group, small size organisms are getting increasing interest related to their unknown diversity and ecological relevance. Here, with remote and infield observations and highly replicated laboratory experiments at community and population levels, we evaluated the effects of copper, as a relevant environmental model pollutant, over the ecology of small eukaryotic plankton. The obtained results indicate that copper act as a homogenizing force to biological diversity among picoeukaryote communities, by decoupling biotic aspects of the abiotic seasonal fluctuations in coastal communities, that leads to a particular microbial community structure. Moreover, picoeukaryote communities exposed to copper pollution, under lab conditions, showed a consistent response though next generation sequencing (NGS) approach, and Mamiellophyceae related taxa showed a differential temporal respond in terms of relative abundance dynamics. Finally to test functional mechanistic hypothesis related to nutritional imbalance, response at protein level in the model organism *Ostreococcus tauri* was tested, ion homeostasis and nitrogen cycling related transport systems were responsive in concentration-dependent manner. Overall, the data suggest a crossed regulation effect with metal homeostasis and nitrogen metabolism with potential ecological and biogeochemical effects.

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List of Abbreviations

ASV	Anodic stripping voltammetry
BAC	Bacteria
Cd	Cadmium
Chl-a	Chlorophyll-a
Co	Cobalt
Cu	Copper
DNA	Deoxyribonucleic acid
dNTP	Desoxinucleotides triphosphates
DOC	Disolved organic carbon
EUK	Eukaryote
Fe	Iron
HCS	High copper site
Humboldt-CS	Humboldt Current System
ICP-MS	Inductively coupled plasma mass spectrometry
Kd490	Attenuation coefficient
LCS	Low copper site

NGS	Next Generation Sequencing
MgCl ₂	Magnesium chloride
Mo	Molybdenum
PAR	Photo active radiation
Pb	Lead
PCR	Polymerase chain reaction
PE	Picoeukaryotes
POC	Particulate organic carbon
PPE	Photosynthetic picoeukaryotes
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal ribonucleic acid
SiO ₄ ⁻⁴	Silicic Acid
Taq	Thermos aquaticus polymerase
Zn	Zinc

Chapter 1

Theoretical framework

1.1 Pico-phytoplankton as model group in disturbance ecology

Coastal environments are of special importance for earth ecosystems and human development. In these regions, roughly half of global primary production takes place, representing a fundamental system for earth biogeochemistry, and ecosystem services. In the case of the Humboldt Current System (Humboldt-CS), Southern Pacific Ocean, the Chilean and Peruvian shores are highly influenced by ocean to land winds, that produce cool and nutrient rich deep waters move to surface layers, phenomena known as upwelling. These features, complemented by a strong seasonal patterns (Blanco et al., 2001; Carr et al., 2002), are traduced in local heterogeneity that is usually related to highly productive systems and low predictive power of oceanographic and climatic conditions (Thiel et al., 2007; Daneri et al., 2000).

Coastal environments are essential for human development since the first human settlements (Martinez et al., 2007; Barbier et al., 2011), by providing the so called ecosystem services, that are activities provided by the environment, that are related to culture, recreation, economic and productive processes (Costanza et al., 1997; Worm et al., 2006). However, human activities have an impact on the environment in several ways, some of them resulting in negative effects

on ecosystems. A fraction of these effects are due to byproducts of different anthropogenic activities, principally associated with agrochemical use, land use modification and mining activities (Halpern et al., 2007; Halpern et al., 2009; Paytan et al., 2009; Nogales et al., 2011). Generally, environmental impact is resulted by waste deposition in coastal waters that reach the coast by industrial wastewaters or aerosol transport (Duce et al., 1991; Paerl, 1997; Paytan et al., 2009; Jordi et al., 2012). Over time, this have produced an imbalance to natural trace metals proportions and availability, which could lead to unknown impacts on biogeochemical cycling, ecosystem functioning, community structure, population dynamics and physiology of organisms that inhabit those regions (James E. Cloern, 2001; Paytan et al., 2009; Shade et al., 2012; Jordi et al., 2012; Mackey et al., 2012; Jacquot & Moffet 2015). In the context of those impacts, trace metals highlights as relevant pollution sources, mainly by been considered persistent pollutants and for their potential toxic effects within the biosphere.

Primary production in coastal systems, like within Humboldt-CS's, are mainly driven by photosynthetic microorganisms that inhabit the water column, known as phytoplankton (Field et al., 1998; Falkowski et al., 2004; Worden et al., 2015). Even though phytoplankton represent less than 1% of the global photosynthetic biomass (Bryant et al., 2003), they play a key role in ocean biogeochemistry, being active players in redox reactions and elemental cycles that are fundamental to ecosystem functioning (Morel & Prince, 2003; Morel, 2008). Both in natural environments and controlled conditions, these microorganisms had shown differential grown dynamics related to nutritional requirements and environmental context (Sunda and Huntsman, 2000; Sunda, 2012). Among the environmental feature that influences phytoplankton, physical features like photo-active radiation (PAR) and superficial sea temperature (SST) have shown to be critical (Sunagawa et al., 2015). Also, chemical features like nutrient availability have shown to be relevant parameters in phytoplankton growth and population dynamics. Macro-nutrients like Carbon, Phosphorous and Nitrogen are known as relevant since stoichiometric observations (Redfield, 1934), and also the so call micro-nutrients, usually in trace concentrations, like iron (Fe), copper(Cu), cadmium (Cd), molybdenum (Mo), among others (de Baar, 1994; W. Sunda, 2012; Twining et al., 2011).

Under the current global change scenario, a series of variations in terms of trace metal distribution and bio-availability is predicted. Future changes will lead to fluctuations in metal distributions in oceanic systems and could alter the proportions in chemical species in the water column, modifying biological availability and exposure rates to those metals (Shi et al., 2010; Millero et al., 2011; Hoffmann et al., 2012). This is the case of copper (Cu), a highly relevant biologically active trace metal which has been related to different functional processes, like iron (Fe) uptake (Peers et al., 2005) and Nitrogen cycling (Jacquot et al., 2013). However, this metal shows a dual behavior: in low concentrations is used as a nutrient that eventually can limit phytoplankton growth (Semeniuk et al., 2015), but at high concentrations produce strong toxic effects in cells (Sunda & Huntsmann 1995; Moffet et al., 1997; Pinto et al., 2003; Morel F., 2008). Experimental data, in global change context scenarios, suggest that free Cu could increase in one order of magnitude, leading to toxic effects to water column biota (Millero et al., 2011; Gledhill et al., 2015; Avendaño et al., 2016).

Trace metals exposition could produce different effects in phytoplanktonic groups, which a common toxic effect haven shown to be the production of reactive oxygen species (ROS) (Pinto et al., 2003). The generation of those ROS lead to effects at different scales like lipid peroxidation, genotoxicity by breakdown of double helix in DNA molecules, electron transport interference in electron transport chain complexes, and protein inactivation (reviewed in Harrison et al., 2007). Further, trace metal availability could affect macro-nutrient assimilation, by interference with molecules that are related to these molecular pathways and coordinate metal ions in their structure. Also, nutrient availability and nutritional state of planktonic cells modify the intracellular trace metal content, especially to zinc (Zn) and Fe (Maldonado et al., 2000; Wang et al., 2001; Glass et al., 2009; Li et al., 2013). Trace metals nutritional unbalance could influence phytoplankton community structure due to disturbances in the functional features of these organisms (Shade et al., 2012; Malone et al., 1996). Moreover, those functional changes could lead to ecosystemic scale disturbance like in primary production and nutrient cycling (Breitburg et al., 1999; Laursen et al., 2002; Le Jeune et al., 2006; Dinsdale et al., 2008; Henríquez-Castillo et al., 2015). Usually, from an eco-toxicology perspective, nutritional unbalances and metabolism

effects showed effects over biomass and pigment composition changes that have been described as a potential disturbance's indicators in planktonic communities (Paerl et al., 2006; Reed et al., 2007; Allison et al., 2008; Strickland et al., 2009).

Recently, small microbial eukaryotes inhabiting in the water column (cells with less than 3 μm in relative diameter) have gained interest due to their unknown and comparatively high diversity in natural environments, and biological novelty of inter-domain relationships (Finkel et al., 2010; de Vargas et al., 2015). Moreover, in global change scenarios proposed for 100 years ahead, it is predicted that these group of organisms could be benefited against bigger cells, because global changes in ocean conditions will be more favorable to organisms with higher nutrient uptake efficiency (Doney et al., 2012; Li et al., 2009; Daufresne et al., 2009; Schaum et al., 2013). These predicted changes in functional features might lead to different ecosystem capabilities in terms of elemental cycles and community stability states, interpreted as community configuration with populations that oscillates near an equilibrium point (Holling, 1973; Beisner et al., 2003; Shade et al., 2012).

Studies targeting microbial eukaryotes in natural environments had described them as diversity rich and with complex patterns in heterotrophic and phototrophic functional groups. Pico-heterotrophic eukaryotes, presumably parasitic for their small size, have been highlighted as relevant for food web structure and energy transport in different ecologically relevant groups (Massana et al., 2004; Ramon Massana, 2015; Zouari et al., 2018). Small size picophytoeukaryotes (PPEs), by their side, have been described as a persistent group through all seasons within a year in coastal environments with cosmopolitan distribution (Buitenhuis et al., 2012; Tragain et al., 2018). In the case of Humboldt-CS, data showed the autotrophic fraction as a persistent group through seasonal changes (Collado-Fabbri et al., 2011). However, functional characterization of these organisms has been challenging due to differential behavior in natural environments where several groups showed not only one functional representative but also mixotrophic lifestyles (Stoecker, 1998; Worden et al., 2015).

Moreover, PPEs have shown a high relevance in ecological systems and Chlorophytes related taxa highlight as relevant organisms in the environment (Not et al., 2012; Tragain et al.,

2018). From an ecological perspective, Chlorophytes -especially the Mamiellophyceae class- have shown a particular pattern of dominance in coastal systems. In surface waters, these organisms have shown high abundances in comparison with other planktonic groups and showed a significant contribution to primary production (Massana et al., 2015; Y. Rii et al., 2016; Hernandez-Ruiz et al., 2018). Also, PPEs have shown to be responsive to seasonal fluctuations in physic-chemical parameters (Wu et al., 2017; Zouari et al., 2018; Lambert et al., 2018; Giner et al., 2018), and also some blooming capabilities with single species dominance (Not et al., 2004; Collado-Fabbri et al., 2011). In coastal systems, this type of organisms dominate the photosynthetic fraction on the water column in terms of abundance by, at least, one of the three main genera described to date: *Ostreococcus*, *Micromonas* and *Bathycoccus* (Vaulot et al., 2008). Recently, *Ostreococcus* spp. have been proposed as a model PPE organisms, mostly due to its ecological relevance as one of the major components of PPE, with similar blooming capacity as the bacterial component in the same size fraction (Chretien-Dinet et al., 1995; O'Kelly et al., 2003; Countwat et al., 2006; Vaulot et al., 2008; Collado-Fabbri et al., 2011). Also, this organism has gain relevance in molecular biology to study the evolutionary path in the green lineage, since the Mamiellales clade represents a basal group within Chlorophytes (Courties et al., 1998; Derelle et al., 2002; Voulot et al., 2008). Also, *Ostreococcus* is considered the smallest free living eukaryote known to date, with the smallest genome (Derelle et al., 2006; Palenik et al., 2007). Moreover, these organisms have been proposed as model organisms to study metal homeostasis (Jancek et al., 2008; Morrissey et al., 2012; Lelandais et al., 2016; Henriquez-Castillo et al., 2018) and nitrogen metabolism in the green algae lineage (Le Bihan et al., 2011).

Due to the presence of bays with different pollution levels in the latitudinal gradient, the Chilean coastal system represents a unique opportunity to evaluate the effects of increased levels of trace metals over biological entities, from a multi-perspective approach. An example of this is the Chañaral Bay, which has been exposed chronically to industrial mining waste with a high concentration of metal residuals, principally (but not exclusively) Cu. This region has been studied before with special emphasis in macroorganisms, and it has been possible to determine an important decrease in biodiversity and differential community structures in the intertidal zone

(J.C. Castilla, 1983; J. Correa et al., 1999; Medina et al., 2007; Lee & Correa, 2007, Moran et al., 2008). In the case of microorganisms, there are data related to microbial groups that are significantly influenced in their abundance by bioavailable Cu concentrations. With this background, it has been possible to determine that Cu is the main pollutant in the area and could influence microbial communities in terms of composition and abundances for planktonic organisms and communities associated to particles or surfaces (Andrade et al., 2006; De la Iglesia et al., 2012; Henríquez-Castillo et al., 2015).

Finally, with the framework exposed here, it is precisely to consider PPEs as a model group that represents a high potential for environmental analysis in a disturbance ecology context. In the present work, multiple approaches are proposed to understand the effect of environmental disturbances on PPEs ecology and physiology. Field observations and characterization plus community level experiments lead this work to a better description and understanding of the effect of metal increases in natural environments, with an ecological perspective. Furthermore, cellular models allowed to explore more mechanistic insights into field observations leading to the development of new hypothesis and interpretation of phenomena observed.

1.2 Hypothesis

"Disturbances by trace metals in coastal systems, especially copper, will produce stable alternative communities of photosynthetic picoeukaryotes, by altering the abundance of specific taxa by direct effects on nutrient metabolism."

1.2.1 General Objective

"Determine trace metals effects over picoeukaryotes community stability, identifying responsive groups to Copper and the effect over nutrient metabolism."

1.2.2 Specific Objectives

1.- To describe the effect of physical-chemical features of water column over picoeukaryotic microbial communities exposed to Copper enrichment's in natural environments in a disturbance ecology framework.

2.- To identify susceptible picoeukaryotic groups over simulated Copper depositions events in microbial communities context.

3.- To evaluate potential functional disturbances over picoeukaryote cellular model physiology under Copper challenges and explore micro and macro nutrients functional interactions.

Chapter 2

Decoupling of biotic and abiotic pattern in coastal areas under chronic trace metals disturbances

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2.1 Abstract

Coastal systems are highly productive marine regions that host a large number of human activities. Unfortunately, since the industrial revolution, levels of trace metals in these regions have increased. Microorganisms use trace metals as micronutrients for key metabolic processes, but in excess, may result in deleterious effects mainly mediated by oxidative stress. In coastal systems, picoeukaryotes represent a diverse and abundant group of microorganisms with widespread distribution and fundamental roles in biogeochemical cycling. In this study, we combined different approaches to explore the seasonal variability of picoeukaryotes in a polluted coastal area affected by chronic trace metal disposure at the south-eastern Pacific Ocean. Through remote and field measurements to monitor environmental conditions, analytical techniques to explore the chemical features of the water column, and 18S rRNA gene sequencing for taxonomical profiling, we determined metal chronic exposure effect on the temporal variability of picoeukaryote communities. Our results revealed the presence of a stable, homogeneous, and constant community, despite the physicochemical seasonal variations, with a high abundance of Bacillariophyta but low abundance of Mamiellophyceae, two common coastal groups. These results suggest that chronic trace metal contamination reduces the temporal heterogeneity of coastal picoeukaryote communities, with a decoupling between abiotic and biotic patterns.

2.2 Introduction

Coastal regions are highly valuable for both global primary productivity and ecosystem services (Antoine et al., 1996; Paytan et al., 2009). However, intensive land use by humans places high pressure on coastal ecosystem functions (Cloern et al., 2016). The concentration of trace metals in coastal environments has increased due to anthropogenic activities, including the use of antifouling paints of ships, fertilizers for agriculture, and intensive mining (Kennish, 2002; Nogales et al., 2011). Trace metals deposited in coastal areas often interact with other chemicals present in seawater and are then transported through ocean currents (Duce et al., 1991; Paerl, 1997; Paytan et al., 2009; Jordi et al., 2012) spreading to surrounding areas. Some coastal regions have endured chronic disturbance due to increases in trace metal concentrations. While chronic disturbances induce large variations in the abundance and composition of macro-organism communities, the effect and magnitude of these impacts on microbial communities are still poorly understood. Trace metals alter the structure and function of the bacterial communities that inhabit these regions, leading to changes in primary productivity (Morán et al., 2008; Nogales et al., 2011).

Trace metals are persistent pollutants and receive specific attention due to their high dispersal capacity and toxic effects that can change the structure of microbial food webs (Sunda and Huntsman, 1995; Sunda and Huntsman, 1998; Nogales et al., 2010). Elevated concentrations of trace metals in seawater decrease the abundance of different microbial groups, such as the cyanobacteria *Synechococcus* and the Prasinophyceae *Prasinoderma* and *Pyramimonas* (Debelius et al., 2009; Debelius et al., 2010; Henríquez-Castillo et al., 2015). Furthermore, changes in the structure of bacterial communities have also been reported (Henríquez-Castillo et al., 2015; Coclet et al., 2017).

Picoeukaryotes (PE) (eukaryotes $< 3 \mu\text{m}$ in cell diameter) are a highly diverse planktonic group, extremely important in open oceans (Shi et al., 2009) and also predominant in coastal zones (Collado-Fabbri et al., 2011; Tragin and Vaultot et al., 2018). They have gained relevance due to their widespread distribution (Masana et al., 2004; Countway Caron 2006; Vaultot et al.,

2008; de Vargas et al., 2015), and critical impact on the carbon cycle and global geochemistry (Joint et al., 1986; Li et al., 1994; Marañón et al., 2003; Worden et al., 2004; Buitenhuis et al., 2012). In most oceanic areas, PE community is dominated by Dinophyceae or Bacillariophyta (de Vargas et al., 2015; Tragin and Vaultot, 2018). Otherwise, coastal systems exhibit a high presence of Chlorophytes from the order Mamiellales and, in certain circumstances, a single Mamiellales specie can outnumber in some environments (Not et al., 2004; Countway Caron 2006; Shi et al., 2009; Collado-Fabbri et al., 2011; Balzano et al., 2012; Ribeiro et al., 2018). PE stably respond to long-term environmental fluctuations, and exhibit seasonal variations that indicate strong coupling between abiotic conditions and the PE community composition (Acevedo-Trejos et al., 2014; Hernández-Ruiz et al., 2018; Giner et al., 2018; Lambert et al., 2019). However, the responses of this highly diverse group to anthropogenic environmental variations are unclear, and our understanding of such responses in environments with a high concentration of trace metals needs to be improved.

In the south-eastern Pacific coast, several bays have been contaminated for the past decades with trace metals from mining activities (Medina et al., 2005). These bays are adjacent to the undisturbed bays within the same region that exhibit similar oceanographic conditions (Correa et al., 1999; Henríquez-Castillo et al., 2015). Therefore, this is an excellent site for assessing the impact of chronic trace metal pollution, as comparison between bays over time allows a robust analysis of the variation of specific microbial groups, such as the PE.

Our study aims to determine if chronic exposure to trace metals affects both the photosynthetic and the heterotrophic components of the PE communities, and how this effect influence the seasonal variability of these communities. We hypothesized that chronic exposure to trace metals will influence the seasonal variability of the coastal PE community structure, and we predict that, in chronically polluted coastal regions, the seasonal influence on the composition and structure of PE will be low, however, in less contaminated sites, the seasonal changes will exceed the effects of the disturbance.

2.3 Material and Methods

2.3.1 Sampling

Sampling campaigns were conducted between January 2011 and March 2014 (N = 11) in two adjacent bays located 5 km apart in the Chañaral area of the northern coast of Chile (Fig. S1). Playa Blanca (26° 13'27" S, 70° 40'2" W) is a coastal site with low copper concentrations (LCS), while Playa La Lancha (26° 10'58,20"S, 70° 39'45,70" W) has high copper concentrations (HCS) (Henríquez-Castillo et al., 2015). During each sampling campaign, seawater samples were collected at a depth of 5 m using 5-L Niskin bottles. The seawater samples were then filtered on land through 20 μm , 3 μm , and 0.2 μm pore-size polycarbonate filters (Millipore, Darmstadt, Germany) using an acid-washed polypropylene tube filtration system.

Samples for determining the heavy metal (copper [Cu], iron [Fe], zinc [Zn], molybdenum [Mo], Cobalt [Co], Cadmium [Cd], Lead [Pb]) and macronutrient (phosphorus [P], nitrogen [N], and silicic acid [SiO_4^-4]) concentrations were collected with 1-L metal-free Kemmerer bottles (maximum depth of 1 m). The physicochemical parameters of seawater were measured in-situ using a conductivity, temperature, depth, and oxygen (CTDO) profiler (SBE19, Seabird electronics, Bellevue, WA, USA).

2.3.2 Satellite Data Analysis

To determine the seasonal variations of environmental parameters in the Chañaral area, level-three AquaMODIS monthly data with a pixel resolution of 4 km were analysed (available online at <https://oceancolor.gsfc.nasa.gov/>). The data files were pre-processed using the SeaDas software (<https://seadas.gsfc.nasa.gov/>) and six NASA products were merged into a one single file: chlorophyll-a (Chl-a), photoactive radiation (PAR), superficial sea temperature (SST), particulate organic carbon (POC), particulate inorganic carbon (PIC), and the diffuse attenuation coefficient at 490 nm (K_d490). After merging, the region of interest was selected and processed to minimize the number of pixels without data, eliminate land pixels, and

identify regional mesoscale processes. This produced a rectangular region of 13×19 pixels (in x, y coordinates). In total, 492 photographs were analysed, corresponding to the six products at level three between January 2010 and October 2016 (82 months). Mean monthly data were used to analyse the seasonal effects using Seasonal-Trend Decomposition Procedure Based on Loess (Cleveland et al., 1990). Additive ($x_t = \text{Trend} + \text{Seasonal} + \text{Random}$) or multiplicative models ($x_t = \text{Trend} \times \text{Seasonal} \times \text{Random}$) were selected by minimizing the sum of the squares of the autocorrelation factors for each satellite time series (the complete original time series data and three-component decompositions are available in Fig. S2).

2.3.3 Chemical Determinations

The macronutrient and total Chl-a concentrations were measured following standard methods (Hansen & Grasshoff, 1983). The total dissolved divalent metals (Cu, Fe, Zn, Mo, Co, Cd, and Pb) were determined by inductively coupled plasma mass spectrometry (ICP-MS; Thermo Fisher Scientific, Waltham, MA, USA). All easily electro-reducible Cu species (labile copper) were measured by square-wave anodic stripping voltammetry (ASV) using a Metrohm 797 VA computrace system (Herisau, Switzerland), as described by Andrade et al. (2006).

2.3.4 Molecular Analysis

The total DNA was directly extracted from the $0.2\text{-}\mu\text{m}$ filters following the phenol:chloroform protocol described by Fuhrman et al. (1988). The integrity and concentration were determined by 1% agarose gel electrophoresis and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), respectively. These parameters were used as a quality index for further analysis. To determine the taxonomic composition of the PE present at each sampling site, 18S rRNA gene sequencing was conducted at the Center for Genomics and Bioinformatics, University Mayor, Chile. The hypervariable region V9 of the 18S rRNA gene was amplified using the 1391f and EukBr primer pair (Amaral-Zettler et al., 2009). The PCR reactions were conducted in a 35-L solution of a Taq buffer with a final concentration of $1 \times, 2 \text{ nM}$ of MgCl_2 , 0.3 nM of dNTPs, 0.3

of M each primer, 2.5 units of GoTaq Flexi DNA Polymerase (Promega, Madison, WI. USA), and 1-5 ng of template DNA. The amplification conditions were as follows: 3 min of initial denaturation at 94 °C, 28 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension of 72 °C for 10 min. For each DNA sample, triplicate amplicons were generated and pooled for further sequencing. The combined amplicons were quantified by conducting a standard qPCR assay using an Illumina Library Quant Kit (KAPA Biosystems, Wilmington, MA USA) following the manufacturer instructions, and then equimolarly pooled and sequenced using an Illumina Miseq following the protocol described by Caporaso et al. (2011). The qPCR-quantified amplicon pool was sequenced in 300 cycles (Illumina Miseq kit, San Diego, CA USA). The raw sequence data were deposited in SRA under BioProject number 00000.

2.3.5 Sequence Data Handling and Statistical Analysis

The sequence assembly, filtering, and operational taxonomic unit (OTU) determination were conducted according to Caporaso et al. (2010). Quality filters were used to determine that the minimum and maximum lengths of the reads were within 100-120 bp, and OTUs were picked following the UCLUST method (Edgar, 2010); the sequences were clustered with threshold values of 97%. The sequences were only aligned against the selected eukaryotic sequences contained in Silva seed v. 123 (Pruesse et al., 2007), and the taxonomy of the OTUs was assigned against the Protist Ribosomal Reference (PR2) v. 4.5_gb203 database (Guillou et al., 2013). An OTU table was generated without considering the reads assigned to Metazoans and unassigned reads, which represented 25% of the dataset. All downstream analyses were conducted in R v. 3.2.1 using the Vegan v. 2.4-1 package. The samples were standardized by trimming the OTU table at an abundance value of 0.03%, and rarefaction analysis was conducted using the vegan package (Oksanen et al., 2008). After rarefaction normalization, all OTUs without reads were discarded from the OTU tables. Clustering analysis was conducted using the Clustsig v. 1.1 package in R (<https://CRAN.R-project.org/package=clustsig>), and SIMPROF

analysis was conducted to test the stochasticity of the grouping patterns. The analyses were conducted following the complete linkage clustering method and by using Bray-Curtis to compute the distances between samples. The algorithm was run using `num.simulated=1000` and `num.expected=1000` arguments. Ordination analysis was conducted with the `vegan` package using the assigned data at different taxonomic levels. Venn Diagrams were produced using the `Venn Diagram` package v. 1.6.17 for the different sampling sites (HCS and LCS), and seasons (Summer and Winter).

2.4 Results

2.4.1 Environmental characterization

Satellite data were used to describe the environmental conditions of the coast in the Chañaral area within the south-eastern Pacific upwelling system. Physical parameters, such as PAR and SST, oscillated seasonally, while Chl-a, PIC, and POC exhibited no clear patterns, but slightly increased during the summer (Fig. 2.1A). The Chl-a concentration and Kd490 were strongly correlated at a regional scale (Pearson coefficient: $r=0.98$; $P<0.05$). Principal component analysis (PCA) showed that these parameters accounted for 79.4% of the variance (Fig. 2.1B). At a local scale, most parameters exhibited a consistent seasonal pattern in which they increased during summer, excluding POC, which exhibited a noisy and almost constant pattern. Furthermore, the eigenvectors related to SST and PAR exhibited an almost perpendicular relationship with the biological variables Chl-a and Kd490, and the PIC related to POC. Overall, the physicochemical and biological variables measured using satellite data exhibited a clear seasonal pattern, and cycles of twelve months occurred consistently throughout the study period (Fig. 2.1B).

The trace metal concentrations in seawater significantly differed between the HCS and LCS samples (Fig. 2.1C), and the total dissolved and biologically available Cu (Cu_{ASV}) were variables that differed most significantly between sites. Other trace metals, such as Fe, Mo, and Zn, also contributed significantly to site differentiation. However, there were no significant variations in the macronutrient (nitrate, phosphate, and silicic acid) concentrations between sites (Table S1).

2.4.2 Biological Characterization

Following the pre-processing and filtering steps, 36,387 reads per sample (N=11) in well-defined rarefaction curves were obtained, with 1,516 OTUs in the dataset (Fig. S3). The PE community present in the area was dominated by Bacillariophyta ($46.5 \pm 8\%$, mean \pm SEM), Mamielophyceae ($30.7 \pm 6.2\%$), Prymnesiophyceae ($26.2 \pm 3.8\%$), Syndiniales ($25.8 \pm 1.8\%$), Marine

stramenopiles -MAST- ($22.1 \pm 3\%$), Dinophyceae ($20.7 \pm 2.7\%$), Spirotrichea ($19.3 \pm 4.7\%$), Basidiomycota ($18 \pm 4.4\%$), Chrysophyceae ($16.4 \pm 4.6\%$), and Dinophyta ($16.1 \pm 5.5\%$) (Fig. 2.2A). The cluster analysis conducted based on the Bray-Curtis coefficient exhibited no clear seasonal patterns. Samples from the same month were grouped, regardless of the sampling site (Fig. 2.2B). When the abundances were decomposed into the top 10 OTUs of the total dataset, per site, and per season, autotrophic organisms were predominant in the Chañaral area. Bacillariophyceae and Mamiellophyceae were the predominant classes and exhibited similar abundances through time. Moreover, in the LCS samples collected during winter, there was a single dominating OTU assigned as *Thalassiosira* ($51.3\% \pm 22.4$ of the total abundance) (Fig. 2.2C). Within the Mamiellophyceae class, the dominant OTU was assigned as *Micromonas*. Dinoflagellates were the predominant heterotrophic taxa over time. Despite this dominance pattern, the diversity and evenness exhibited no significant differences between seasons and sampling sites (Shannon max entropy index and Pielou's evenness index, data not shown).

The OTUs that were constantly represented spatiotemporally were analysed further, and it was revealed that the core PE community in the Chañaral area was composed of 807 OTUs that represented 53.2% of all the OTUs in the dataset and 98.7% of the total reads, with a clear predominance of autotrophic OTUs assigned to Bacillariophyta (OTU1-*Thalassiosira* and OTU2-*Chaetoceros*, Fig. 2.3A and 2.3B). The variable community was represented by 709 OTUs, corresponding to 46.7% of the OTUs and 1.3% of the total reads. This variable community was mainly composed of heterotrophic or mixotrophic taxa, particularly the Syndiniales order and Dinophyceae class (Fig. 4C). Significant changes in the relative abundances of 6.13% of the OTUs in the dataset were detected between seasons and/or sites (93 low-abundance OTUs). Among those, only 10 OTUs significantly differed between sites (Table S2).

The trace metals concentration strongly influenced the distribution of samples, according to the analysis of the interactions between the OTU abundances and in-situ environmental parameters, which explained 41% of the total variance (Fig. 2.4). The concentrations of Zn, Cu, and Cu_ASV had the highest contributions to the observed variance. The effects of Cu and Cu_ASV on the samples collected in summer were more profound, regardless of the sampling area.

2.5 Discussion

Despite the limitations of using remote sensing in nearshore environments, the predicted seasonal pattern proposed for this area is consistent with the in-situ reports and modelling approaches available for the northern part of the Humboldt-CS, indicating that remote-sensing data can be used for the long-term monitoring of coastal environments (Penven et al., 2005; Thiel et al., 2007). Moreover, the patterns described here are consistent with seasonal descriptions of marine systems, such as temperature fluctuations and interactions with biological components, including the Chl-a concentration. Thus, seasonal geophysical variations influence the biological features of these environments (Vargas et al., 2007; Echevin et al., 2008; Calvo-Díaz et al., 2008). However, the concentrations of trace metals differed consistently between sites, regardless of the season. This indicates that the trace metals were not transported by the coastal northward circulation of the Humboldt-CS to the adjacent bays. The geomorphology of the enclosed bays and sedimentation rate may prevent the transport of metals to nearby unpolluted areas. The reported heavy metal concentrations, particularly those of the different Cu forms, are within the range of previously reported values for the study area (Andrade et al., 2006; Henríquez-Castillo et al., 2015). Therefore, this chronic disturbance is temporally stable.

The PE community structure patterns in the global ocean highlight the dominance of the heterotrophic component of microbial assemblages, in terms of their abundance and diversity. The contributions of the presumably parasitic MALV I-II and Dynophyceae to this functional group are important, as they are widespread in oceanic waters and coastal environments and their ecological relevance is increasing (Masana et al., 2004; Worden, 2006; Masana et al., 2015; Song et al., 2017; Hernández-Ruiz et al., 2018; Zouari et al., 2018). In contrast, the PE composition of the chronically disturbed area studied here shows that the most sequences were concentrated in autotrophic taxa. However, a large portion of the detected OTUs was associated with heterotrophic taxa, which is in concordance with previous studies conducted in Humboldt-CS (Parris et al., 2014; Vargas et al., 2007).

Our results show that the PPEs in the Chañaral area is dominated by Bacilliarophyta, and

the contributions of the Mamiellophyceae class are lower. This is in contrast to the trends observed in other Humbolt-CS sites, where Mamiellophyceae taxa are predominant in the PPE, and coastal waters worldwide (Collado-Fabbri et al., 2011; Balzano et al., 2012; Cabello et al., 2016; Tragin and Vaultot, 2018; Wu et al., 2017; Ribeiro et al., 2018). Furthermore, the composition of the community in the Chañaral area was stable over time, exhibiting little variation both between seasons and sites. This suggests that the composition of the PE assembly is decoupled from seasonal environmental forcing, which is likely due to the pressure exerted by the chronic copper exposure faced by the PE assembly that exceeds the pressure exerted by seasonal factors. However, it is imperative to evaluate the alternative hypothesis to elucidate the actual area affected by pollution and the potential changes in ecosystem structures and functioning at global and local scales. Under the current scenario of global development and the growth of the human population and economy, the change rate in coastal systems will increase (Cloern et al., 2016). This highlights the importance of understanding how anthropogenic pressures alter the structure and functioning of the microbial communities that inhabit coastal environments. In this study, by combining remote sensing, and chemical and biological in-situ characterization through profiling the taxonomic structure PE communities, we demonstrated that chronic Cu disturbance in a coastal area resulted in particular community patterns that differ to those in previous reports (Masana et al., 2015; Cabello et al., 2016; Wu et al., 2017; Hernández-Ruiz et al., 2018). Moreover, these effects exert a pressure that can uncouple the community features from abiotic seasonal variations, such as the abundance and composition of PE assembles. Furthermore, the effect of trace metal pollution on the PE composition extended beyond the area where the metals were effectively detectable, suggesting that the area of influence of metal pollution could exceed the area within which the trace metal concentrations in the water column were higher. The spatiotemporal homogeneity of the PE community composition and structure suggest that these communities were decoupled from natural oscillations in environmental conditions.

From the perspective of multiple alternative states in community ecology (Holling, 1973; Scheffer and Carpenter, 2003), our results suggest that the studied PE communities exhibit a new and different stable state, where seasonal changes in the environmental conditions have im-

perceivable effects on the community structure (Shade et al., 2012). This type of chronic effect should be studied in more detail and, if accurate, should be considered in predictive models of coastal edge usage. The decrease in the natural heterogeneity of communities renders them prone to large declines in abundance and diversity following catastrophic events, and local and regional extinctions tend to be equally high. That is, chronic contamination has shifted the community from its initial state to an alternative state, which has decreased the community's spatial heterogeneity, and the effects of natural variations in environmental conditions are surpassed by those of the chronic disturbance. Under the current scenario of global changes and the growth of the human population and economy, the change rate in coastal systems will increase (Cloern et al., 2016). This highlights the importance of understanding how anthropogenic pressures alter the structure and functioning of the microbial communities that inhabit coastal environments.

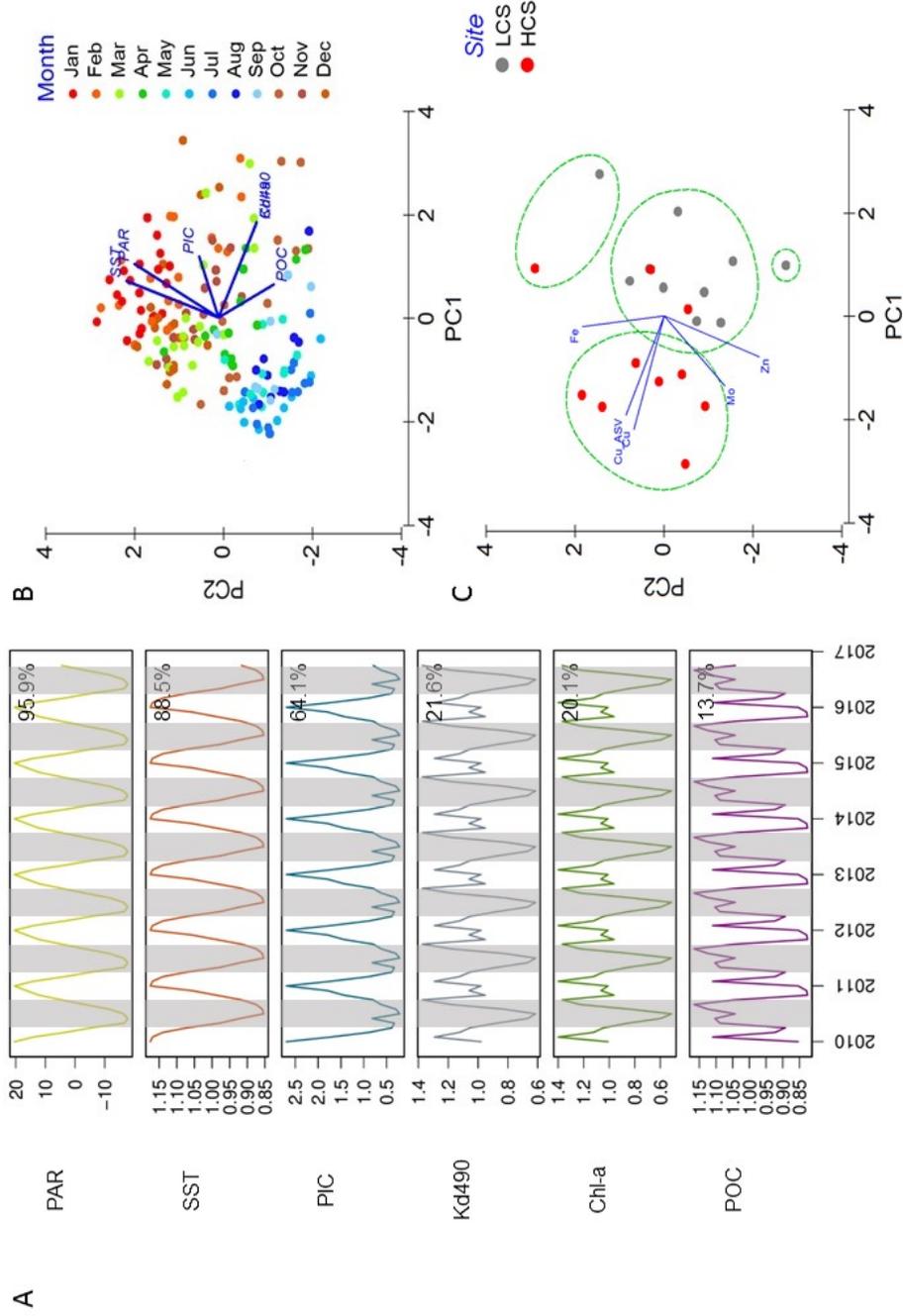


Figure 2.1: Characteristics of the study sites. (a) Decomposed seasonal pattern between January 2010 and October 2016. The time series dataset was collected from AquaMODIS mission of NASA as monthly data. The white and grey boxes represent the summer and winter data, respectively. (b) PCA of the study period. The points represent one month of a 9×9 -pixel grid within the Atacama grid and the arrows represent the eigenvectors of each parameter analysed. (c) PCA of the trace metal concentrations for each sampling site.

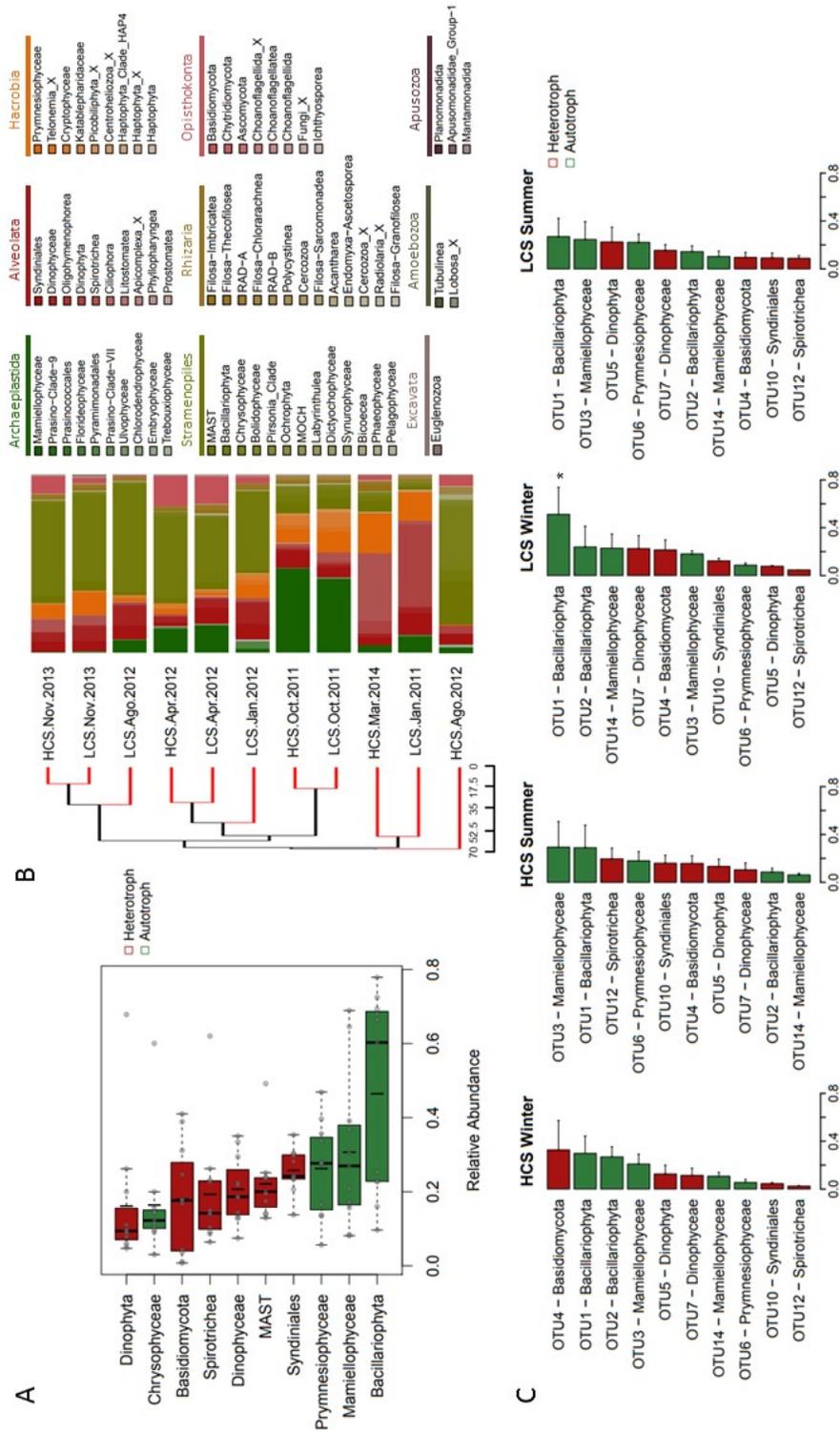


Figure 2.2: Community description and similarity analysis. (a) Boxplot of the corrected relative abundance of all OTUs assigned at PR2 level 4 (Order). The PR2 was ranked by the mean of each category (small vertical line). The values of each sample are exhibited as grey dots. (b) Relative abundance plots showing the sorted cluster relationships based on the Bray-Curtis dissimilarities and assessed by the SIMPROF test. The red branches represent significant SIMPROF groups. (c) Ten taxa that were pre-ranked with the highest abundances, assigned at PR2 level 4 and classified as autotrophic (green) and heterotrophic (red) (standard errors are shown).

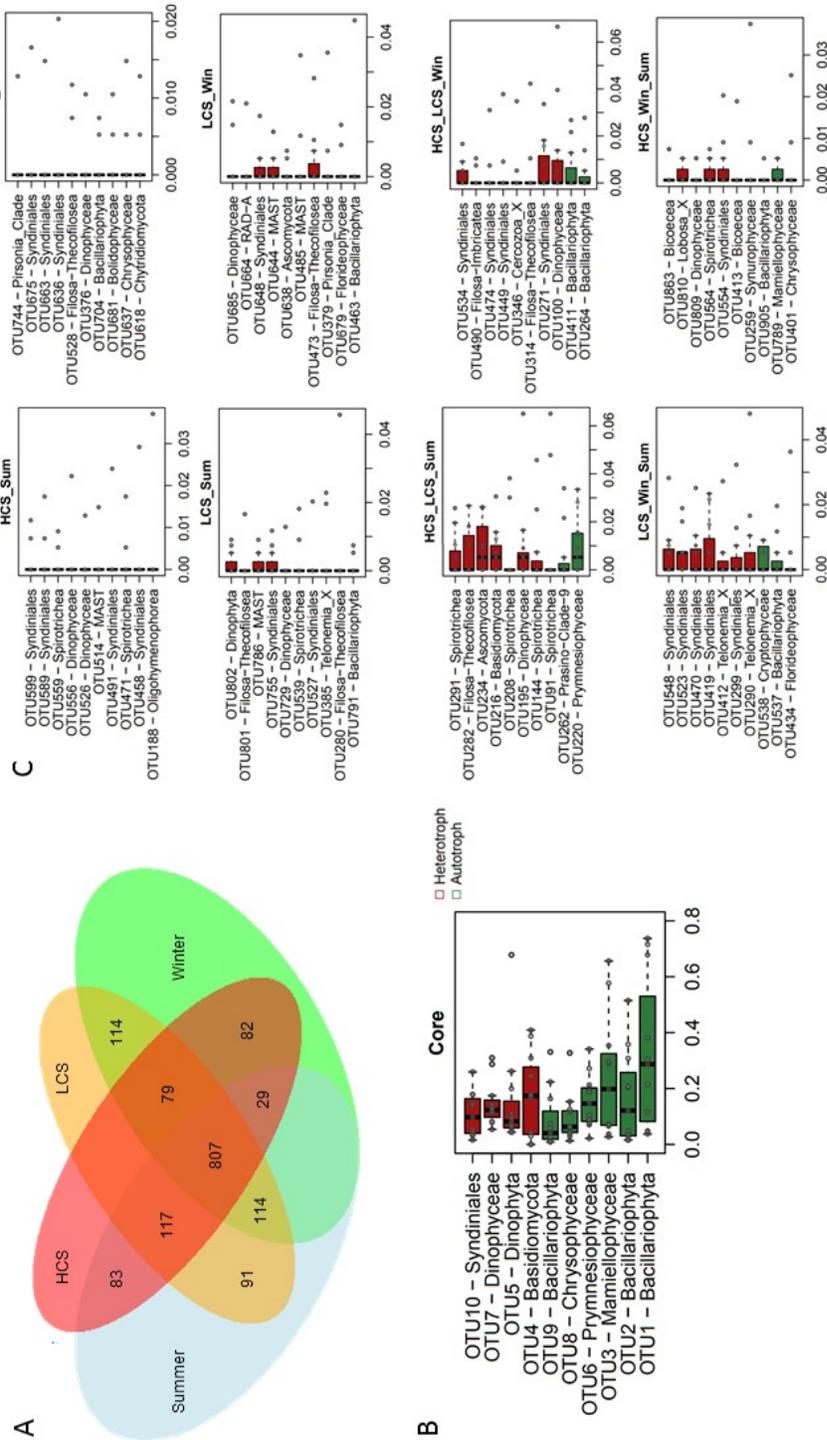


Figure 2.3: Categorical composition analysis. (a) Venn diagrams of the seasonal categories and sampling sites. The numbers represent the number of OTUs per category. (b, c) Boxplots of the corrected relative abundances of the top ten OTUs per category assigned at PR2 level 4. The ranking of the OTUs is also shown.

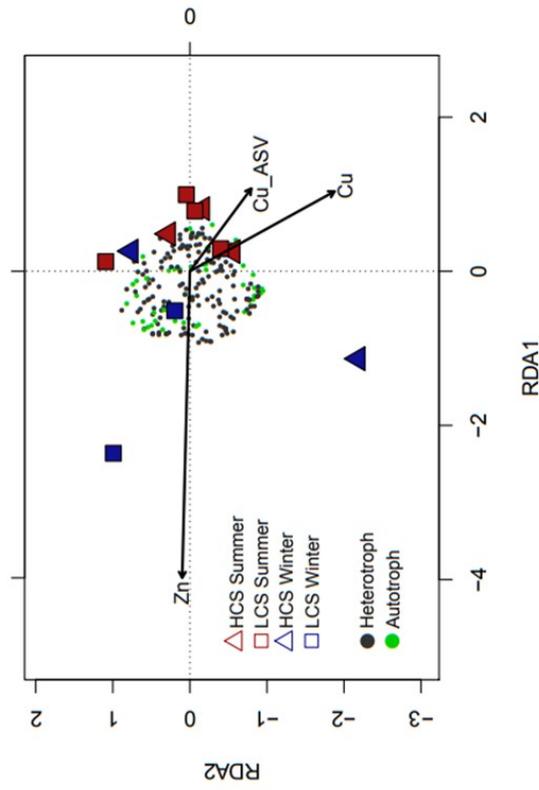


Figure 2.4: Redundancy analysis conducted for OTUs assigned at PR2 level 6, capturing 41.8% of the total variance. The assigned OTUs are shown as green (autotrophic) and black (heterotrophic) dots and the centroids of the LCS and HCS samples are indicated by squares and triangles, respectively. The winter and summer samples are indicated in blue and red, respectively. The significant variables are also shown as eigenvariables (Zn: $P=0.005$, Cu: $P=0.03$ and Cu_{ASV} : $P = 0.055$)

Chapter 3

Differential responses of coastal Mamiellophyceae to copper challenges

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3.1 Abstract

Today's ocean shows a clear footprint of human activities, with some of them causing a profound effect on ecosystem structures and functioning. Copper residuals have emerged as a highly concerning pollutant due to its toxic effects and an increase in its availability, by anthropogenic origin disposals and global change future scenarios. Because different human activities related to coastal line like differential land usage and waste transport, coastal environments are a region with high susceptibility to chemical pollutants. Further, the effect of coastal copper pollution on eukaryotic microbial communities, a critical coastal component for global carbon cycle, environmental services, and human activities, are still not well understood. Microcosms experiments appear as a suitable tool to test eukaryotic microbial community dynamics with toxic challenges, providing data for the development of theoretical frameworks that will allow constructing predictive models based on identifying microbial sentinels. Our results showed taxa-dependent responses to copper pollution in a model coastal photosynthetic picoeukaryotic community and differential dynamic responds patters related to copper inputs. Moreover, ecological relevant Mamiellophyceae taxa showed significant differential effects in two highly relevant genera: *Ostreococcus* highly sensitive and *Micromonas* resistant to copper challenges at community level.

3.2 Introduction

Today, no area of the world's ocean can be pointed out as free of human influence, and anthropogenic pressures on marine ecosystems have been shown to be increasing in magnitude. Pollutant addition due to waste transport and disposal as byproduct of anthropogenic inland activities, is a major concern in modern oceans, with coastal regions showing the highest impact indices of pollution (Halpern et al., 2009; Halpern et al., 2015). In some coastal areas, historical industrial activities have had recurrent waste disposal events leading to major concerns in environmental and human safety due to the toxic nature of the mixtures released into the environment (Correa et al., 1999; Kennish, 2002; de Souza Machado et al., 2016). Among the most common mining wastes, copper (Cu) stands out because of its ambivalence regarding the biology of the organisms. The role of copper as part of protein structure, electron transfer reactions, enzymatic reactions and other key biological functions is opposed to its toxic effects that influence fundamental processes, like photosynthesis, nutrient uptake and cellular growth (Peers & Price, 2006; Biwas et al., 2013; Lelong et al., 2013; Pillai et al., 2014). Moreover, it can lead to changes in higher organization levels, like communities and ecosystems, by diversity and specie richness loss (Le Jeune et al., 2006; Le Jeune et al., 2007; De la Iglesia et al., 2012; Henriquez-Castillo et al., 2015). Additionally, according to current ocean models, free copper ion in the sea water could increase by a factor of up to ~ 2 by 2100 (Millero et al., 2011; Hoffmann et al., 2012), increasing concerns by its toxic effects (Kenneth H. Coale, 1991; Debelius et al., 2009). The importance of understanding how different coastal taxa responds to copper disturbances emerges in perspective of the globally distributed impact of this pollutant in a changing ocean.

One essential group of organisms inhabiting coastal oceans are eukaryotic phytoplankton, a group known to fulfill fundamental ecological roles in aquatic food webs, biogeochemical cycles, and Earth's climate system (Azam & Worden, 2004; Lima-mendez et al., 2015; Worden et al., 2015). Eukaryotic phytoplanktonic assemblages have shown copper-mediated changes in abundance and community structure, making them a good biological indicator for environmental

disturbances (Le Jeune et al., 2006; Paytan et al., 2009; Henriquez-Castillo et al., 2015). Inside this group, photosynthetic picoeukaryotes (PPEs, cells with 3 - 0.2 μm diameter) have been found to possess a high and unexplored diversity (Worden et al., 2006; Vaultot et al., 2008; de Vargas et al., 2015), a meaningful contribution to coastal biomass and trophic carbon transport (Jardillier et al., 2010; Rii et al., 2016), and a high contribution to primary production, up to $\sim 73\%$ in coastal waters (Worden et al., 2004; Orsi et al., 2018). Besides, PPEs have shown to be a particularly responsive group to copper pollution (Coclet et al., 2017), making them a suitable study group for this type of environmental disturbances. The advent of high throughput sequencing made recently possible to recognize taxa that shape PPEs assemblages (Amaral-Zettler et al., 2009), highlighting the relevance of the Mamiellophyceae class in terms of relative abundances in coastal and surface waters of the modern ocean, with the genus *Ostreococcus*, *Micromonas* and *Bathycoccus* as the most abundant members of this *taxa* (Guillou et al., 2004; Tragin & Vaultot, 2018).

Community level studies can lead to valuable and more realistic insights than single organism or population level studies when it comes to understanding the effects of anthropogenic pollution over environments (Clemets & Rohr, 2009; Cravo-Laureau et al., 2017). Microcosm approaches represent a suitable tool to study and analyze the ecological changes on microbial communities produced by fluctuations in physicochemical parameters in fluid environments, allowing to test hypothesis in ecological context (Escaravage et al., 1996; le B. Williams & Egge, 1998; de le Broise & Palenik, 2007). However, the sampling and the microcosms approach itself represent a major force of change that should be controlled statistically. Thus, the design of well-replicated experiments is critical to enable a statistical differentiation of this effect from the one of interest (Petersen et al., 1997; Berg et al., 1999). Moreover, to test effect over assemblage composition in relation to environmental disturbance, a previous description of the previous states are needed to quantify the effect (Shade et al., 2012; Tett et al., 2013). Also, the identification of the assemblage components (*taxa*) that change or do not change after copper disturbance could become a cornerstone for the construction of prediction models regarding how ecosystem processes will be affected (Worden et al., 2015), and the selection of suitable cellular

models to test for more physiological responses.

In this work, the changes generated in PPEs assemblies against acute exposure to copper were analyzed to understand how this pollutant modify coastal PPEs community structure. By the use of a highly replicated experiment, flow cytometry and next generation sequencing (NGS) analysis of 18S rRNA gene, variation of the PPEs community was follow through time. Besides an evident decrease of cellular abundance associated with copper exposure, our data indicate a differential response of coastal PPEs. Mamiellophyceae related taxa being dominant in early tested samples, in terms of two mayor genera detected *Ostrococcus* and *Micromonas* showed a differential temporal response pattern in fluctuation of relative abundance respect total community assemble.

3.3 Materials and Methods

3.3.1 Sample collection and microcosm experiment

Surface seawater was sampled from the southern limit of the Coastal Marine Research Station, ECIM, Pontificia Universidad Católica de Chile (33° 30' 7.00" S 71° 37' 56.62" O) on August 2017. 115 L of 200 μm pre-filtered seawater were collected in six, previously acid-washed (10% v/v HCl) and rinsed with Mili-Q water, 20 L bottles. Immediately after sampling, the 115 L liters of seawater were first homogenized and then, separated into 100 3L polycarbonate transparent bottles, previously acid-washed (10% v/v HCl) and rinsed with Mili-Q water. Each 3L bottle was filled with 1.15 L of collected seawater (experimental units). From the 100 experimental units, 50 were randomly assigned to *copper incubated* condition, to which copper chloride solution was added in order to achieve the 25 $\mu\text{g/L}$ final concentration. The 50 remaining experimental units correspond to the *control* condition, which were incubated without the addition of any compound. The bottles were incubated under a 12:12 photoperiod in cold room at 18°C.

3.3.2 Flow cytometry cell abundance measurements

In order to estimate the variations in photosynthetic picoeukaryote cellular abundances in response to copper disturbance, 1.35 mL were sampled into cryovials from three randomly chosen experimental units for each conditions at 4, 20, 44 and 68 hours after Cu amendment. These samples were fixed with 1% paraformaldehyde/0.5% glutaraldehyde solution in borate buffer, pH 8.4 adjusted, immediately freezed in liquid nitrogen and later stored at -40 °C until analysis. The samples were analyzed using an Influx flow cytometer (Instituto Milenio de Oceanografía, Universidad de Concepción, Concepción, Chile). Samples were run for 5 minutes at a flow rate of 25 $\mu\text{L}/\text{min}$ and through a combination of 457 nm and 532 nm lasers with a 692/40 nm filter. Picophytoeukaryote cell count was done based on the cell optical properties, using the FlowJo software (Tree Star, Ashland, OR, USA).

3.3.3 DNA extraction and molecular analysis

At 4, 20, 44 and 68 hours from the start of the incubation, 10 experimental units per condition were in serie filtered through 20 (nylon), 3 (polycarbonate) and 0.2 μm (polyethersulfone) pore size filter, in order to collect the picoeukaryote cells. Filters were stored in 2.0 mL Eppendorf tubes at $-20\text{ }^{\circ}\text{C}$ until DNA extraction. Tubes containing 0.2 μm filters were filled with lysis buffer (20% p/v sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 8.3). Subsequently, DNA extraction was made following the Phenol:Chloroform Fuhrman et al., 1988 modified protocol. DNA integrity was evaluated by 1% agarose gel electrophoresis and DNA was quantified using Qubit®2.0 Fluorometer (Thermo Fisher Scientific). Picoeukaryote extracted DNA was sent to the *Center for Comparative Genomics and Evolutionary Bioinformatics* (Dalhousie University, Halifax, Nova Scotia, Canada) for V4 region of 18S rRNA gene (400 bp) amplification and sequencing. Amplicons were generated following the Comeau et al., 2011 protocol, using the primer set E572F (5'-CYGCGGTAATTCCAGCTC-3') and E1009R (5'-AYGGTATCTRATCRTCTTYG-3') with Illumina adapters. Sequencing was performed on an Illumina MiSeq platform, using a 2 x 300 bp paired-end configuration.

3.3.4 Sequence data processing and analysis

Sequences were provided by the *Center for Comparative Genomics and Evolutionary Bioinformatics* in fastq format, with primers and adapters previously removed. Subsequent sequence processing was done with Mothur v.1.40.5 (Scholss et al., 2009). Reads were selected between 400 bp and 450 bp sequence length, containing no ambiguous bases and with as maximum of 8 homopolymers. Also, all singletons were removed. The remaining reads were aligned to SILVA seed (release 128) eukaryote subset and then the alignment was proceeded by trimming and gap removal. After the removal of chimeras, datasets were pre-clustered and Operational Taxonomic Units (OTUs) were built at 97% similarity with taxonomy assigned against the PR² database (Guillou et al., 2013). Further processing, was made in R environment v.3.4.1 (<http://www.R-project.org/>) and consist in Rarefaction analysis (Fig. S1) and raw abundances per sample (Fig.

S2), which conclude in the use of raw data after abundance filter evaluation at 0.005% . Final processing consisted in removal of OTUs classified as Metazoa, Eukaryota_unclassified, Dinophyta, Opisthokonta_unclassified and Fungi. For the analysis of the photosynthetic component, a subset database was selected with Chlorophyta, Haptophyta, Cryptophyta, Ochrophyta, Rhodophyta, and Streptophyta_unclassified classified OTUs only.

3.3.5 Statistical analyses

All statistical analyses were performed using the R v.3.4.1 software (<http://www.R-project.org/>). Ordination analysis was run directly over abundance matrices using vegan package as well as distance computed metrics. Data management were made in R environment and Primer 6 software (Primer-E, Plymouth, UK). To identify dynamic changes over community structure Analysis of similarities (ANOSIM) was performed between samples belonging to different conditions and OTUs presenting statistically significant differences between *Control* and *Copper incubated* conditions, in at least one measured time were selected for population dynamics analysis. This resulted in 68 OTUs (equivalent to 93.211 reads), which represented a 27.27% of the total photosynthetic OTUs and 10.16% of the total OTUs within the data set. Then, these were grouped in 33 taxonomic categories according to level 7 PR² database. Also, SIMPROF analysis was made to find relationships among samples and taxa obtained by molecular approaches. These techniques were complemented with analysis of variance (ANOVA) as appropriate in order to identify significantly treatment effect in abundance changes and dynamic patterns. Finally, visualization was made in R environment with ggplot, gplot and base R packages.

3.4 Results

3.4.1 Copper effect over cellular abundances and groups dominance

Effect of acute copper exposure over PPEs cell abundance showed that mean cell counts of *Control* condition showed a constant trend in which cellular abundance were increasing throughout the evaluated time window. On the other hand, cell numbers in *Copper incubated* condition decreased drastically after 48 hrs of exposure to copper (Fig. 1, Upper). Even more when a clear effect of copper over cell abundance was observed, interaction between experimental condition and time was showed a significantly effect over PPEs cell abundance ($P < 0.05$). Also, a decreasing signal in 692nm laser channel were observed as well as a more diffuse pattern in forward scatter channel in comparison with *Control* condition over evaluated time window (Fig. 1, Bottom).

Moreover, to understand how changes in observed cell abundances are reflected in PPEs taxonomic distribution, the reads associated with photosynthetic OTUs were considered and selection resulted in 93,211 reads that were classified in 250 OTUs (74.4% and 37.37% of total reads and OTUs in the dataset, respectively). In terms of taxa proportions within samples, in early measured replicates composition (4 hours of exposition) at class taxonomic level between Conditions was Mamiellophyceae ($60.97 \pm 8.37\%$ for *Control* and $50.10 \pm 8.26\%$ for *Copper incubated*), Prymnesiophyceae ($20.80 \pm 6.26\%$ for *Control* and $50.10 \pm 8.26\%$ for *Copper incubated*), Cryptophyceae ($5.61 \pm 1.37\%$ for *Control* and $6.44 \pm 1.87\%$ for *Copper incubated*) and Bacillariophyta ($2.76 \pm 1.51\%$ for *Control* and $7.54 \pm 3.33\%$ for *Copper incubated*) dominated both disturbed and undisturbed systems (Fig. 2A).

Overtime incubation produce that *Control* condition remain with no differences for at least 44 hours. By contrast, *Copper incubated* condition shown a gradual relative decrease in Mamiellophyceae relative abundances, and an increase in Prymnesiophyceae groups (Fig. 2A). Dominance patterns of taxa ranked and classify by Class taxonomic level in final time evaluated were in *Control* condition as Prasino-Clade-VII ($37.67 \pm 17.41\%$), Trebouxiophyceae ($23.23 \pm 4.78\%$),

Mamiellophyceae ($15.82 \pm 14.95\%$) and Prymnesiophyceae ($11.41 \pm 9.42\%$); otherwise, for *Copper incubated* as Prymnesiophyceae ($51.95 \pm 0.54\%$), Mamiellophyceae ($14.29 \pm 5.40\%$), Trebouxiophyceae ($7.70 \pm 6.95\%$) and Bacillariophyta ($7.44 \pm 6.07\%$)(Fig. 2A).

3.4.2 Copper exposure over PPEs structure and temporal dynamics.

Effects of copper over PPEs community structure were analyzed through Bray-Curtis based NDMS analysis which showed a clear differential responses pattern over *Control* and *Copper incubated* condition (Fig. 2B). Replicates tended to remain closer in initial time evaluated for both experimental conditions. *Control* and *Copper incubated* treatment differentiation occurred from 4 hours of incubation onwards, followed by an dispersion increase among experimental units. Samples variation was clearly guided by on going time and treatment. Overtime differentiation resulted into two defined groups. (ANOSIM; $R = 0.862$, $P < 0.05$ for Time effect and $R = 0.529$, $P < 0.05$ for Condition effect).

Further analysis was carried out to examine response patterns at OTU level. Clustering analysis based on Bray-Curtis dissimilarity revealed that assemblages differentiate for *Control* over *Copper incubated* condition, and final times with differential grouping patterns for each treatment were observed. Within *Control* and *Copper incubated* conditions all replicates showed a similar pattern of relative abundance and OTUs dominance (Fig. 3A). Moreover, *Ostreococcus* dominance in *Control* condition was observed over Mamiellophyceae and other photoautotrophic related OTUs. Nevertheless, *Copper incubated* condition a different dominance pattern was observed, where *Micromonas* related OTUs dominated the photoautotrophic fraction, even more, *Ostreococcus* related OTUs showed a decrease in abundance over all copper treated samples. Additionally, *Bathycoccus* and *Phaeocystis* related OTUs showed a distinguishable dynamic effect in copper treated samples (Fig. 3A), in which a relative increase of abundance was observed. Further, at 68 hours of incubation a differential response pattern was also observed, in which at the end of the experiment *Prasino-Clade-VII-B-1* and *Phaeocystis* related OTUs dominated *Control* and *Copper incubated* conditions respectively, showing a clear deleterious effect

over Chlorophytes related taxa.

In terms of taxa grouping pattern, a clear differentiation among taxa was observed, in which relatively dominant taxa are distinguishable from low abundant taxa. For high relative abundance taxa, which account 89.81% of total relative abundance, only *Bathycoccus* and *Phaeocystis* showed a similar behaviour for experimental conditions over time, where other taxa showed an specific response pattern. This was also observed over less abundant taxa with a less clear grouping pattern.

Each of the above mentioned responsive and dominant taxa behavior through time was graphed for *Control* and *Copper incubated* conditions (Fig.3B). Most of the high abundance taxas evaluated showed an initial relative abundance comparable between experimental conditions. Differential dynamic behavior was observed in comparison among conditions for all top 6 PPE grouped taxa, with the exception of *Micromonas* and *Chrysochromulina* that showed a similar dynamic patter with initial difference conserved through time. Also, a higher decreasing rate in relative abundances was observed as caused by copper treatment for *Ostreococcus*, *Bathycoccus* and *Pyramimonas*. A rapid increase in abundance was observed only for *Minutocellus*, which showed a rapid response before 20 hours of exposure. Finally, a late maximal response group was identify, composed by *Phaeocystis* and *Prasino-Clade-VII-B-1* that showed an opposite response dynamic pattern, where for *Phaeocystis* related OTUs a consistent decreasing pattern was observed until 68 hours of incubation in which a favorable effect of copper treatment was observed. As opposed, *Prasino-Clade-VII-B-1* related OTUs, a consistent increased in relative abundances until 68 hours of exposure in which deleterious copper effect and a clear increase under control condition was observed.

3.5 Discussion

The study of photosynthetic picoeukaryote assemblage responses to copper disturbances has gained importance during the last decade (Stauber et al., 2005; Henriquez-Castillo et al., 2015; Coclet et al., 2017), due to the constant concern about chemical changes (here copper) over modern marine ecosystems and further potential changes of trace metal availability forced by anthropogenic activities and changing patterns in global system (Millero et al., 2011; Hoffmann et al., 2012). This microcosms study show a new insight, with high resolution, on the response of the specific components that shape PPEs community composition in coastal sea water.

Measurements of PPEs cell abundances showed significantly differences between *Control* and *Copper incubated* conditions through the interaction among time and treatments, suggesting that there was indeed an effect of copper over PPEs cell abundance. The effect observed in cellular abundances was a clear decrease due to copper exposition that increase overtime, as found before in previous works (Henriquez-Castillo et al., 2015; Coclet et al., 2017). Decreasing cellular abundances, in this experimental set up, suggest that if in natural environments an important decrease in cellular abundance and biomass is not observed, a population turnover and residential times of water masses that transport cells through currents became relevant as resilience mechanism to stabilized microbial communities. Moreover, taken into account that the copper concentration applied to the *Copper incubated* experimental units (25 $\mu\text{g/L}$) has been actually measured in contaminated coastal systems (Andrade et al., 2006), the decrease in cell abundances as a consequence of copper pollution could result in a modification of elemental cycles with unknown effects over ecosystems and eventual risk for services delivered by coastal ecosystems.

The stable assemble composition until 44 hours of incubation in *Control* condition was consistent with stable trend in cell abundances. Although there was a change in *Control* composition during the first 44 hours was in lower magnitude than occurring changes in composition for *Copper incubated* assemblages. Hence, it was also possible to differentiate between changes in composition mediated by copper. The last confirming microcosms approach as a valid experi-

mental setup to test disturbance hypothesis over microbial ecology.

The initial dominance of Mamiellophyceae classified OTUs within the microcosms was concordant with what has been previously described in coastal PPEs assemblages (Collado-Fabbri et al., 2011; Lambert et al., 2018). This dominance pattern remained unaltered in *Control* condition for at least 44 hours of incubation. However, a drastic change in composition was registered over 68 hours of incubation, which consisted in a radical decrease of Mamiellophyceae and increase of Prasino-Clade-VII and Trebouxiophyceae related OTUs. Prasino-Clade-VII has been found to be the dominant group of green algae in oligotrophic oceanic waters (Dos Santos et al., 2017). This pattern could be associated with a competitive advantage of this group of PPEs, given by possible nutrient depletion related to long term incubation in a microcosm experiment. Hence, microcosms approach to ecotoxicology should consider effects over controlled time windows.

Furthermore, a reorganization of the assemblage structure was observed. The rapid response and following gradual decrease of Mamiellophyceae in *Copper incubated* condition indicated susceptibility to copper inputs. The sensitivity of green algae, in particular of Prasinophytes, had already been demonstrated (Henriquez-Castillo et al., 2015), but different responses within this clade had not been explored in details. Our results suggests that copper disturbances could alter ecological patterns favoring organisms that are usually not dominant in this size fraction. Thus, this observation could be related to physiological factors in planktonic cells like nutrition and chemical species availability in water column and/or biological interactions like competitive advantages (alternative strategies) over specific functional groups that were present in the assemblage and modified when exposed to copper. A suitable hypothesis over copper increasing availability is that Prymnesiophytes result favored in natural environments leading to functional changes in ecosystems due to these organisms influences over carbon cycle in blooming season (Liu et al., 2015).

A drastic effect over Mamiellophyceae members was measured in the time window evaluated over the experimental setup, that shown a drastic dominance shift between *Ostreococcus* and *Micromonas* before 44hours of incubation. Especially, a rapid *Ostreococcus* decrease in relative abundance and a temporal dominance of *Micromonas* until 68 hours of incubation

which showed a general Chlorophytes decrease in relation with Haptophytes. The underlying factors that explain this pattern between these closely related taxa are unknown. Nevertheless, copper concentration dependent growth inhibition and potential coupling between Cu and Fe metabolisms have been proposed for *Ostreococcus* (Henriquez-Castillo et al., 2018). Furthermore, physiological and ecological distinctions between these two assemblage-dominant taxa like distribution along the water column, biogeography and motility (Tragin & Vauot, 2018) could have both an influence on their differential response.

Copper is still considered to be fundamental for economic and social development in modern societies. However, the management of wastes that contain copper are still problematic and closely related to coastal environments and the effects on marine biodiversity and productivity are still not well understood. This microcosms study reveals a rapid re-shaping, as a product of an artificial copper disturbance, in natural PPEs assemblage by altering the dominance patterns of key components. The identification of responsive components at genus level could lead to the eco-physiological mechanistic interpretations of field observations. Thus, allowing to generate predictive models in natural environments and identification of natural sentinels as key indicators for disturbance ecology.

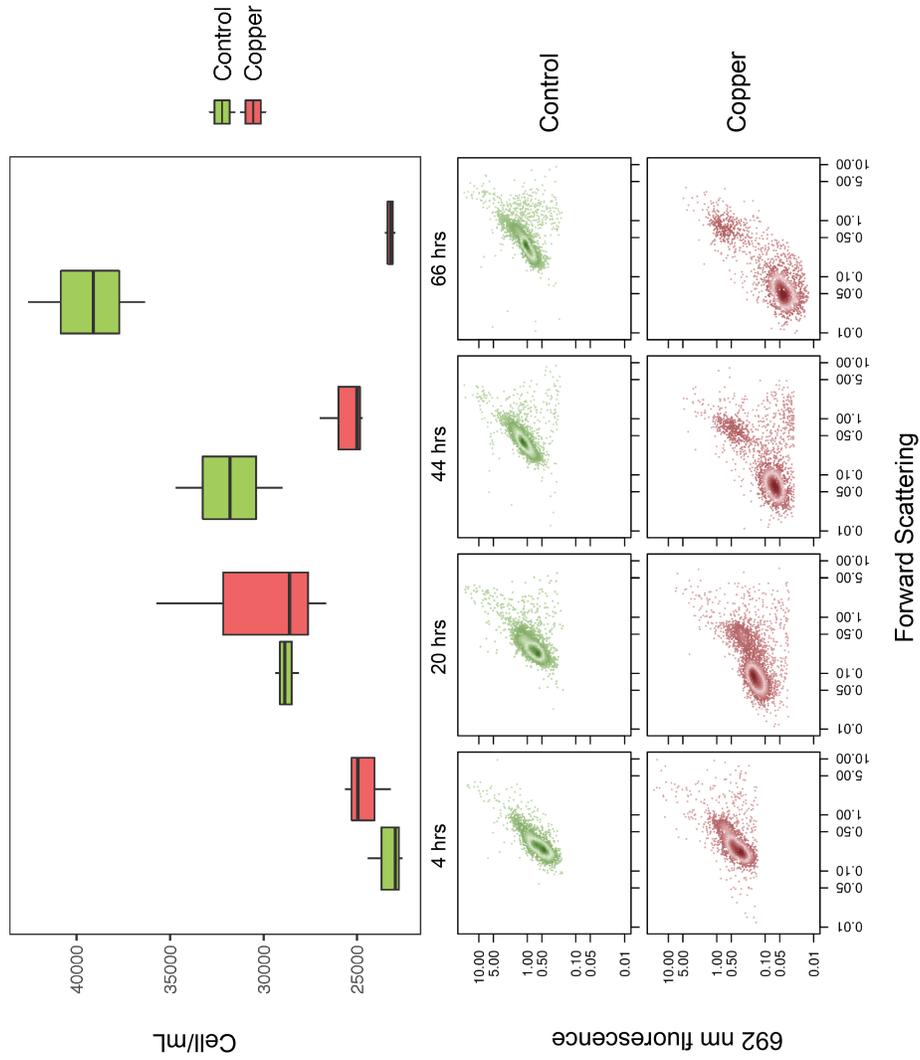


Figure 3.1: Cell abundances and Cytometry analysis. (upper) Cellular abundance variability in each experimental condition for correlative sampled time points. (bottom) Representative cytograms per experimental condition over time in concordance to sampling points showing fluorescence particles exited at 692nm as a proxy to Chlorophyll concentration per particle and Forward scatter signal as a proxy of cell size distribution.

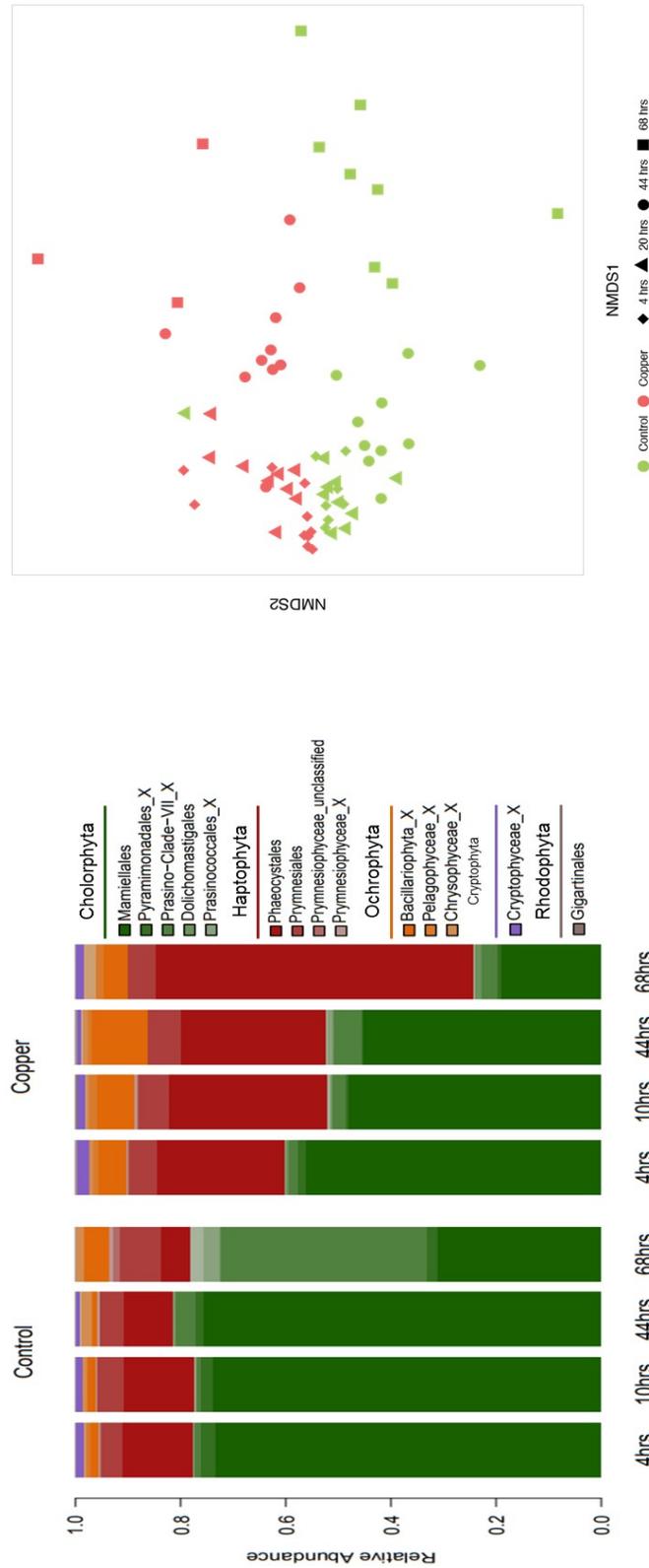


Figure 3.2: Taxonomic abundance patterns and assembly structure. (Right) Photosynthetic selected OTUs grouped by taxonomic origin indicated as colors. Mean relative abundances of taxa at level 2 PR² data base of this subset is shown. (Left) Non Metric Multidimensional Scaling (NMDS) analysis of community structure over time. All replicates are plotted and *Control* condition is shown in green color and *Copper incubated* condition is shown in light red. Point shape represent temporal variation.

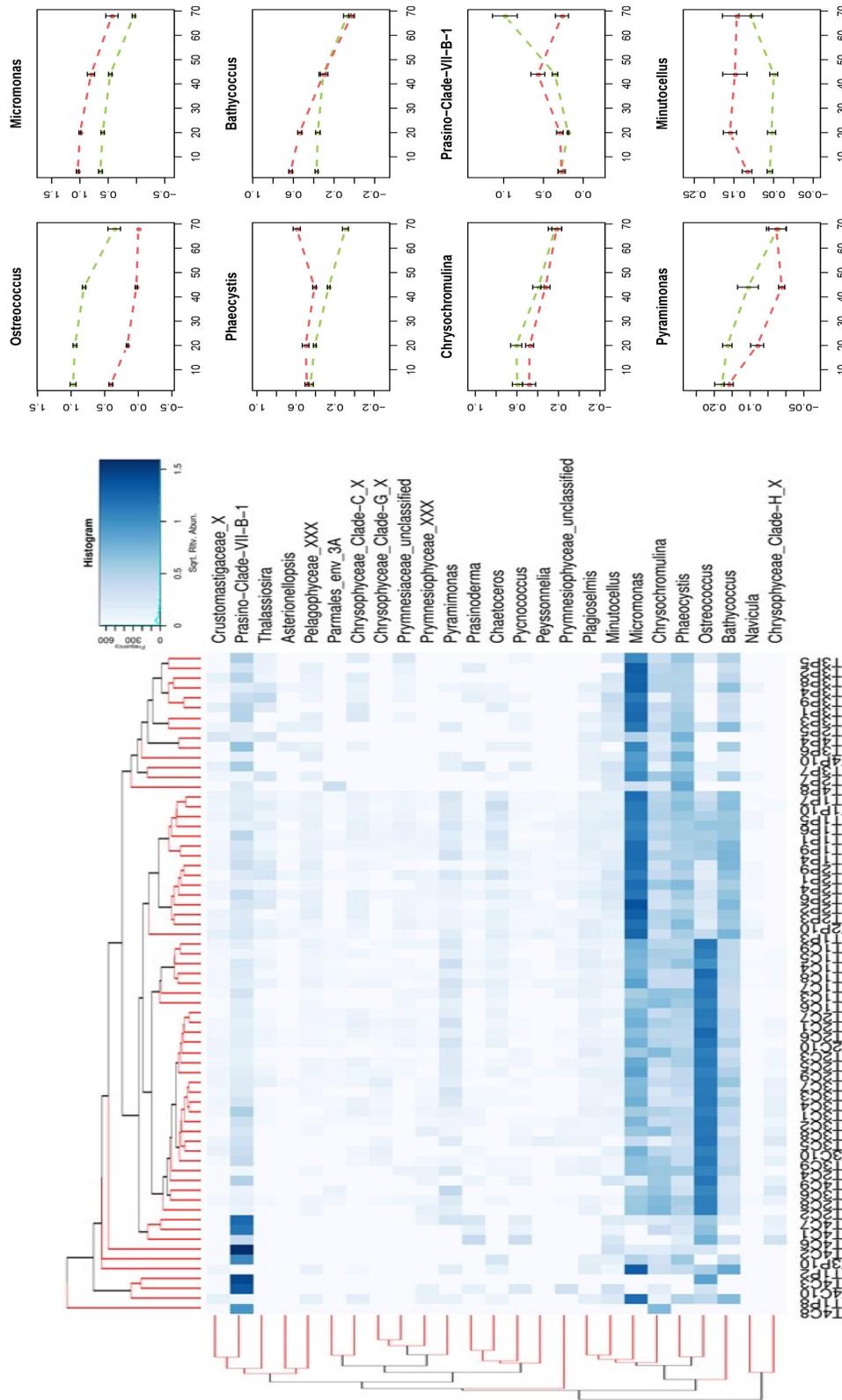


Figure 3.3: Photosynthetic OTUs Temporal Behavior. Summed level 7 PR² assign OTUs and their sample distribution with selected OTUs by photosynthetic lineages and p<0.05 t-student test between *Control* and *Copper incubated* conditions. (A) Cluster similarity analysis and heatmap of all samples with 33 statistically selected groups are shown clustered by Bray-Curtis similarity. SIMPROF bootstrapped algorithm was used and results are indicated in red branches in each tree. (B) Temporal dynamics of the eight most abundant statistically selected groups. Average of square root relative abundances are shown per sampling point with standard error (Control (Green) and Copper treated (Red)).

Chapter 4

Metal for life: Cross-talk metabolisms between trace metal and nitrogen in the model marine Chlorophyte *Ostreococcus tauri*

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4.1 Abstract

Planktonic cells in the modern ocean are susceptible to changes in their chemical environment. Fluctuation in micro- and macro-nutrient exposure highly influence plankton growth. Thus, trace metal availability might lead to differential nutritional states in plankton cells, interacting as well with macro-nutrients requirements. Nitrogen metabolism represents an essential part of planktonic phototrophic eukaryotes molecular machinery and physiology. A large number of proteins related to these molecular pathways contain metal ions in their structure, making this process susceptible to ion homeostasis and availability. Here, with molecular tools approach, we test copper and iron interaction over nitrogen metabolism and ion homeostasis transmembrane transport proteins. Using engineered *Ostreococcus tauri* strains, a model organism of small size phytoplankton, we test the protein production under different copper and iron growing conditions. Our results on copper induction over nitrogen transport protein suggest that copper exposure increase nitrogen nutritional requirements, by a relative increase in nitrate transport protein production. Moreover, copper and iron interaction were observed in a synergistic way, increasing protein production signal. Overall, our results suggest that copper and iron interact over nitrogen metabolism in green phototrophic eukaryotes, representing a potential mechanistic response to copper toxicity in natural environments.

4.2 Introduction

The modern ocean is susceptible to changes in different aspects. One of them is fluctuations of chemical species in the water column due to natural sources or anthropogenic origins (Halpern et al., 2009). It is in this context where planktonic cells play a key role in ecosystem structure and functioning, representing half of the total primary production of ocean systems (Azam et al., 1983; Falkowski et al., 1998), and one of the key responsive groups to environmental pollution (Karl E. Havens, 1994; Islam et al., 2004). Within the phototrophic fraction, the small size eukaryotic plankton (i.e. photosynthetic picoeukaryotes, PPEs) highlights due to it have been described as persistent overall season through the year, playing functional roles in elemental cycles especially in carbon fluxes (Calvo-Diaz et al., 2008; Collado-Fabbri et al., 2010; Jardillier et al., 2010). Moreover, this group has shown high taxonomic diversity and ecological relevance (de vargas et al., 2015). In recent years *Ostreococcus* spp. has been proposed as model organisms for Chlorophytes within this size fraction, because favorable growing conditions in culture media, easy genetic manipulation and ecological relevance being dominant in this size fractions, even in comparison to phototrophic bacteria, representing an opportunity to explore cellular mechanisms based from infield observations (Six et al., 2005; Lozano et al., 2014; Lelandais et al., 2016).

PPEs nutritional requirements are connected with macro and micro-nutrients availability in natural environments. The elemental composition of planktonic cells is related to environmental conditions, cellular functions, and plasticity (William Sunda, 2012; Guo et al., 2012). From macro-nutrients, nitrogen chemical species represent a vital substrate for growth and development in algae and highlight as an important nutritional source that can shape biological communities, modulate microbial metabolism and limited algal growth in natural systems (Morel & Price, 2003; Schoffman et al., 2016; Guo et al., 2012; Twining et al., 2015). Moreover, with nitrogen cycle perspective, several key elements of molecular machinery that had evolved related to acquire and processing of nitrogen sources it has been described as proteins that coordinate metal ions in their structure, representing then a cornerstone in microbial metabolism (Glass &

Orphan, 2012; Blaby-Haas & Merchant, 2012; Sanz-Lueque et al., 2015). Moreover, this structural feature represents an interconnection among cellular components and processes. Further, macro-nutrients requirements have shown significant influences in structuring microbial communities, mostly due to dual effects as nutrients at low concentrations but as toxicant at greater amounts (Stauber & Florence, 1987; Twining et al., 2011).

A key step in nitrogen and trace metal acquisition in photosynthetic eukaryotes nutrition are membrane transporters, that move passively or actively solutes into the cell. These membrane transporters represent the first layer of regulation in cellular content and stoichiometry (Blaby-Haas & Merchant, 2012; Guo et al., 2012). Several nitrogenous forms are present in the water column in natural environments, being nitrate an important nitrogen source in the global ocean, especially for the PPEs subgroup (Fouilland et al., 2004), that have a preference for nitrogenous small solutes (Morado & Capone, 2018). Nitrate, in their assimilation process, requires an enzymatic reaction that produces reduced species as nitrite, which then could be assimilated into the central metabolism in a process dependent on Molybdenum. This makes ion homeostasis an important factor regulating nitrogen metabolism and productivity in PPEs (Fouilland et al., 2004). Then, ion homeostasis represents a vital process that regulate inner equilibrium in cells.

In this context, Iron (Fe) and Copper (Cu) are of especial interest as micro-nutrients due to their relation to biological processes. Both ions play key roles in cellular metabolism, related to nutrients acquisition and homeostasis (Peers & Price, 2006; Schoffman et al., 2016). Further, it has been shown that those elements interact in cellular context modulating metabolism and cell growth, especially in Fe limited environments (Peers et al., 2005; Biwas et al., 2013; Semeniuk et al., 2015). Metal ions could interact in biological process context limiting transport, influence availability or ion displacement (Haferburg & Kothe, 2007; Lemire et al., 2013). These interactions result in changes in organism physiology with unexplored ecological nor biogeochemical effects.

The aim of this work was to test the effects of Fe and Cu availability on key transmembrane transporters that participate in nitrogen metabolism and ion homeostasis in the ecologically relevant PPE marine model, *Ostreococcus tauri*. We evaluate the potential effects of Cu and Fe

though engineered strains, using fusion proteins as indicators of protein production through fluorescence, in different growth conditions. The hypothesis leading this work was that Cu toxic effects interact with Fe availability and modulate nitrogen metabolism related transporters at protein level. We predict that Cu induced transport proteins in a Fe availability regulated manner, and at high concentrations this effect would be disrupted due to Cu toxic effects.

4.3 Materials and Methods

4.3.1 Strains and Culture Condition

Ostreococcus tauri strain RCC745 (Clade C) was grown in plastic flasks or 96 well plates (white colored), under constant light at an intensity of $20 \mu\text{mol quanta cm}^{-2}\text{sec}^{-1}$. Cells were grown in Keller-based Artificial Sea Water ASW (WERNER BioAgents) medium. This modified Keller medium contained was modify as follows: 24.55 gL^{-1} NaCl, 0.75 gL^{-1} KCl, 4.07 gL^{-1} $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1.47 gL^{-1} $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 6.04 gL^{-1} $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.21 gL^{-1} NaHCO_3 , 0.138 gL^{-1} NaH_2PO_4 and 0.75 gL^{-1} NaNO_3 . Cell counting was performed by flow cytometry (Acurri C6, BD Biosciences, NJ, USA) and cells were fixed in 0.25 % glutaraldehyde for 10 min before measuring.

4.3.2 Gene selection and Informatic approach

Selection of Cu early responsive transmembrane annotated genes from *Ostreococcus tauri* were selected based on the work of Hernandez-Castillo et al., 2015 and KEEG annotation. Three genes were picked up from early responsive patterns to Cu challenge, and engineered strains were made as described in Lozano et al., 2014 to produce translational reporters as described elsewhere and consisting in using modified version of vector known as pOtLuc (Millar et al., 1992; Strayer et al., 2000; Corellou et al., 2009) containing multi-restriction recognition site followed by *luc* gen upstream, also containing *kan* selective cassette with H4 gene promoter. Annotations were corroborated and compared with previous KEGG (<https://www.genome.jp/kegg/>) and pfam (<https://pfam.xfam.org/>) annotations. Also, annotations computed and integrated within Inter-pro (<https://www.ebi.ac.uk/interpro/beta/>) web service and PANTHER (<http://pantherdb.org/>) server were used to establish protein identity.

4.3.3 Cu and natural water assays

Cells were refreshed in ASW with a EDTA free trace metal cocktail, as described in Botebol et al., 2015. Cell cultures at exponential growth phase were transferred into 96 white colored well plate at 1 million cells per mL of cell density, in a final volume of 200 mL of Cu free culture media. Then, Luciferin was added in a ratio of 15 μL of stock solution ($1\mu\text{M}$ final concentration) per 3 mL of culture. Overnight incubation take place and Cu Sulfate was added at $1\mu\text{M}$, $5\mu\text{M}$, $7.5\mu\text{M}$, $15\mu\text{M}$, $20\mu\text{M}$ prior to measurement. For Fe-Cu interactions, culture media with different amounts of Fe Citrate were made previous to the assay. Growing concentrations were as follows: $1\mu\text{M}$, $2\mu\text{M}$, $4\mu\text{M}$, $8\mu\text{M}$, $16\mu\text{M}$. Independent experimental units ($n=3$) were place in independent plates to be read in a Berthold microplate luminometer LB 960. Natural water assay was performed with the same methodology, however, *Ostrococcus tauri* in culture strains were first diluted and then mixed with pre-filtered natural sea water in order to have same cell densities per well.

4.4 Results

4.4.1 Cu response pattern

Under laboratory conditions, cells were exposed to growing concentrations of CuSO_4 . Engineered strain with Nitrate (NO_3^-) transporter (OT_ostta10g00950) indicator responded quick, and in a concentration dependent pattern (Fig.1A). Concentrations greater than $10 \mu\text{M}$ showed a pick signal close to 2 hours after Cu exposure, followed by a continuous decrease of the signal. However, lower concentrations showed a continuous increase of the signal, reaching maximal response after 9 hours of Cu exposure. Engineered strain with the P-ATPase substrate transporter (OT_ostta05g03500) indicator, showed rapid responses with a maximum signal related to $7.5 \mu\text{M}$ Cu, at around an hour after Cu exposure (Fig.1B). After that, all conditions showed strong inhibition of the signal, in a time dependent manner. Also, Molybdate transporter showed an activation to Cu inputs, but at low signal (less than 50% in signal responses respected to control condition, Fig.1C). However, consistent temporal pattern was observed and all concentrations tested increased signal over a consistent time window of around 9 hours.

In the case of the test with naturally polluted seawater, all the engineered strains tested here shown a decrease in the signal during the first hour of the experiment. After that, a consistent increase in the fluorescent signal was observed, in a concentration dependent manner, followed by a stationary state and an increased signal reaching values greater than 200% respected to control condition (Fig.1 D-F). This pattern was clear for engineered strain with (NO_3^-) transporter (OT_ostta10g00950, Fig.1 D) and engineered strain with Molybdenum transporter (OT_ostta10g00960, Fig.1 F). For the engineered strain with P-ATPase (OT_ostta05g03500, Fig.1 E) before 3 hours of exposure a noisy signal was observed. However, exposure to 175/25 proportion of natural water showed a maximal responded signal compared to other engineered strains tested here at around 6 hours. Other protein specific patterns were detected under this experimental conditions. The nitrate transporter response was faster than other proteins reaching pick signal at around 5 hours of exposure and maximal intensity was observed in Molybdate

transporter at 6.4 hours of exposure reaching 260% of signal respect to control condition.

4.4.2 Fe and Cu interaction with Nitrate transporter.

Cu assay in *Ostreococcus tauri* engineered strain with (NO_3^-) transporter (OT_ostta10g00950) was performed under increasing Fe concentration in culture media. A consistent response was observed in relation with control condition (Fig.2 A-J). Maximum rate of signal increase was observed in first 30 min of exposure and pick signal was observed around 2 hours after the beginning of the assay (Fig2. A-E). In increasing conditions of Fe in culture media, Cu induction of protein production showed an overall growing signal pattern, which reach maximum values at $16\mu\text{M}$ Fe without modifying general response (Fig.2 A-E). Nevertheless, maximal Cu concentration used ($20\mu\text{M}$ Cu) showed a saturation behavior at concentrations greater than $1\mu\text{M}$ Fe, resulting in less signal than lower Cu concentration ($15\mu\text{M}$ Cu condition). Further, when Fe was tested in Cu fixed conditions Fig2. F-J), a low intensity induction was observed, that reach its pick at $10\mu\text{M}$ Cu with $8\mu\text{M}$ Fe at 4 hours of exposure (Fig2. H). At low Cu concentrations in media, the signal remain lower than 50% of induction respect to control condition (Fig2. F-G). Moreover, at high Cu exposure ($15\mu\text{M}$ Cu and $20\mu\text{M}$ Cu, Fig.2 I-J) a common pattern of induction was observed before 2 hours of exposure and comparable to $10\mu\text{M}$ Cu, but an opposite behavior was observed in following time window and concentrations dependent inhibition after two hours of measurement was observed.

4.5 Discussion

From a cellular metabolism perspective, the results presented here showed how micro-nutrients and macro-nutrients interact in cellular plasticity and adaptation in a culture media context. First, protein responses to Cu, in all the strains tested, were fast and clear with a clear induction after 3 hours of Cu exposure. This pattern suggests that organisms of this size fraction are resilient to changes in their chemical environment and are prepared to respond with molecular tools to fluctuations in their surrounded chemical environment. In the case of the nitrate transporter, a consistent increase of the signal was observed for all concentrations, until $7.5\mu\text{M}$ Cu ions. This result suggests that Cu might increase nitrogen source requirements for planktonic algae. Moreover, previous data suggest that metal interactions with nitrogen related enzymes, pigment profiles and energy metabolisms occur in green lineage in terms of cellular physiology and functional performance (Botbol et al., 2015; Lelandais et al., 2016; Scheiber et al., 2019). The other proteins tested here showed a more diffuse pattern, but a clear response, suggesting that potential other controls and regulated mechanisms are involved. From ion homeostasis perspective, Molybdate transporter and P-ATPase induction patterns suggest that planktonic cells are prepared to deal with ionic changes in media and in emulated natural conditions responses do not require heavy production of more proteins suggesting an energy-efficient mechanism.

In natural water assays, a biphasic response pattern was observed, suggesting that in natural seawater multiple inductors could be presented, making the sensors reacts in a different way when compared to artificial seawater. In that sense, the availability of those inductors could be affecting in a different way the cellular physiology. As well as in the previous assay, the response of the strains to the different molecules present in the seawater was fast, even less than hours of exposure. If the observed induction is considered just by one single chemical species, it suggests that multiple complexation states play a key role in algae metabolisms. This highlighting the importance of chemical environments and biological context for algal cells were production of complex molecules and posterior release to surrounded media could influence metabolisms and nutritional requirements of adjacent cells in natural environments (Bruland et al., 1991;

Gonzalez-Davila, 1995; Stauber et al., 2005). Exposure with water from heavy Cu-loaded place showed the greater signal suggesting that complex mixtures are present with biological activity and lead to changes in nutritional requirements of plankton cells in natural environments.

In the Fe-Cu interaction assay, a synergistic effect was observed suggesting that those ions influence cellular metabolism and homeostasis play a key role in macro-nutrient acquisition (William G. Sunda, 2012). An increase of signal was observed especially in Cu inductions assays followed by a heavy decrease in signal, suggesting that potential toxic effects in natural environments might be incremented under potential Fe depositions. This effect could be relevant in contexts of coastal management and potential Fe fertilization attempts and must be evaluated carefully (Sunda, & Huntsman, 1995; Timmermans et al., 1998; Lelong et al., 2013). Now, in Fe induction assay, a low magnitude induction was observed suggesting that cells were adapted to Fe availability variations not so for Cu assay showing a more narrow range of variation. However, a maximum induction was observed at $10\mu\text{M}$ of Cu sulfate in culture media suggesting that cells are adapted to a limited amount of Cu exposition. Moreover, highest ion concentrations tested here showed that potential Fe-Cu competition might occur. This observation is based on the greater concentration in Cu tested here ($20\mu\text{M}$) was inhibited in relation to $15\mu\text{M}$ in Cu assays, and same pattern was observed in Fe induction assays.

The patterns discussed became even more relevant in the present time, where chemical changes in modern ocean are observed at global scale (Foster & Charlesworth, 1996; Paytan et al., 2009; Nogales et al., 2011; Zeng et al., 2015). Chemical pollution and coastal environment usage represent a novel input of trace metals directly in the environments at no clear rates. This might result in unknown effects over microbial local ecology with unknown effects in biogeochemical cycles and food webs. In future global scenarios for global change prediction, Cu availability is expected to increase and Fe availability might decrease potentially leading to more restricted growth conditions for planktonic cells and with eventual toxic effect by Cu availability (Millero et al., 2011; Bijma et al., 2013). These effects might result in unknown ecological impacts on microbial communities and ecosystem functions.

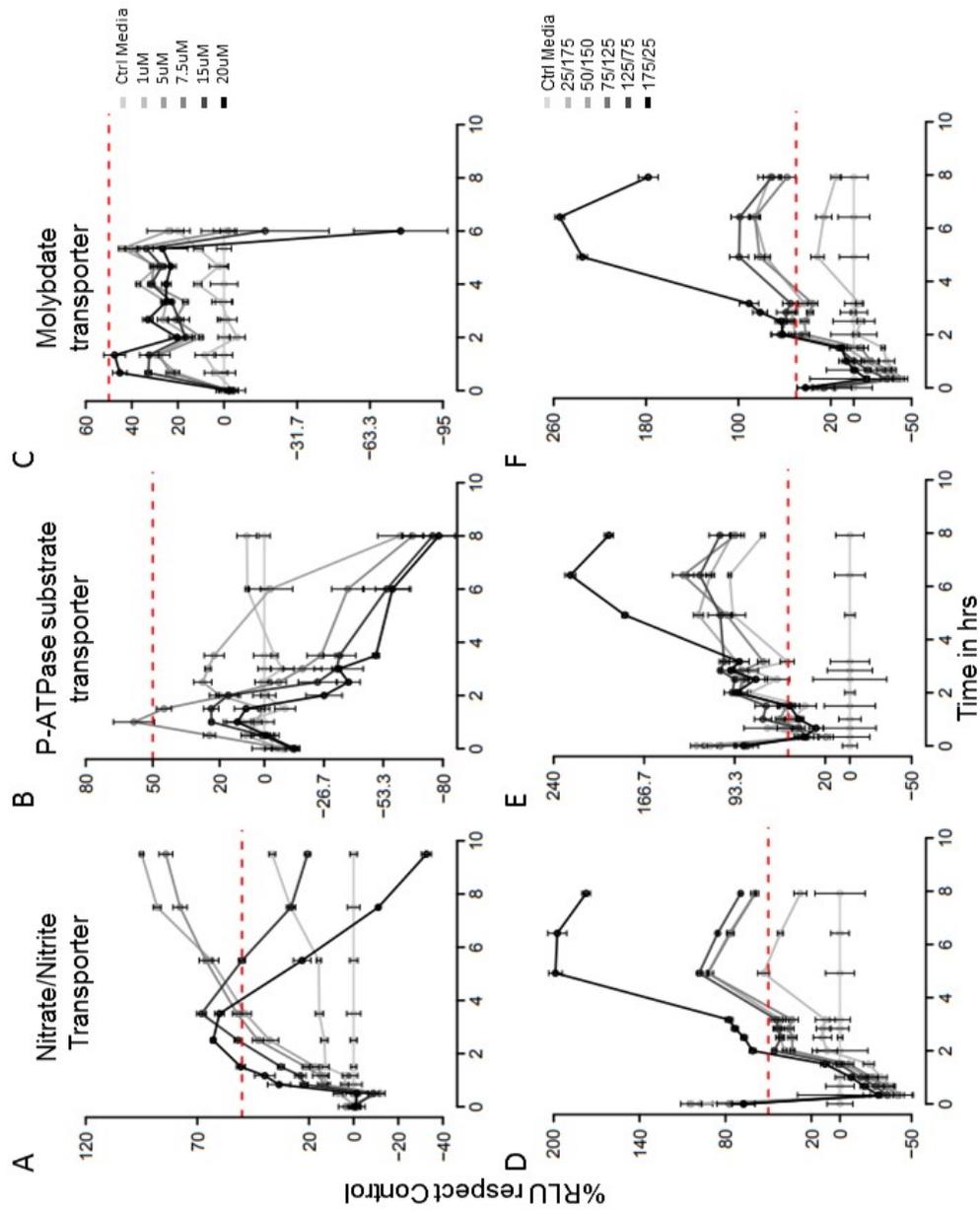


Figure 4.1: Cu response assay. Selected genes response under different metal exposure measured as relative luminescence units (RLU) as translation reporter and in percentages related to control condition. Also, 50% increase related to control condition is shown as red line. (Upper panel, A-C) Protein production response pattern under culture conditions (ASW media) in increasing Cu concentrations. (Lower panel, D-F) Protein production response under exposure to Natural water from Chañaral area.

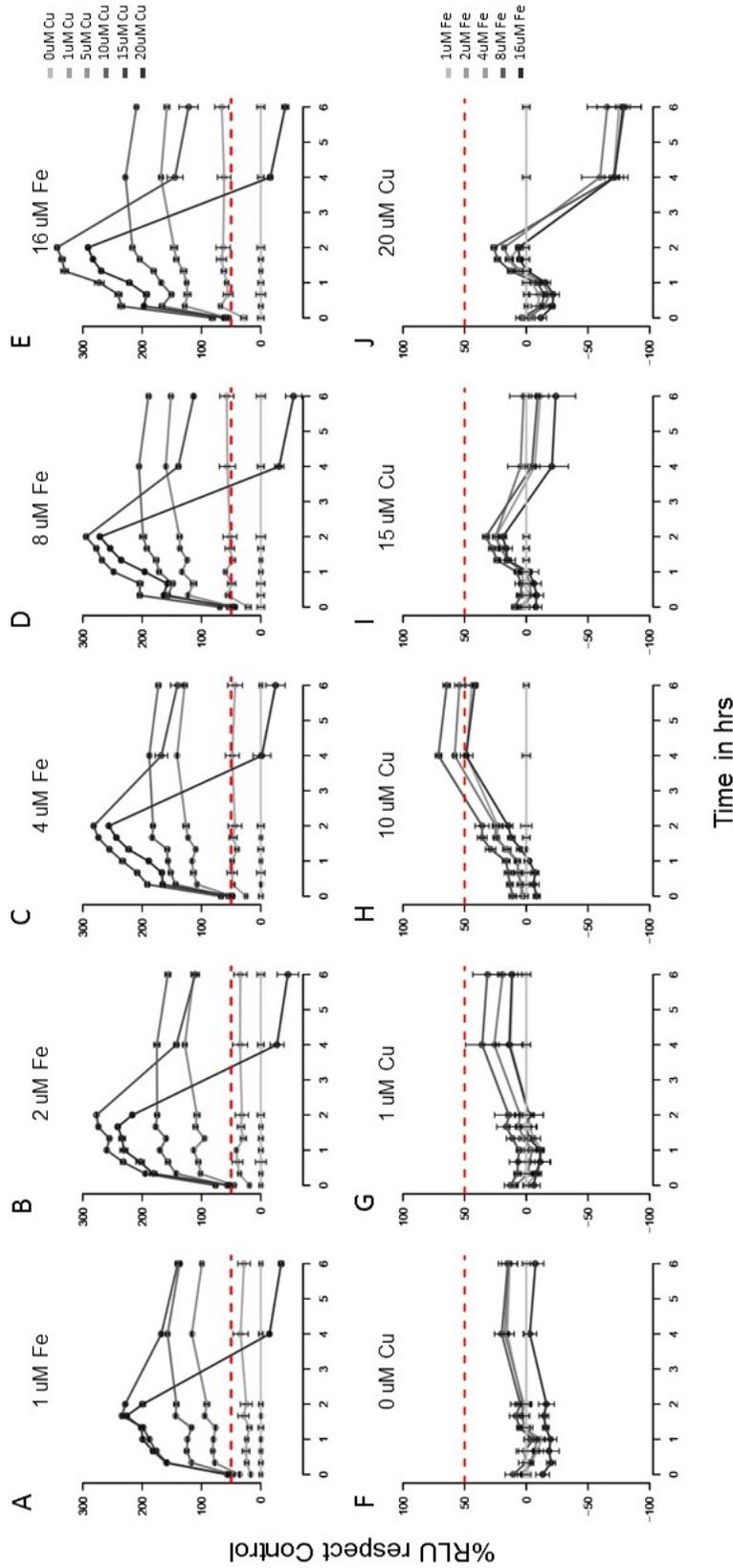


Figure 4.2: Cu-Fe interactions assays on Nitrate transporter. OT_ostfa10g00950 gene response under different metal exposure measured as relative luminescence units (RLU) and in percentages related to control condition. Also, 50% increase related to control condition is shown as red line. (Upper panel, A-E) Protein production response to Cu under different basal Fe concentrations in ASW culture media. (Lower panel, F-J) Protein response to Fe induction under different basal Cu Concentrations in ASW culture media.

Chapter 5

General Discussion

As from the data generated by this thesis work, community ecology with emphasis on environmental patterns was evaluated, leading to a better understanding of disturbances on natural environments, from a microbial ecology perspective. With community ecology outlook, field data showed a particular temporal behavior of picoeukaryotic communities under trace metals disturbances, with special focus on Copper ions in the dissolved fraction. Commonly in scientific literature, microbial planktonic communities are described as hetero/mixotrophic dominated and highly sensitive to physical and chemical environmental fluctuations (de Vargas et al., 2015; Connell et al., 2017; Martin-Platero et al., 2018). Nevertheless, in the Chañaral area, a reference site to study environmental disturbances, a diffuse pattern was described in which (i) seasonal fluctuations in community ensembles were non-clear or nonexistent and (ii) functional and taxonomic dominance patterns were disturbed in comparison with previous studies documented to date (Alexandra Z. Worden, 2006; Rii et al., 2016), shifting heterotrophic dominance to autotrophic dominance. Furthermore, within the autotrophic fraction, the commonly coastal dominance of Mamiellophyceae was changed to Bacilliarophyta related taxa. This observation leads to the main conclusion in which trace metals, specifically Copper, can act as active picoeukaryotic communities shaper and disrupt community sensitivity to physic-chemical features.

Field observations represent an invaluable information source over how natural environment

and ecological systems develop and work overtime. However, in-field approximations to microbial ecology have methodological and theoretical limitations. From a disturbance ecology point of view, and taking statistical designs into account, address real effects over environmental fluctuations in a temporal framework require in-detail description of previous stable or transient states (B. E. Beisner et al., 2003; Shade et al., 2012). The last is in order to produce quantifiable interpretations over the phenomena and with a proper sampling design quantify effect sizes. In real scenarios, rely on previous data is not always possible due to lack of data or methodological feasibility. This problem leads to comparisons in which the lack of previous states avoids the possibility to generate solid predictive models over natural environments. Nevertheless, those comparisons and descriptions could generate over time a solid framework from which develop null models and interpretations from direct observations (Karsenti et al., 2011).

Due to the above mention limitations, an alternative approach became necessary and ecological experimentation emerge as a tool to test specific questions. Thus, to test Cu effects over picoeukaryote populations and community, a non-disturbed microbial assemblage was used with a microcosms approach, under laboratory conditions. This approximation to environmental disturbances allows us to identify and quantify Cu effects over non-disturbed populations in a microbial community context, as a simulation of copper artificial depositions. Ecologically relevant groups were identified as susceptible and responsive to copper inputs. In scientific literature, it is well accepted that Mamiellophyceae related taxa, with at least one of main three species (*Ostreococcus*, *Micromonas* and *Bathycoccus*), largely dominate water column environments in coastal regions in the eukaryotic small size fraction (Not et al., 2004; Lambert et al., 2018). Humboldt-CS's coast is not an exception and *Ostreococcus* related taxa have been proposed as dominant in non-disturbed, microbial assemblage in the microcosms approach (Collado-Fabbri et al., 2011). Fast responses were observed, in which *Ostreococcus* related taxa relative abundances diminish significantly after four hours of Cu exposure, suggesting that Cu inputs in natural environments generate immediate effects over microbial populations, shifting in unknown ways the functional properties and features of natural ecosystems. Moreover, a taxa dependent differential temporal behavior was observed, suggesting that further analyses

are required to understand the specific responses of key taxa, within microbial ensembles, with ecological significance in disturbance ecology context. In spite of the fact that this approach allows identifying key responsive pattern in a consistent way, methodological limitations prevent to develop quantitative models which can be applied and tested in other environmental conditions. Temporal resolution related to real-time responses, multiple initial states related to identification of common responsive patterns over water column picoeukaryotic communities and multiple environmental parameterizations were identified as main limitations related to predictive model generation in wide scenarios. However, and despite these limitations, general responsive pattern was clear and consistent overall sampling design. *Ostreococcus* relative abundances strong decrease indicate that this model organism could represent an opportunity to test mechanistic hypothesis related to disturbance ecology.

Ostreococcus had been propose as model organism to study metal metabolism in marine picoeukaryotic plankton (Palenik et al., 2007; Lozano et al., 2014; Lelandais et al., 2016; Botbol et al., 2017; Scheiber et al., 2019) as part of green lineage (Chlorophyta). Moreover, with the Cu susceptibility observed in community context, this model organisms highlight as a suitable model to study Cu disturbances in an ecologically relevant context associated with coastal environments. In this line, dynamical copper responses in *Ostreococcus* physiological responses could lead to interpretations of how environmental disturbances affect microbial communities, ecosystemic functions and eventually may lead to bioengineering interventions to environmental disasters. Multi-nutrient interactions and their impacts on cellular physiology are canonical common-knowledge in scientific literature (William G. Sunda, 1989; Morel et al., 1991; William G. Sunda, 2012; Twining et al, 2015), but in unicellular Chlorophytes, and especially in marine models, little is known in-details about physiological responses in copper metabolism (Guo et al., 2012; Scheiber et al., 2019). Interactions among metal's cellular management and nitrogenous sources have been proposed, and membrane transporters response has been explored (Derelle et al., 2006; Blaby-Haas Merchant, 2012; Blaby-Haas Merchant, 2017). The data presented here suggest a rapid response to ion homeostasis and a hypothetical increasing requirement of nitrogen sources in a synergistic manner with iron availability under copper challenges.

However, the cellular approach in spite of generating in-details data with a mechanistic perspective has associated limitations and challenges as well. Generalize from cellular processes and physiological features to an environmental system, and assume comparable properties from model cellular organisms to a broad range of organisms without taking to account an evolutionary perspective, have been consider the principal challenges in this approximation and one of the main modern ecological questions (Sutherland et al., 2013; Boyd et al., 2018). Eventually, cyclical processes are required to generate consistent models that take into account data from cellular processes to ecosystem features (Worden et al., 2015). In an iterative processes, cellular data can be used to assess and explore environmental variability and identify common trends and particularities directly from the environment.

The understanding of environmental phenomena has become an imperative in our time, mostly by changing perspectives about the relevance of ecological and physical processes next to the growing human footprint in natural systems (Crain et al., 2008; Halpern et al., 2009; Halpern et al., 2015), but also due to emerging of new technologies that allows to explore natural environments with new approaches. Trace metals disturbances, by its side, have been explored extensible in the literature from a bacterio-centric point of view and little is known from cellular nor ecological responses of eukaryotic microbes (Harrison et al., 2007 Dupont et al., 2011). Moreover, from an environmental perspective, unclear consequences are known for local deposits that generally consider the ocean dilution factor as gravitating without considering local variability and understanding the details of environmental local processes (Michael J. Kennish, 2002; Pinto et al., 2003). Thus, such features related to local variability and processes should be included in management and environmental policy frameworks in order to evaluate future scenarios with higher accuracy and precision.

Appendix A

Chapter 2 Supplementary Material

Sample ID	Cu	Fe	Zn	Mo	Co	Cd	Pb	Cu_ASV	N	P	Si	T	Sal.
LCS.Jan.2011	2.998	7.651	0.656	9.709	0.006	0.036	0.144	0.200	12.779	1.513	9.144	14.972	34.464
LCS.Ago.2012	2.538	0.912	3.903	9.496	0.379	0.108	0.361	0.302	13.656	2.193	8.633	13.880	34.036
LCS.Oct.2011	5.623	0.010	2.023	11.170	0.200	0.200	0.200	0.830	9.001	2.359	9.235	13.347	34.695
LCS.Jan.2012	1.573	2.275	2.570	11.317	0.015	0.073	0.028	0.710	5.179	1.294	8.115	18.200	34.619
LCS.Apr.2012	0.608	0.523	8.531	8.450	0.008	0.043	0.086	0.140	4.726	1.277	6.929	17.739	34.613
LCS.Nov.2013	4.200	5.531	0.824	10.060	0.008	0.077	0.059	0.070	-	-	-	-	-
LCS.Mar.2014	2.386	3.822	0.873	9.517	0.873	0.077	0.014	0.045	-	-	-	-	-
HCS.Oct.2011	21.583	0.030	2.473	12.267	0.200	0.200	0.200	8.780	14.510	2.479	18.903	13.799	34.648
HCS.Jan.2012	4.540	2.980	3.195	11.697	0.020	0.076	0.026	3.570	5.449	1.650	6.752	18.100	34.671
HCS.Apr.2012	3.154	0.432	2.150	4.490	0.005	0.041	0.010	2.035	6.051	1.413	11.642	17.261	34.618
HCS.Ago.2012	9.085	1.045	5.166	8.208	0.037	0.054	0.103	1.335	12.594	2.201	7.917	14.052	34.660
HCS.Nov.2013	7.781	1.873	1.682	10.109	0.018	0.073	1.207	3.651	-	-	-	-	-
HCS.Mar.2014	13.349	3.913	0.510	9.022	0.024	0.079	0.012	5.163	-	-	-	-	-

Table A.1: Physic-Chemical Characterization. Measured Trace Metals in samples and other parameters. Concentration of dissolved fraction of each metal analyzed in this study. Also Bioavailable copper was included measured by Anodic stripping voltammetry (ASV)

ID	Level	Level	Level	Level	Level	Level	Level	Level	P value Season	P value Site	Mean R.A ± SEM
	1	2	3	4	5	6	7	8			
OTU327	Eukaryota	Alveolata	Dinophyta	Dinophyceae	Dinophyceae_X	Dinophyceae_XX	Madanidinium	Madanidinium_loirii	0.514	0.045	0.012±0.003
OTU407	Eukaryota	Alveolata	Dinophyta	Dinophyta	Dinophyta	Dinophyta	Dinophyta	Dinophyta	0.144	0.048	0.006±0.001
OTU117	Eukaryota	Alveolata	Dinophyta	Dinophyceae	Dinophyceae_X	Dinophyceae_XX	Prorocentrum	Prorocentrum	0.742	0.045	0.039±0.017
OTU505	Eukaryota	Alveolata	Dinophyta	Dinophyta	Dinophyta	Dinophyta	Dinophyta	Dinophyta	0.185	0.011	0.005±0.002
OTU1113	Eukaryota	Alveolata	Dinophyta	Syndiniales	Dino-Group-II	Dino-Group-II-Clade-17	Dino-Group-II-Clade-17_X	Dino-Group-II-Clade-17_X_sp.	0.350	0.027	0.002±0.001
OTU135	Eukaryota	Alveolata	Dinophyta	Dinophyceae	Dinophyceae_X	Dinophyceae_XX	Dinophyceae_XXX	Dinophyceae_XXX_sp.	0.596	0.031	0.079±0.021
OTU792	Eukaryota	Alveolata	Dinophyta	Dinophyceae	Dinophyceae_X	Dinophyceae_XX	Dinophyceae_XXX	Dinophyceae_XXX_sp.	0.560	0.035	0.001±0.001
OTU1330	Eukaryota	Alveolata	Dinophyta	Dinophyceae	Dinophyceae_X	Dinophyceae_XX	Dinophyceae_XXX	Dinophyceae_XXX_sp.	0.526	0.018	0.001±0.001
OTU324	Eukaryota	Hacrobia	Centrohelioczoa	Centrohelioczoa_X	Pterocystida	Pterocystida_X	Pterocystida_XX	Pterocystida_XX_sp.	0.609	0.028	0.009±0.003
OTU114	Eukaryota	Stramenopiles	Ochrophyta	Chrysophyceae	Chrysophyceae_X	Chrysophyceae_Clade-H	Clade-H_X	Clade-H_X_sp.	0.732	0.019	0.058±0.018

Table A.2: OTUs with significant effect by Site. OTUs were selected by significance in Site abundances comparisons. Also, each OTUs taxonomy is indicated with mean relative abundances respect o total dataset.

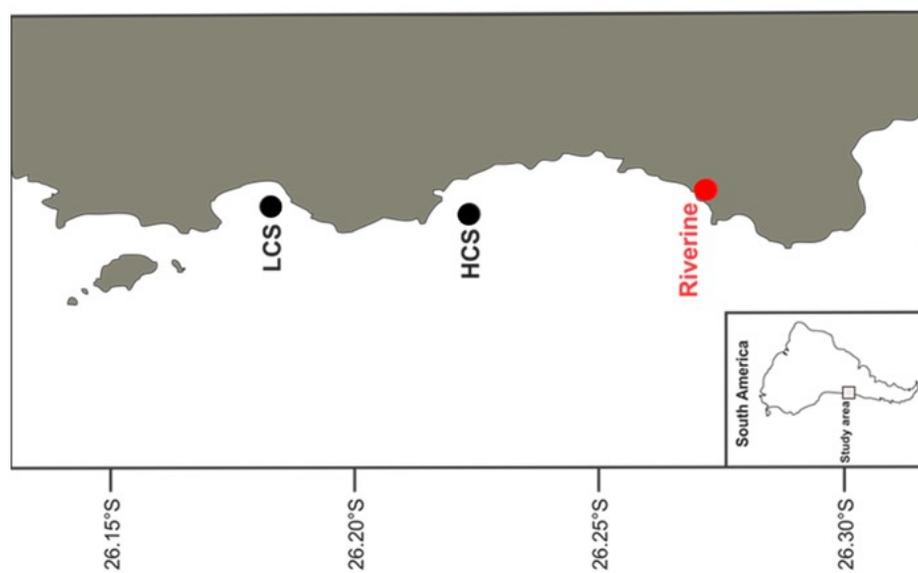


Figure A.1: Study Site. Latitudinal zoom of study site in Atacama region. Discharge river is indicated in red and sample sites are shown as black dots. Also inset of south America is shown as context for the region.

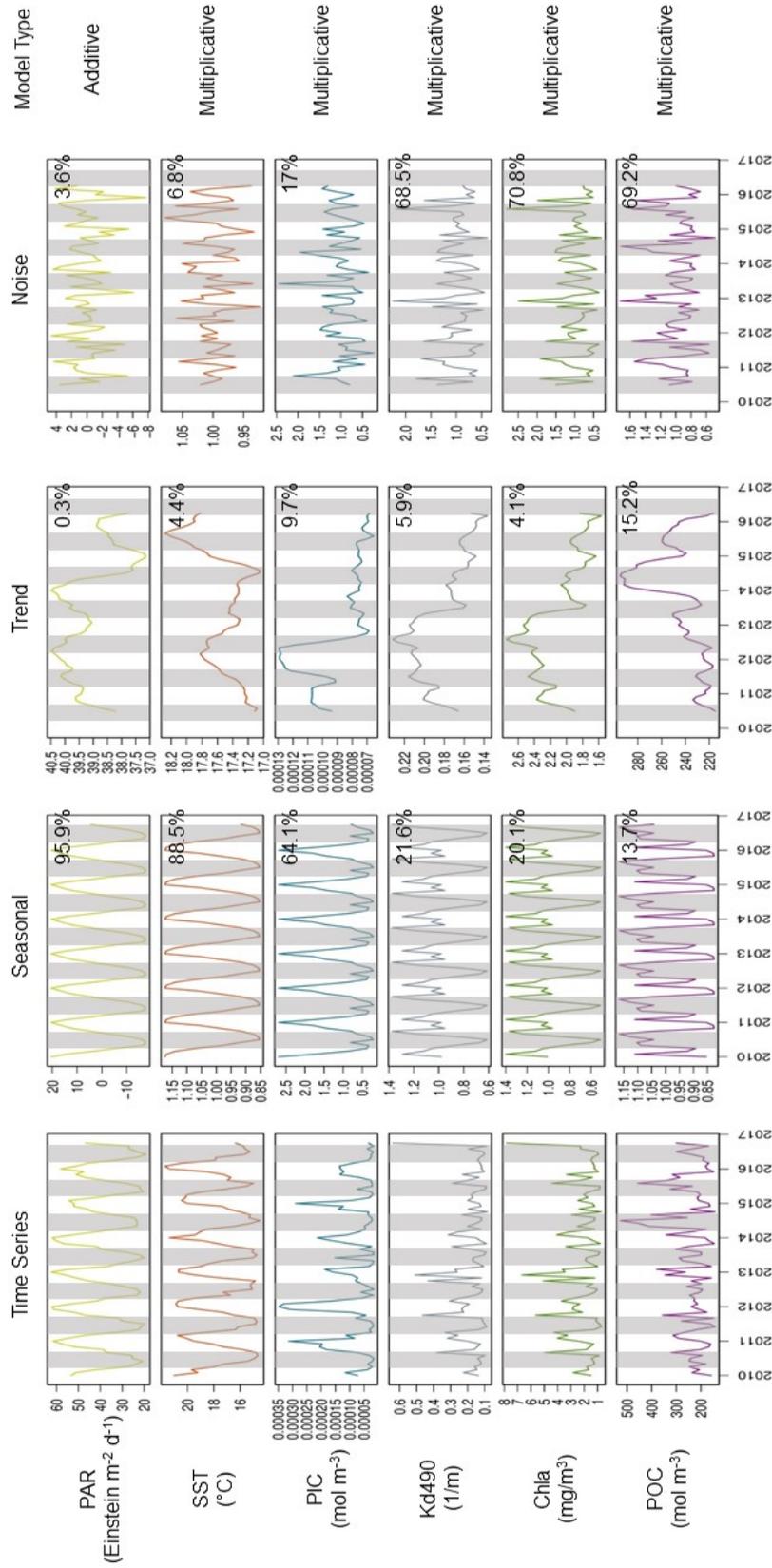


Figure A.2: Complete time series decomposition. Six parameters are shown decomposed by Additive or Multiplicative method. Original time series is indicated in the first column and components are represented by next three columns. Percentages of variance contribution to the original time series are shown in each panel.

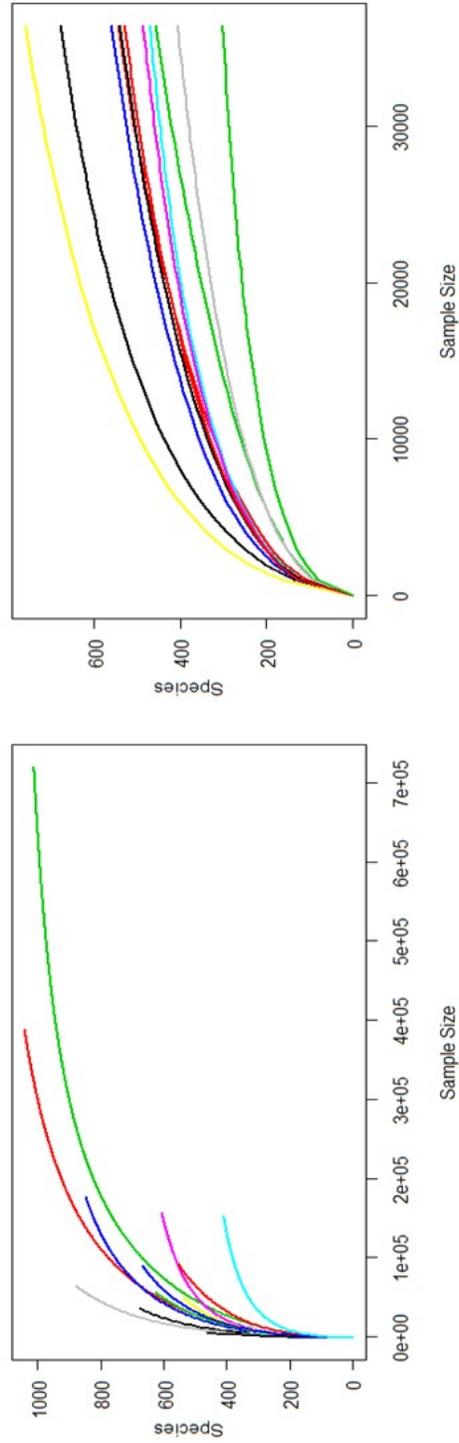


Figure A.3: Rarefaction Analysis. Curves computed with Vegan packages. (left) Original Data. (right) Post rarefaction where all samples were normalized to 36387 reads per sample.

Appendix B

Chapter 3 Supplementary Material

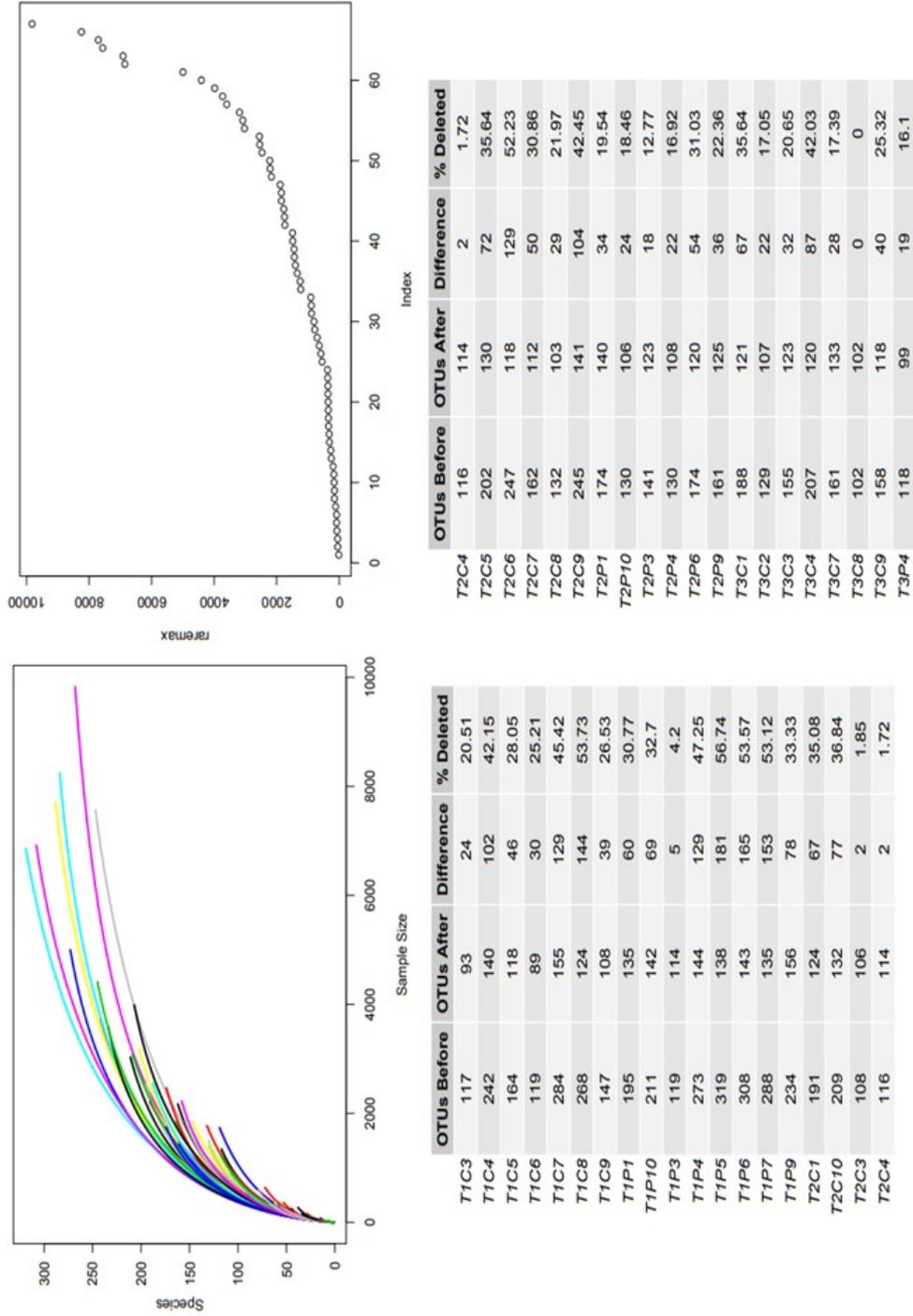


Figure B.1: Rarefaction and raw Abundance Analysis. Curves computed with Vegan packages. (Topleft) Original Data. (TOPright) ranked reads abundances per sample. (Bottom) Post rarefaction data per sample in tabular format indicating percentages of OTUs deleted if Control condition replicate 8 (T3C8) samples is used as reference.

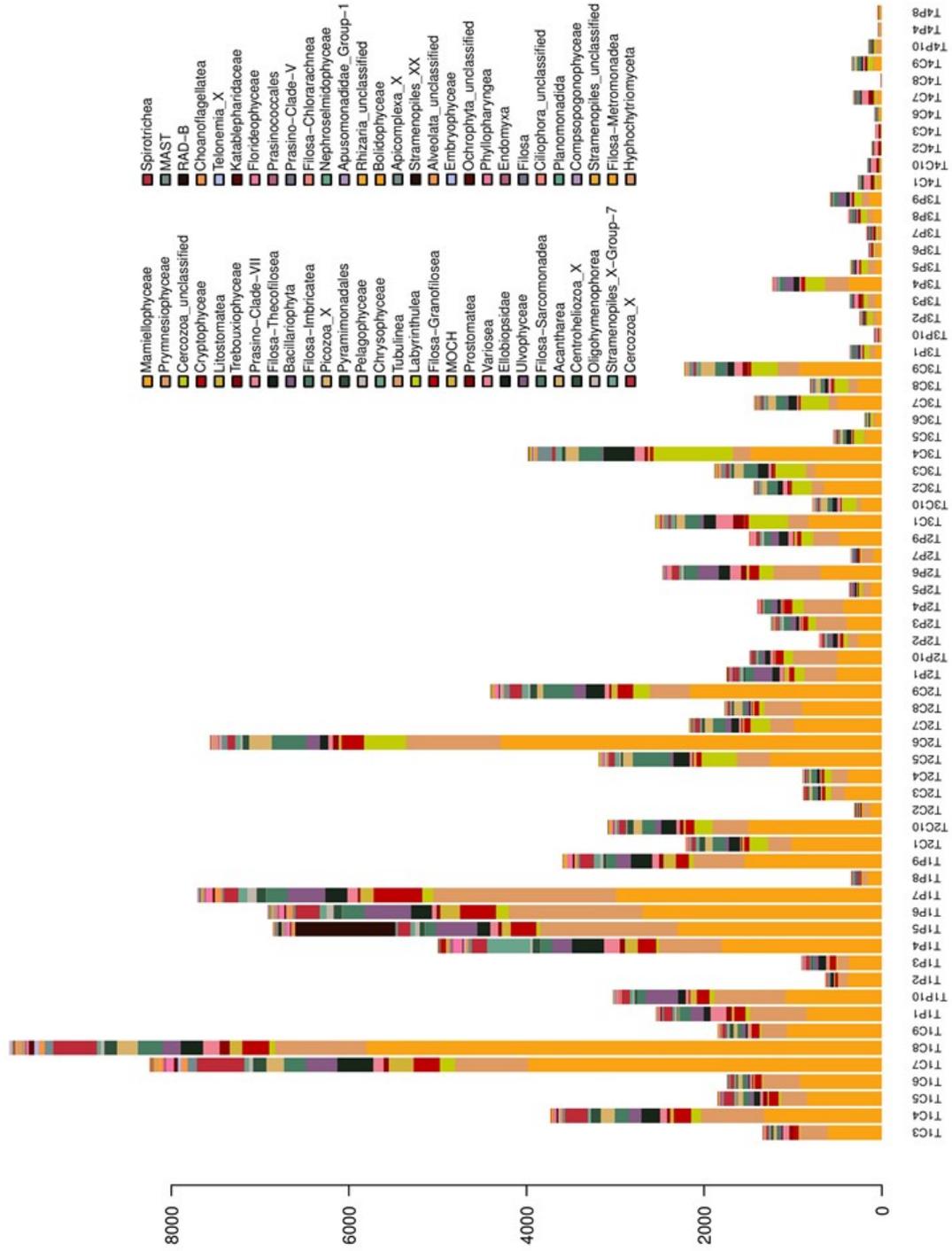


Figure B.2: Raw Abundance per sample. Reads abundances per sample and all replicates. Also taxonomic assignment at Level 4 PR² Data base is shown

Appendix C

Chapter 4 Supplementary Material

Genome	OT_ostta10g00950	OT_ostta05g03500	OT_ostta10g00960
Protein Accession Number	XP_022839760.1	XP_003079457.2	XP_003081530.1
KEGG Annotation	Nitrate/nitrite transporter	ATP-binding cassette subfamily G member2	Sulfate permease SulP Family
Homologous Domains	MFS Facilitator superfamily	P-loop containing nucleoside triphosphate hydrolase	
Domain	Mayor Facilitator superfamily domain	ABC Transporter-like AAA+ ATPase Domain ABC-2 type transporter	
Tmhelix/transmembrane	12/12	9/6	9/7
Panther	High-affinity Nitrate Transporter 2.1 Related (PTHR23515:SF2)	ATP-Binding cassette Transporter (PTHR19241)	Molybdate Transporter 1 (PTHR31970:SF0)

Table C.1: Gene selection. Different annotations for three genes selected based on computed information for sequence level made with Inter-pro tool

Bibliography

- [1] Esteban Acevedo-Trejos, Gunnar Brandt, and Agostino Merico. “Mechanisms shaping phytoplankton community structure and diversity in the ocean”. In: *Scientific Reports* 5.8918 (2014), pp. 17–20.
- [2] Linda a. Amaral-Zettler, Elizabeth a. McCliment, Hugh W. Ducklow, and Susan M. Huse. “A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA Genes”. In: *PLoS ONE* 4.7 (2009), pp. 1–9.
- [3] Santiago Andrade, James Moffett, and Juan A. Correa. “Distribution of dissolved species and suspended particulate copper in an intertidal ecosystem affected by copper mine tailings in Northern Chile”. In: *Marine Chemistry* 101 (2006), pp. 203–212.
- [4] David Antoine, Jean-Michele André, and André Morel. “Estimation at global scale from satellite (coastal zone color scanner) chlorophyll”. In: *Global Biogeochemical Cycles* 10.1 (1996), pp. 57–69.
- [5] Lizeth Avendaño, Martha Gledhill, Eric P. Achterberg, Victoire M. C. Rérolle, and Christian Schlosser. “Influence of Ocean Acidification on the Organic Complexation of Iron and Copper in Northwest European Shelf Seas; a Combined Observational and Model Study”. In: *Frontiers in Marine Science* 3.April (2016).
- [6] F. Azam, T. Fenchel, J.G. Field, J.S. Gray, L.A. Meyer-Reil, and F. Thingstad. “The ecological role of water-column microbes in the sea”. In: *Marine Ecology Progress Series* 10 (1983), pp. 257–263.

- [7] Farooq Azam and Alexandra Z. Worden. “Microbes, Molecules, and Marine Ecosystems”. In: *Science* 303.March (2004), pp. 1622–1624.
- [8] H.J.W de Baar. “Von Liebig’s law of the minimum and plankton ecology”. In: *Progress in Oceanography* 33 (1994), pp. 347–386.
- [9] Sergio Balzano, Dominique Marie, Priscillia Gourvil, and Daniel Vaultot. “Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples”. In: *The ISME Journal* 6 (2012), pp. 1480–1498.
- [10] EB Barbier, SD Hacker, and C Kennedy. “The value of estuarine and coastal ecosystem services”. In: *Ecological Monographs* 81.2 (2011), pp. 169–193.
- [11] B. E. Beisner, D. T. Haydon, and K. Cuddington. “Alternative Stable States in Ecology”. In: *Frontiers in Ecology and the Environment* 1.7 (2003), pp. 376–382.
- [12] Gry Mine Berg, Patricia M. Glibert, and Chung Chi Chen. “Dimension effects of enclosures on ecological processes in pelagic systems”. In: *Limnology and Oceanography* 44.5 (1999), pp. 1331–1340.
- [13] Haimanti Biswas, Debasmita Bandyopadhyay, and Anya Waite. “Copper addition helps alleviate iron stress in a coastal diatom: Response of *Chaetoceros gracilis* from the Bay of Bengal to experimental Cu and Fe addition”. In: *Marine Chemistry* 157 (2013), pp. 224–232.
- [14] Crysten E. Blaby-Haas and Sabeeha S. Merchant. “The ins and outs of algal metal transport”. In: *Biochimica et Biophysica Acta - Molecular Cell Research* 1823.9 (2012), pp. 1531–1552.
- [15] Crysten E Blaby-haas and Sabeeha S Merchant. “Regulating cellular trace metal economy in algae”. In: *Current Opinion in Plant Biology* 39 (2017), pp. 88–96.

- [16] J L Blanco, A C Thomas, M Carr, and P T Strub. “Seasonal climatology of hydrographic conditions in the upwelling region off northern Chile”. In: *Journal of Geophysical Research* 106.C6 (2001), pp. 11451–11467.
- [17] Hugo Botebol, Gaelle Lelandais, Christophe Six, Emmanuel Lesuisse, Arnaud Meng, Stéphane Lecrom, Robert Sutak, Jean-claude Lozano, and Philippe Schatt. “Acclimation of a low iron adapted *Ostreococcus* strain to iron limitation through cell biomass lowering”. In: *Scientific Reports* October 2016 (2017), pp. 1–11.
- [18] Hugo Botebol, Emmanuel Lesuisse, Robert Šuták, Christophe Six, Jean-Claude Lozano, Philippe Schatt, Valérie Vergé, Amos Kirilovsky, Joe Morrissey, Thibaut Léger, Jean-Michel Camadro, Audrey Gueneugues, Chris Bowler, Stéphane Blain, and François-Yves Bouget. “Central role for ferritin in the day/night regulation of iron homeostasis in marine phytoplankton”. In: *Proceedings of the National Academy of Sciences* 112.47 (2015), pp. 14652–14657.
- [19] Philip W. Boyd, Sinead Collins, Sam Dupont, Katharina Fabricius, Jean Pierre Gattuso, Jonathan Havenhand, David A. Hutchins, Ulf Riebesell, Max S. Rintoul, Marcello Vichi, Haimanti Biswas, Aurea Ciotti, Kunshan Gao, Marion Gehlen, Catriona L. Hurd, Haruko Kurihara, Christina M. McGraw, Jorge M. Navarro, Göran E. Nilsson, Uta Passow, and Hans Otto Pörtner. “Experimental strategies to assess the biological ramifications of multiple drivers of global ocean change-A review”. In: *Global Change Biology* December 2017 (2018), pp. 2239–2261.
- [20] Denis de la Broise and Brian Palenik. “Immersed in situ microcosms: A tool for the assessment of pollution impact on phytoplankton”. In: *Journal of Experimental Marine Biology and Ecology* 341.2 (2007), pp. 274–281.
- [21] Brul, Odonat J R Kw, and Hutchins Da. “Interactive influences of bioactive trace metals on biological production in oceanic waters”. In: *Limnology and Oceanography* 36.8 (1991), pp. 1555–1577.

- [22] Donald a Bryant. “The beauty in small things revealed.” In: *Proceedings of the National Academy of Sciences of the United States of America* 100.17 (2003), pp. 9647–9649.
- [23] E. T. Buitenhuis, William K. W. Li, Daniel Vaultot, M. W. Lomas, M. R. Landry, F. Partensky, D. M. Karl, O. Ulloa, L. Campbell, S. Jacquet, F. Lantoiné, F. Chavez, D. Macias, M. Gosselin, and G. B. McManus. “Picophytoplankton biomass distribution in the global ocean”. In: *Earth System Science Data* 4 (2012), pp. 37–46.
- [24] Ana M. Cabello, Mikel Latasa, Irene Forn, Xosé Anxelu G. Morán, and Ramon Mas-sana. “Vertical distribution of major photosynthetic picoeukaryotic groups in stratified marine waters”. In: *Environmental Microbiology* 18.5 (2016), pp. 1578–1590.
- [25] Christine Cagnon. *Microbial Ecotoxicology*. 2017, p. 362.
- [26] Alejandra Calvo-díaz, Xosé Anxelu G Morán, and Luis Ángel Suárez. “Seasonality of picophytoplankton chlorophyll a and biomass in the central Cantabrian Sea , southern Bay of Biscay”. In: *Journal of Marine Systems* 72 (2008), pp. 271–281.
- [27] J Gregory Caporaso, Justin Kuczynski, Jesse Stombaugh, Kyle Bittinger, Frederic D Bushman, Elizabeth K Costello, Noah Fierer, Antonio Gonzalez Peña, Julia K Goodrich, Jeffrey I Gordon, Gavin A Huttlely, Scott T Kelley, Dan Knights, Jeremy E Koenig, Ruth E Ley, Catherine A. Lozupone, Daniel Mcdonald, Brian D Muegge, Meg Pirrung, Jens Reeder, Joel R Sevinsky, Peter J Turnbaugh, William A Walters, Jeremy Widmann, Tanya Yatsunenko, Jesse Zaneveld, and Rob Knight. “QIIME allows analysis of high-throughput community sequencing data”. In: *Nature Methods* 7.5 (2010), pp. 335–336.
- [28] Mary-elena Carr, P Ted Strub, and Andrew C Thomas. “Evolution of 1996–1999 La Niña and El Niño conditions off the western coast of South America: A remote sensing perspective”. In: *Journal of Geophysical Research* 107.C12 (2002), (29) 1 –15.
- [29] Juan C. Castilla. “Environmental impact in sandy beaches of copper mine tailings at Chañaral, Chile”. In: *Marine Pollution Bulletin* 14.12 (1983), pp. 459–464.

- [30] Juan C. Castilla and E. Nealler. “Marine environmental impact due to mining activities of El Salvador copper mine, Chile”. In: *Marine Pollution Bulletin* 9.3 (1978), pp. 67–70.
- [31] William H. Clements and Jason R. Rohr. “Community responses to contaminants: Using basic ecological principles to predict ecotoxicological effects”. In: *Environmental Toxicology and Chemistry* 28.9 (2009), pp. 1789–1800.
- [32] Rober B. Cleveland, William S. Cleveland, Jean E. McRae, and Irma Terpenning. “STL: A seasonal-trend decomposition procedure based on Loess”. In: *Journal of Official Statistics* 6 (1990), pp. 3–73.
- [33] J. E. Cloern. “Our evolving conceptual model of coastal eutrophication problem”. In: *Marine Ecology Progress Series* 210 (2001), pp. 223–253.
- [34] James E Cloern, Paulo C Abreu, Jacob Carstensen, Laurent Chauvaud, Ragnar Elmgren, Jacques Grall, Holly Greening, and John Olov Roger. “Human activities and climate variability drive fast-paced change across the world ’ s estuarine – coastal ecosystems”. In: *Global Change Biology* 22 (2016), pp. 513–529.
- [35] Kenneth H. Coale. “Effects of iron , manganese , and zinc enrichments on productivity and biomass in the subarctic Pacific”. In: *Limnology and Oceanography* 36.8 (1991), pp. 1851–1864.
- [36] Clément Coclet, Cédric Garnier, Floriane Delpy, Dominique Jamet, Gaël Durrieu, Christophe Le Poupon, Marie Mayer, and Benjamin Misson. “Trace metal contamination as a toxic and structuring factor impacting ultraphytoplankton communities in a multicontaminated Mediterranean coastal area”. In: *Progress in Oceanography* (2017).
- [37] Silvana Collado-Fabbri, Daniel Vaultot, and Osvaldo Ulloa. “Structure and seasonal dynamics of the eukaryotic picophytoplankton community in a wind-driven coastal upwelling ecosystem”. In: *Limnology and Oceanography* 56.6 (2011), pp. 2334–2346.

- [38] Paige E. Connell, Victoria Campbell, Alyssa G. Gellene, Sarah K. Hu, and David A. Caron. “Planktonic food web structure at a coastal time-series site: II. Spatiotemporal variability of microbial trophic activities”. In: *Deep-Sea Research Part I: Oceanographic Research Papers* 121. July 2016 (2017), pp. 210–223.
- [39] Florence Corellou, Christian Schwartz, Jean Paul Motta, El Batoul Djouani-Tahri, Frédéric Sanchez, and François Yves Bougeta. “Clocks in the green lineage: Comparative functional analysis of the circadian architecture of the picoeukaryote *ostreococcus*”. In: *Plant Cell* 21.11 (2009), pp. 3436–3449.
- [40] Juan A. Correa, Juan C. Castilla, Marco Ramirez, Manuel Varas, Nelson Lagos, Sofia Vergara, Alejandra Moenne, Domingo Roman, and Murray T. Brown. “Copper, copper mine tailings and their effect on marine algae in northern Chile”. In: *Journal of Applied Phycology* 11.1 (1999), pp. 57–67.
- [41] Robert Costanza, Ralph Arge, Rudolf DeGroot, Stephen Farberk, Monica Grasso, Bruce Hannon, Karin Limburg, Shahid Naeem, Robert V O Neill, Jose Paruelo, Robert G Raskin, and Paul. Sutton. “The value of the world’s ecosystem services and natural capital”. In: *Nature* 387. May (1997), pp. 253–260.
- [42] Peter D. Countway and David A. Caron. “Abundance and distribution of *Ostreococcus* sp. in the San Pedro channel, California, as revealed by quantitative PCR”. In: *Applied and Environmental Microbiology* 72.4 (2006), pp. 2496–2506.
- [43] Caitlin Mullan Crain, Kristy Kroeker, and Benjamin S. Halpern. “Interactive and cumulative effects of multiple human stressors in marine systems”. In: *Ecology Letters* 11.12 (2008), pp. 1304–1315.
- [44] Giovanni Daneri, Victor Dellarossa, Renato Quiñones, Barbara Jacob, Paulina Montero, and Osvaldo Ulloa. “Primary production and community respiration in the Humboldt Current System off Chile and associated oceanic areas”. In: *Marine Ecology Progress Series* 197 (2000), pp. 41–49.

- [45] Martin Daufresne, Kathrin Lengfellner, and Ulrich Sommer. “Global warming benefits the small in aquatic ecosystems.” In: *Proceedings of the National Academy of Sciences of the United States of America* 106.31 (2009), pp. 12788–12793.
- [46] Anderson Abel de Souza Machado, Kate Spencer, Werner Kloas, Marco Toffolon, and Christiane Zarfl. “Metal fate and effects in estuaries: A review and conceptual model for better understanding of toxicity”. In: *Science of the Total Environment* 541 (2016), pp. 268–281.
- [47] B. Debelius, J. M. Forja, Á. DelValls, and L. M. Lubián. “Toxic effect of copper on marine picophytoplankton populations isolated from different geographic locations”. In: *Scientia Marina* 74.S1 (2010), pp. 133–141.
- [48] B. Debelius, J. M. Forja, T. A. Delvalls, and L. M. Lubián. “Toxicity of copper in natural marine picoplankton populations”. In: *Ecotoxicology* 18 (2009), pp. 1095–1103.
- [49] Evelyne Derelle, Conchita Ferraz, Stephane Rombauts, Pierre Rouzé, Alexandra Z. Worden, Steven Robbens, Frédéric Partensky, Sven Degroeve, Sophie Echeynié, Richard Cooke, Yvan Saeys, Jan Wuyts, Kamel Jabbari, Chris Bowler, Olivier Panaud, Benoît Piégu, Steven G Ball, Jean-Philippe Ral, François-Yves Bouget, Gwenael Piganeau, Bernard De Baets, André Picard, Michel Delseny, Jacques Demaille, Yves Van de Peer, and Hervé Moreau. “Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features.” In: *Proceedings of the National Academy of Sciences of the United States of America* 103.31 (2006), pp. 11647–11652.
- [50] Scott C. Doney, Mary Ruckelshaus, J. Emmett Duffy, James P. Barry, Francis Chan, Chad a. English, Heather M. Galindo, Jacqueline M. Grebmeier, Anne B. Hollowed, Nancy Knowlton, Jeffrey Polovina, Nancy N. Rabalais, William J. Sydeman, and Lynne D. Talley. “Climate change impacts on marine ecosystems”. In: *Annual Review of Marine Science* 4.1 (2012), pp. 11–37.

- [51] Robert A . Duce and Neil W . Tindale. “Atmospheric transport of iron and its deposition in the ocean”. In: *Limnology and Oceanography* 36.8 (1991), pp. 1715–1726.
- [52] Christopher L. Dupont, Gregor Grass, and Christopher Rensing. “Copper toxicity and the origin of bacterial resistance—new insights and applications”. In: *Metallomics* 3.11 (2011), p. 1109.
- [53] V Echevin, O Aumont, J Ledesma, and G Flores. “The seasonal cycle of surface chlorophyll in the peruvian upwelling system: A modelling study”. In: *Progress in Oceanography* 79 (2008), pp. 167–176.
- [54] Robert C Edgar. “Search and clustering orders of magnitude faster than BLAST”. In: *Bioinformatics* 26.19 (2010), pp. 2460–2461.
- [55] V. Escaravage, T. C. Prins, A. C. Smaal, and J. C.H. Peeters. “The response of phytoplankton communities to phosphorus input reduction in mesocosm experiments”. In: *Journal of Experimental Marine Biology and Ecology* 198.1 (1996), pp. 55–79.
- [56] P. G. Falkowski. “Biogeochemical controls and feedbacks on ocean primary production”. In: *Science* 281.5374 (1998), pp. 200–206.
- [57] P. G. Falkowski, Miriam E Katz, Andrew H Knoll, Antonietta Quigg, John a Raven, Oscar Schofield, and F J R Taylor. “The evolution of modern eukaryotic phytoplankton.” In: *Science (New York, N.Y.)* 305.5682 (2004), pp. 354–360.
- [58] Christopher B. Field, Michael J. Behrenfeld, James T. Randerson, and P. G. Falkowski. “Primary production of the biosphere: Integrating terrestrial and oceanic components”. In: *Science* 281.5374 (1998), pp. 237–240.
- [59] Zoe V. Finkel, John Beardall, Kevin J. Flynn, Antonietta Quigg, T. Alwyn V Rees, and John a. Raven. “Phytoplankton in a changing world: Cell size and elemental stoichiometry”. In: *Journal of Plankton Research* 32.1 (2010), pp. 119–137.
- [60] I D L Foster and S M Charlesworth. “Heavy metals in the hydrological cycle: trends and explanation”. In: *Hydrological Processes* 10.August 1994 (1996), pp. 227–261.

- [61] E. Fouilland, C. Descolas-Gros, C. Courties, Y. Collos, A. Vaquer, and A. Gasc. “Productivity and growth of a natural population of the smallest free-living eukaryote under nitrogen deficiency and sufficiency”. In: *Microbial Ecology* 48.1 (2004), pp. 103–110.
- [62] Catherine Gérikas Ribeiro, Adriana Lopes Dos Santos, Dominique Marie, Frederico Pereira Brandini, and Daniel Vaultot. “Small eukaryotic phytoplankton communities in tropical waters off Brazil are dominated by symbioses between Haptophyta and nitrogen-fixing cyanobacteria”. In: *ISME Journal* (2018), pp. 1–15.
- [63] Caterina R Giner, Vanessa Balagué, Anders K Krabberød, Isabel Ferrera, Albert Reñé, Esther Garcés, Josep M Gasol, Ramiro Logares, and Ramon Massana. “Quantifying long term recurrence in planktonic microbial eukaryotes”. In: *Molecular Ecology* October (2018), pp. 923–935.
- [64] Jennifer B. Glass and Victoria J. Orphan. “Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous Oxide”. In: *Frontiers in Microbiology* 3.February (2012), pp. 1–20.
- [65] Martha Gledhill, Eric P. Achterberg, Keqiang Li, Khairul N. Mohamed, and Micha J A Rijkenberg. “Influence of ocean acidification on the complexation of iron and copper by organic ligands in estuarine waters”. In: *Marine Chemistry* 177 (2015), pp. 421–433.
- [66] M. Gonzalez-Davila. “The role of phytoplankton cells on the control of heavy metal concentration in seawater”. In: *Marine Chemistry* 48.3-4 (1995), pp. 215–236.
- [67] Laure Guillou, Dipankar Bachar, Stéphane Audic, David Bass, Cédric Berney, Lucie Bittner, Christophe Boutte, Gaétan Burgaud, Colomban De Vargas, Johan Decelle, Javier Del Campo, John R. Dolan, Micah Dunthorn, Bente Edvardsen, Maria Holzmann, Wiebe H.C.F. Kooistra, Enrique Lara, Noan Le Bescot, Ramiro Logares, Frédéric Mahé, Ramon Massana, Marina Montresor, Raphael Morard, Fabrice Not, Jan Pawlowski, Ian Probert, Anne Laure Sauvadet, Raffaele Siano, Thorsten Stoeck, Daniel Vaultot, Pascal Zimmermann, and Richard Christen. “The protist ribosomal reference database (PR2):

- A catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy”. In: *Nucleic Acids Research* 41 (2013), pp. 597–604.
- [68] Laure Guillou, Wenche Eikrem, Marie-Josèphe Chrétiennot-Dinet, Florence Le Gall, Ramon Massana, Khadidja Romari, Carlos Pedrós-Alió, and Daniel Vaultot. “Diversity of picoplanktonic prasinophytes assessed by direct nuclear SSU rDNA sequencing of environmental samples and novel isolates retrieved from oceanic and coastal marine ecosystems.” In: *Protist* 155.2 (2004), pp. 193–214.
- [69] Jian Guo, Suzanne Lapi, Thomas J. Ruth, and Maria T. Maldonado. “The effects of iron and copper availability on the copper stoichiometry of marine phytoplankton”. In: *Journal of Phycology* 48.2 (2012), pp. 312–325.
- [70] Götz Haferburg and Erika Kothe. “Microbes and metals: Interactions in the environment”. In: *Journal of Basic Microbiology* 47.6 (2007), pp. 453–467.
- [71] Benjamin S. Halpern, Melanie Frazier, John Potapenko, Kenneth S. Casey, Kellee Koenig, Catherine Longo, Julia Stewart Lowndes, R. Cotton Rockwood, Elizabeth R. Selig, Kimberly A. Selkoe, and Shaun Walbridge. “Spatial and temporal changes in cumulative human impacts on the world’s ocean”. In: *Nature Communications* 6.May (2015), pp. 1–7.
- [72] Benjamin S. Halpern, Kimberly A. Selkoe, Fiorenza Micheli, and Carrie V. Kappel. “Evaluating and ranking the vulnerability of global marine ecosystems to anthropogenic threats”. In: *Conservation Biology* 21.5 (2007), pp. 1301–1315.
- [73] Benjamin S. Halpern, Shaun Walbridge, Kimberly A. Selkoe, Carrie V. Kappel, Fiorenza Micheli, Caterina D’Agrosa, John F. Bruno, Kenneth S. Casey, Colin Ebert, Helen E. Fox, Rod Fujita, Dennis Heinemann, Hunter S. Lenihan, Elizabeth M. P. Madin, Matthew T. Perry, Elizabeth R. Selig, Mark Spalding, Robert Steneck, and Reg Watson. “A global map of human impact on marine ecosystems”. In: *Science* 948.2008 (2009), pp. 948–953.

- [74] Joe J Harrison, Howard Ceri, and Raymond J Turner. “Multimetal resistance and tolerance in microbial biofilms.” In: *Nature reviews. Microbiology* 5.12 (2007), pp. 928–938.
- [75] Karl E. Havens. “Structural and functional responses of a freshwater plankton community to acute copper stress”. In: *Environmental Pollution* 86.3 (1994), pp. 259–266.
- [76] Carlos Henríquez-castillo, Hugo Botebol, and Adelaide Mouton. “Ostreococcus tauri luminescent reporter lines as biosensors for detecting pollution from copper-mine tailing effluents in coastal environments”. In: *Frontiers of Environmental Science* 6.May (2018), pp. 1–11.
- [77] Carlos Henríquez-Castillo, Susana Rodríguez-Marconi, Francisca Rubio, Nicole Trefault, Santiago Andrade, and Rodrigo De la Iglesia. “Eukaryotic picophytoplankton community response to copper enrichment in a metal-perturbed coastal environment”. In: *Phycological Research* 63 (2015), pp. 1–9.
- [78] Marta Hernández-Ruiz, Esther Barber-Lluch, Antero Prieto, Xosé Antón Álvarez-Salgado, Ramiro Logares, and Eva Teira. “Seasonal succession of small planktonic eukaryotes inhabiting surface waters of a coastal upwelling system”. In: *Environmental Microbiology* 20.8 (2018), pp. 2955–2973.
- [79] Linn J Hoffmann, Eike Breitbarth, Philip W Boyd, and Keith A Hunter. “Influence of ocean warming and acidification on trace metal biogeochemistry”. In: *Marine Ecology Progress Series* 470 (2012), pp. 191–205.
- [80] C. S. Hollings. “Resilience and stability of ecological systems”. In: *Annual Review Ecology and Systematics* 4 (1973), pp. 1–23.
- [81] Rodrigo de la Iglesia, Daniel Valenzuela-Heredia, Santiago Andrade, Juan Correa, and Bernardo González. “Composition dynamics of epilithic intertidal bacterial communities exposed to high copper levels”. In: *FEMS Microbiology Ecology* 79.3 (2012), pp. 720–727.

- [82] Shahidul Islam and Masaru Tanaka. “Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis”. In: *Marine Pollution Bulletin* 48 (2004), pp. 624–649.
- [83] Jeremy E Jacquot, Yoshiko Kondo, Angela N Knapp, and James W Moffett. “The speciation of copper across active gradients in nitrogen-cycle processes in the eastern tropical South Pacific”. In: *Limnology and Oceanography* 58.4 (2013), pp. 1387–1394.
- [84] Ludwig Jardillier, Mikhail V Zubkov, John Pearman, and David J Scanlan. “Significant CO₂ fixation by small prymnesiophytes in the subtropical and tropical northeast Atlantic Ocean.” In: *The ISME journal* 4.9 (2010), pp. 1180–1192.
- [85] I. R. Joint, N. J. P. Owens, and A. J. Pomeroy. “Seasonal production of photosynthetic picoplankton and nanoplankton in the Celtic Sea”. In: *Marine Ecology Progress Series* 28 (1986), pp. 251–258.
- [86] Antoni Jordi, Gotzon Basterretxea, Antonio Tovar-Sánchez, Andrés Alastuey, and Xavier Querol. “Copper aerosols inhibit phytoplankton growth in the Mediterranean Sea.” In: *Proceedings of the National Academy of Sciences of the United States of America* 109.52 (2012), pp. 21246–9.
- [87] Eric Karsenti, Silvia G. Acinas, Peer Bork, Chris Bowler, Colomban de Vargas, Jeroen Raes, Matthew B. Sullivan, Detlev Arendt, Francesca Benzoni, Jean Michel Claverie, Mick Follows, Gaby Gorsky, Pascal Hingamp, Daniele Iudicone, Olivier Jaillon, Stefanie Kandels-Lewis, Uros Krzic, Fabrice Not, Hiroyuki Ogata, Stéphane Pesant, Emmanuel Georges Reynaud, Christian Sardet, Michael E. Sieracki, Sabrina Speich, Didier Velayoudon, Jean Weissenbach, and Patrick Wincker. “A holistic approach to marine Eco-systems biology”. In: *PLoS Biology* 9.10 (2011), pp. 7–11.
- [88] Michael J Kennish. “Environmental threats and environmental future of estuaries”. In: *Environmental Conservation* 29.1 (2002), pp. 78–107.

- [89] Amy R Kirkham, Cécile Lepère, Ludwig E Jardillier, Fabrice Not, Heather Bouman, Andrew Mead, and David J Scanlan. “A global perspective on marine photosynthetic picoeukaryote community structure.” In: *The ISME journal* 7 (2013), pp. 922–936.
- [90] Stefan Lambert, Margot Tragin, Jean-claude Lozano Jean-françois Ghiglione, Daniel Vaultot, and François-yves Bouget Pierre E Galand. “Rhythmicity of coastal marine picoeukaryotes , bacteria and archaea despite irregular environmental perturbations”. In: *The ISME Journal* (2018).
- [91] P.J. le B. Williams and J.K. Egge. “The management and behaviour of the mesocosms”. In: *Estuarine, Coastal and Shelf Science* 46.2 (1998), pp. 3–14.
- [92] Anne Hélène Le Jeune, Marie Charpin, Véronique Deluchat, Jean Francois Briand, Jean Francois Lenain, Michel Baudu, and Christian Amblard. “Effect of copper sulphate treatment on natural phytoplanktonic communities”. In: *Aquatic Toxicology* 80.3 (2006), pp. 267–280.
- [93] Anne Hélène Le Jeune, Marie Charpin, Denis Sargos, Jean François Lenain, Véronique Deluchat, Nadine Ngayila, Michel Baudu, and Christian Amblard. “Planktonic microbial community responses to added copper”. In: *Aquatic Toxicology* 83.3 (2007), pp. 223–237.
- [94] Gaëlle Lelandais and Emmanuel Lesuisse. “Ostreococcus tauri is a new model green alga for studying iron metabolism in eukaryotic phytoplankton”. In: *BMC Genomics* 17.319 (2016), pp. 1–23.
- [95] A Lelong, Eva Bucciarelli, H Hégaret, and Philippe Soudant. “Iron and copper limitations differently affect growth rates and photosynthetic and physiological parameters of the marine diatom *Pseudo-nitzschia delicatissima*”. In: *Limnology and Oceanography* 58.2 (2013), pp. 613–623.

- [96] Joseph a Lemire, Joe J Harrison, and Raymond J Turner. “Antimicrobial activity of metals: mechanisms, molecular targets and applications.” In: *Nature reviews. Microbiology* 11.6 (2013), pp. 371–84.
- [97] William K. W. Li. “Primary production of Prochlorophytes, Cyanobacteria, and Eucaryotic ultraphytoplankton: Measurements from Flow Cytometric Sorting”. In: *Limnology and Oceanography* 39.1 (1994), pp. 169–175.
- [98] William K. W. Li, Fiona a McLaughlin, Connie Lovejoy, and Eddy C Carmack. “Smallest algae thrive as the Arctic Ocean freshens.” In: *Science* 326.5952 (2009), p. 539.
- [99] Gipsi Lima-mendez, Karoline Faust, Nicolas Henry, Johan Decelle, Sébastien Colin, Fabrizio Carcillo, Samuel Chaffron, J Cesar Ignacio-espinoza, Simon Roux, Flora Vincent, and Lucie Bittner. “Determinants of community structure in the global plankton interactome”. In: *Science* 348.6237 (2015), pp. 1–10.
- [100] Hua Xue Liu, Hong Hui Huang, Shan Nan Xu, Ming Dai, and Ping Ping Shen. “Planktonic community structure during a harmful bloom of *Phaeocystis globosa* in a subtropical bay, with special reference to the ciliate assemblages”. In: *Ecotoxicology* 24.7-8 (2015), pp. 1419–1429.
- [101] Adriana Lopes Dos Santos, Thibaut Pollina, Priscillia Gourvil, Erwan Corre, Dominique Marie, José Luis Garrido, Francisco Rodríguez, Mary Hélène Noël, Daniel Vaultot, and Wenche Eikrem. “Chloropicophyceae, a new class of picophytoplanktonic prasinophytes”. In: *Scientific Reports* 7.1 (2017), pp. 1–20.
- [102] Jean-Claude Lozano, Philippe Schatt, Hugo Botebol, Valérie Vergé, Emmanuel Lesuisse, Stéphane Blain, Isabelle a. Carré, and François-Yves Bouget. “Efficient gene targeting and removal of foreign DNA by homologous recombination in the picoeukaryote *Ostreococcus*”. In: *Plant Journal* 78.6 (2014), pp. 1073–1083.
- [103] Katherine R M Mackey, Kristen N. Buck, John R. Casey, Abigail Cid, Michael W. Lomas, Yoshiki Sohrin, and Adina Paytan. “Phytoplankton responses to atmospheric metal

- deposition in the coastal and open-ocean Sargasso Sea”. In: *Frontiers in Microbiology* 3.OCT (2012), pp. 1–15.
- [104] Emilio Marañón, Michael J Behrenfeld, Natalia González, Beatriz Mouriño, and Mikhail V Zubkov. “High variability of primary production in oligotrophic waters of the Atlantic Ocean : uncoupling from phytoplankton biomass and size structure”. In: *Marine Ecology Progress Series* 257 (2003), pp. 1–11.
- [105] M. L. Martínez, A. Intralawan, G. Vázquez, O. Pérez-Maqueo, P. Sutton, and R. Landgrave. “The coasts of our world: Ecological, economic and social importance”. In: *Ecological Economics* 63.2-3 (2007), pp. 254–272.
- [106] Antonio M. Martin-Platero, Brian Cleary, Kathryn Kauffman, Sarah P. Preheim, Dennis J. McGillicuddy, Eric J. Alm, and Martin F. Polz. “High resolution time series reveals cohesive but short-lived communities in coastal plankton”. In: *Nature Communications* 9.1 (2018), pp. 1–11.
- [107] Ramon Massana. *Marine Protists*. 2015, pp. 3–21.
- [108] Ramon Massana, Angélique Gobet, Stéphane Audic, David Bass, Lucie Bittner, Christophe Boute, Aurélie Chambouvet, Richard Christen, Jean Michel Claverie, Johan Decelle, John R. Dolan, Micah Dunthorn, Bente Edvardsen, Irene Forn, Dominik Forster, Laure Guillou, Olivier Jaillon, Wiebe H.C.F. Kooistra, Ramiro Logares, Frédéric Mahé, Fabrice Not, Hiroyuki Ogata, Jan Pawlowski, Massimo C. Pernice, Ian Probert, Sarah Romac, Thomas Richards, Sébastien Santini, Kamran Shalchian-Tabrizi, Raffaele Siano, Nathalie Simon, Thorsten Stoeck, Daniel Vaultot, Adriana Zingone, and Colomban de Vargas. “Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing”. In: *Environmental microbiology* 17.10 (2015), pp. 4035–4049.

- [109] M. Medina, S. Andrade, S. Faugeron, N. Lagos, D. Mella, and J. A. Correa. “Biodiversity of rocky intertidal benthic communities associated with copper mine tailing discharges in northern Chile”. In: *Marine Pollution Bulletin* 50.4 (2005), pp. 396–409.
- [110] Andrew J. Millar, Sharla R. Short, Nam Hai Chua, and Steve A. Kay. “A novel circadian phenotype based on firefly luciferase expression in transgenic plants”. In: *Plant Cell* 4.9 (1992), pp. 1075–1087.
- [111] Frank Millero, Ryan Woosley, Benjamin DiTrollo, and Jason Waters. “Effect of ocean acidification on the speciation of metals in seawater”. In: *Oceanography* 22.4 (2011), pp. 72–85.
- [112] James W. Moffett, Larry E Brand, Peter L Croot, Katherine A Barbeau, James W Moffett, Larry E Brand, and I Peter L Croot. “Cu speciation and cyanobacterial distribution in harbors subject to anthropogenic Cu input”. In: *Limnology and Oceanography* 42.5 (1997), pp. 789–799.
- [113] Ana C Morán, Martha B Hengst, Rodrigo De La Iglesia, Santiago Andrade, Juan a Correa, and Bernardo González. “Changes in bacterial community structure associated with coastal copper enrichment.” In: *Environmental toxicology and chemistry* 27.11 (2008), pp. 2239–2245.
- [114] Michael Morando and Douglas G Capone. “Direct utilization of organic nitrogen by phytoplankton and its role in nitrogen cycling within the southern California bight”. In: *Frontiers in Microbiology* 9.September (2018), pp. 1–13.
- [115] François M. M. Morel. “The co-evolution of phytoplankton and trace element cycles in the oceans”. In: *Geobiology* 6.3 (2008), pp. 318–324.
- [116] François M. M. Morel, Robert J M Hudson, and Neil M. Price. “Limitation of productivity by trace metals in the sea”. In: *Limnology and Oceanography* 36.8 (1991), pp. 1742–1755.

- [117] François M. M. Morel and Neil M. Price. “The biogeochemical cycles of trace metals in the oceans”. In: *Science* 300.5621 (2003), pp. 944–947.
- [118] Balbina Nogales, Mariana P. Lanfranconi, Juana M. Piña-Villalonga, and Rafael Bosch. “Anthropogenic perturbations in marine microbial communities”. In: *FEMS Microbiology Reviews* 35 (2011), pp. 275–298.
- [119] Fabrice Not, Mikel Latasa, Dominique Marie, Thierry Cariou, Daniel Vaultot, and Nathalie Simon. “A single species, *Micromonas pusilla* (Prasinophyceae), dominates the eukaryotic picoplankton in the western english channel”. In: *Applied and Environmental Microbiology* 70.7 (2004), pp. 4064–4072.
- [120] Fabrice Not, Raffaele Siano, Wiebe H C F Kooistra, Nathalie Simon, Daniel Vaultot, and Ian Probert. “Diversity and ecology of eukaryotic marine phytoplankton”. In: *Advances in Botanical Research*. Vol. 64. 2012, pp. 1–53.
- [121] William D. Orsi, Susanne Wilken, Javier del Campo, Thierry Heger, Erick James, Thomas A. Richards, Patrick J. Keeling, Alexandra Z. Worden, and Alyson E. Santoro. “Identifying protist consumers of photosynthetic picoeukaryotes in the surface ocean using stable isotope probing”. In: *Environmental Microbiology* 20.2 (2018), pp. 815–827.
- [122] Hans W Paerl. “Coastal eutrophication and harmful algal blooms : Importance of atmospheric deposition and groundwater as “ new ” nitrogen and other nutrient sources”. In: *Limnology and Oceanography* 42.5 (1997), pp. 1154–1165.
- [123] Brian Palenik, Jane Grimwood, Andrea Aerts, Pierre Rouzé, Asaf Salamov, Nicholas Putnam, Christopher L. Dupont, Richard Jorgensen, Evelyne Derelle, Stephane Rombauts, Kemin Zhou, Robert Otilar, Sabeeha S Merchant, Sheila Podell, Terry Gaasterland, Carolyn Napoli, Karla Gendler, Andrea Manuell, Vera Tai, Olivier Vallon, Gwenael Piganeau, Séverine Jancek, Marc Heijde, Kamel Jabbari, Chris Bowler, Martin Lohr, Steven Robbens, Gregory Werner, Inna Dubchak, Gregory J Pazour, Qinghu Ren, Ian Paulsen, Chuck Delwiche, Jeremy Schmutz, Daniel Rokhsar, Yves Van de Peer,

- Hervé Moreau, and Igor V Grigoriev. “The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation.” In: *Proceedings of the National Academy of Sciences of the United States of America* 104.18 (2007), pp. 7705–7710.
- [124] Darren J. Parris, Sangita Ganesh, Virginia P. Edgcomb, Edward F. DeLong, and Frank J. Stewart. “Microbial eukaryote diversity in the marine oxygen minimum zone off northern Chile”. In: *Frontiers in Microbiology* 5 (2014), pp. 1–11.
- [125] Adina Paytan, Katherine R M Mackey, Ying Chen, Ivan D Lima, Scott C Doney, Natalie Mahowald, Rochelle Labiosa, and Anton F Post. “Toxicity of atmospheric aerosols on marine phytoplankton.” In: *Proceedings of the National Academy of Sciences of the United States of America* 106.12 (2009), pp. 4601–4605.
- [126] Graham Peers and Neil M. Price. “Copper-containing plastocyanin used for electron transport by an oceanic diatom.” In: *Nature* 441.7091 (2006), pp. 341–4.
- [127] Graham Peers, Sarah-Ann Quesnel, and Neil M. Price. “Copper requirements for iron acquisition and growth of coastal and oceanic diatoms”. In: *Limnology and Oceanography* 50.4 (2005), pp. 1149–1158.
- [128] P Penven, V Echevin, J Pasapera, F Colas, and J Tam. “Average circulation , seasonal cycle , and mesoscale dynamics of the Peru Current System : A modeling approach”. In: *Journal of Geophysical Research* 110.C10021 (2005), pp. 1–21.
- [129] John E. Petersen, Chung Chi Chen, and W. Michael Kemp. “Scaling aquatic primary productivity: Experiments under nutrient- and light-limited conditions”. In: *Ecology* 78.8 (1997), pp. 2326–2338.
- [130] Smitha Pillai, Renata Behra, Holger Nestler, Marc J-F Suter, Laura Sigg, and Kristin Schirmer. “Linking toxicity and adaptive responses across the transcriptome, proteome, and phenotype of *Chlamydomonas reinhardtii* exposed to silver.” In: *Proceedings of the National Academy of Sciences of the United States of America* 111.9 (2014), pp. 3490–5.

- [131] Ernani Pinto, T. C. S. Sigaud- Kutner, M. a. S. Leitaó, O. K. Okamoto, David Morse, and Pio Colepicolo. “Heavy metal-induced oxidative stress in algae”. In: *Journal Phycology* 39 (2003), pp. 1008–1018.
- [132] Elmar Pruesse, Christian Quast, Katrin Knittel, Bernhard M Fuchs, Frank Oliver Glo, and Wolfgang Ludwig. “SILVA : a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB”. In: *Nucleic acids research* 35.21 (2007), pp. 7188–7196.
- [133] Alfred C. Redfield. “On the proportions of organic derivatives in sea water and their relation to the composition of plankton”. In: *James Johnstone Memorial Volume, Liverpool University Press*. 1934, pp. 176–192.
- [134] Yoshimi M Rii, Solange Duhamel, Robert R Bidigare, David M Karl, Daniel J Repeta, and Matthew J Church. “Diversity and productivity of photosynthetic picoeukaryotes in biogeochemically distinct regions of the South East Pacific Ocean”. In: *Limnology and Oceanography* 61 (2016), pp. 806–824.
- [135] Emanuel Sanz-luque, Alejandro Chamizo-ampudia, Angel Llamas, Aurora Galvan, and Emilio Fernandez. “Understanding nitrate assimilation and its regulation in microalgae Overview of Nitrate Assimilation”. In: *Frontiers in Plant Science* 6.October (2015), pp. 1–17.
- [136] Elisa Schaum, Björn Rost, Andrew J. Millar, and Sinéad Collins. “Variation in plastic responses of a globally distributed picoplankton species to ocean acidification”. In: *Nature Climate Change* 3.3 (2013), pp. 298–302.
- [137] Marten Scheffer and Stephen R Carpenter. “Catastrophic regime shifts in ecosystems: linking theory to observation”. In: *Trends in Ecology and Evolution* 18.12 (2003), pp. 648–656.
- [138] Ivo Florin Scheiber, Jana Pilátová, Ronald Malych, Eva Kotabova, Matyáš Krijt, Daniel Vyoral, Jan Mach, Thibaut Léger, Jean-Michel Camadro, Ondřej Prášil, Emmanuel

- Lesuisse, and Robert Sutak. “Copper and iron metabolism in *Ostreococcus tauri* – the role of phytoferritin, plastocyanin and a chloroplast copper-transporting ATPase”. In: *Metallomics* (2019).
- [139] Patrick D. Schloss, Sarah L. Westcott, Thomas Ryabin, Justine R. Hall, Martin Hartmann, Emily B. Hollister, Ryan A. Lesniewski, Brian B. Oakley, Donovan H. Parks, Courtney J. Robinson, Jason W. Sahl, Blaz Stres, Gerhard G. Thallinger, David J. Van Horn, and Carolyn F. Weber. “Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities”. In: *Applied and Environmental Microbiology* 75.23 (2009), pp. 7537–7541.
- [140] Hanan Schoffman, Hagar Lis, Yeala Shaked, and Nir Keren. “Iron–nutrient interactions within phytoplankton”. In: *Frontiers in Plant Science* 7.August (2016), pp. 1–12.
- [141] David M. Semeniuk, Randelle M. Bundy, Chris D. Payne, Katherine A. Barbeau, and Maria T. Maldonado. “Acquisition of organically complexed copper by marine phytoplankton and bacteria in the northeast subarctic Pacific Ocean”. In: *Marine Chemistry* 173 (2015), pp. 222–233.
- [142] Ashley Shade, Hannes Peter, Steven D. Allison, Didier L. Baho, Mercè Berga, Helmut Bürgmann, David H. Huber, Silke Langenheder, Jay T. Lennon, Jennifer B H Martiny, Kristin L. Matulich, Thomas M. Schmidt, and Jo Handelsman. “Fundamentals of microbial community resistance and resilience”. In: *Frontiers in Microbiology* 3 (2012), pp. 1–19.
- [143] Dalin Shi, Yan Xu, Brian M Hopkinson, and François M. M. Morel. “Effect of ocean acidification on iron availability to marine phytoplankton.” In: *Science* 327.5966 (2010), pp. 676–679.
- [144] Xiao Li Shi, Dominique Marie, Ludwig Jardillier, David J. Scanlan, and Daniel Vaultot. “Groups without Cultured Representatives Dominate Eukaryotic Picophytoplankton in the Oligotrophic South East Pacific Ocean”. In: *PLoS ONE* 4.10 (2009), e7657.

- [145] Christophe Six, Alexandra Z. Worden, Francisco Rodríguez, Hervé Moreau, and Frédéric Partensky. “New insights into the nature and phylogeny of prasinophyte antenna proteins: *Ostreococcus tauri*, a case study”. In: *Molecular Biology and Evolution* 22.11 (2005), pp. 2217–2230.
- [146] Xue Song, Zhimeng Xu, Qian Liu, Yan Li, Yu Ma, Jian Wang, Mengran Sun, Hongbing Shao, Hui Sun, Gill Malin, Yong Jiang, and Min Wang. “Comparative study of the composition and genetic diversity of the picoeukaryote community in a Chinese aquaculture area and an open sea area”. In: *Journal of the Marine Biological Association of the United Kingdom* 97.1 (2017), pp. 151–159.
- [147] J. L. Stauber, S. Andrade, M. Ramirez, M. Adams, and J. a. Correa. “Copper bioavailability in a coastal environment of Northern Chile: Comparison of bioassay and analytical speciation approaches”. In: *Marine Pollution Bulletin* 50.11 (2005), pp. 1363–1372.
- [148] Diane K. Stoecker. “Conceptual models of mixotrophy in planktonic protists and some ecological and evolutionary implications”. In: *European Journal of Protistology* 34.3 (1998), pp. 281–290.
- [149] C. Strayer, T. Oyama, T. F. Schultz, R. Raman, D. E. Somers, P. Mas, S. Panda, J. A. Kreps, and S. A. Kay. “Cloning of the Arabidopsis clock gene TOC1, an autoregulatory response regulator homolog”. In: *Science* 289.5480 (2000), pp. 768–771.
- [150] William G. Sunda. “Feedback interactions between trace metal nutrients and phytoplankton in the ocean”. In: *Frontiers in Microbiology* 3.JUN (2012), pp. 1–22.
- [151] William G Sunda. “Trace Metal Interactions with Marine Phytoplankton”. In: *Biological Oceanography* 6.5-6 (1989), pp. 411–442.
- [152] William G. Sunda and Susan A. Huntsman. “Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems”. In: *Science of the Total Environment* 219.2-3 (1998), pp. 165–181.

- [153] William G. Sunda and Susan A. Huntsman. “Regulation of copper concentration in the oceanic nutricline by phytoplankton uptake and regeneration cycles”. In: *Limnology and Oceanography* 40.1 (1995), pp. 132–137.
- [154] William J. Sutherland, Robert P. Freckleton, H. Charles J. Godfray, Steven R. Beissinger, Tim Benton, Duncan D. Cameron, Yohay Carmel, David A. Coomes, Tim Coulson, Mark C. Emmerson, Rosemary S. Hails, Graeme C. Hays, Dave J. Hodgson, Michael J. Hutchings, David Johnson, Julia P.G. Jones, Matt J. Keeling, Hanna Kokko, William E. Kunin, Xavier Lambin, Owen T. Lewis, Yadvinder Malhi, Nova Mieszkowska, E. J. Milner-Gulland, Ken Norris, Albert B. Phillimore, Drew W. Purves, Jane M. Reid, Daniel C. Reuman, Ken Thompson, Justin M.J. Travis, Lindsay A. Turnbull, David A. Wardle, and Thorsten Wiegand. “Identification of 100 fundamental ecological questions”. In: *Journal of Ecology* 101.1 (2013), pp. 58–67.
- [155] P Tett, R J Gowen, S J Painting, M Elliott, R Forster, D K Mills, E Bresnan, E Capuzzo, T F Fernandes, J Foden, R J Geider, L C Gilpin, M Huxham, S J Malcolm, T Platt, and M Racault. “Framework for understanding marine ecosystem health”. In: *Marine Ecology Progress Series* 494 (2013), pp. 1–27.
- [156] Martin Thiel, Erasmo C. Macaya, Enzo Acuña, Wolf E. Arntz, Horacio Bastias, Katherina Brokordt, Patricio A. Camus, Juan C. Castilla, Leonardo R. Castro, Maritza Cortés, Clement P. Dumont, Ruben Escribano, Miriam Fernandez, Jhon A. Gajardo, Carlos F. Gaymer, Ivan Gomez, Andrés E. González, Humberto E. González, Pilar A. Haye, Juan-Enrique Illanes, Jose L. Iriarte, Domingo A. Lancellotti, Guillermo Luna-Jorquera, Carolina Luxoro, Patricio H. Manriquez, Víctor Marín, Praxedes Muñoz, Sergio A. Navarrete, Eduardo Perez, Elie Poulin, Javier Sellanes, Hector H. Sepúlveda, Wolfgang Stotz, Fadia Tala, Andrew Thomas, Cristian A. Vargas, Julio A. Vasquez, and J.M. Alonso Vega. “The Humboldt Current System of northern and central Chile oceanographic processes, ecological interactions and socioeconomic feedback”. In: *Oceanography and Marine Biology: An Annual Review* 45 (2007), pp. 195–344.

- [157] K R Timmermans, M Gledhill, R F Nolting, M J W Veldhuis, H J W de Baar, and C M G van den Berg. “Responses of marine phytoplankton in iron enrichment experiments in the northern North Sea and northeast Atlantic Ocean”. In: *Marine Chemistry* 61.3-4 (1998), pp. 229–242.
- [158] Margot Tragin and Daniel Vaultot. “Green microalgae in marine coastal waters: The ocean sampling day (OSD) dataset”. In: *Scientific Reports* September (2018), pp. 1–12.
- [159] Benjamin S. Twining and Stephen B. Baines. “The trace metal composition of marine phytoplankton.” In: *Annual review of marine science* 5 (2013), pp. 191–215.
- [160] Benjamin S. Twining, Sara Rauschenberg, Peter L. Morton, and Stefan Vogt. “Metal contents of phytoplankton and labile particulate material in the north Atlantic ocean”. In: *Progress in Oceanography* 137 (2015), pp. 261–283.
- [161] Colomban De Vargas, Stéphane Audic, Nicolas Henry, Johan Decelle, Frédéric Mahé, Ramiro Logares, Enrique Lara, Cédric Berney, Noan Le Bescot, Ian Probert, Margaux Carmichael, Julie Poulain, and Sarah Romac. “Eukaryotic plankton diversity in the sunlit ocean”. In: *Science* 348.6237 (2015), pp. 1–12.
- [162] Cristian A Vargas, Rodrigo A Martí, L Antonio Cuevas, Marcelo A Pavez, Carolina Cartes, Humberto E Gonza, and Giovanni Daneri. “The relative importance of microbial and classical food webs in a highly productive coastal upwelling area”. In: *Limnology and Oceanography* 52.4 (2007), pp. 1495–1510.
- [163] Daniel Vaultot, Wenche Eikrem, Manon Viprey, and Hervé Moreau. “The diversity of small eukaryotic phytoplankton ($\leq 3 \mu\text{m}$) in marine ecosystems”. In: *FEMS Microbiology Reviews* 32.5 (2008), pp. 795–820.
- [164] Alexandra Z. Worden. “Picoeukaryote diversity in coastal waters of the Pacific Ocean”. In: *Aquatic Microbial Ecology* 43 (2006), pp. 165–175.

- [165] Alexandra Z. Worden, M. J. Follows, S. J. Giovannoni, S. Wilken, a. E. Zimmerman, and P. J. Keeling. “Rethinking the marine carbon cycle: Factoring in the multifarious lifestyles of microbes”. In: *Science* 347.6223 (2015), (1257594) 1–10.
- [166] Alexandra Z. Worden, Jessica K. Nolan, and B. Palenik. “Assessing the dynamics and ecology of marine picophytoplankton: The importance of the eukaryotic component”. In: *Limnology and Oceanography* 49.1 (2004), pp. 168–179.
- [167] Boris Worm, Edward B. Barbier, Nicola Beaumont, J. Emmett Duffy, Carl Folke, Benjamin S. Halpern, Jeremy B.C. Jackson, Heike K. Lotze, Fiorenza Micheli, Stephen R. Palumbi, Enric Sala, Kimberley A. Selkoe, John J. Stachowicz, and Reg Watson. “Impacts of biodiversity loss on ocean ecosystem services”. In: *Science* 314.5800 (2006), pp. 787–790.
- [168] Wenxue Wu, Lei Wang, Yu Liao, Songli Xu, and Bangqin Huang. “Spatial and seasonal distributions of photosynthetic picoeukaryotes along an estuary to basin transect in the northern South China Sea”. In: *Journal of Plankton Research* 39.3 (2017), pp. 423–435.
- [169] Xiangfeng Zeng, Xijuan Chen, and Jie Zhuang. “The positive relationship between ocean acidification and pollution”. In: *Marine Pollution Bulletin* 91.1 (2015), pp. 14–21.
- [170] Amel Bellaaj Zouari, Malika Bel Hassen, Vanessa Balagué, Emna Sahli, Mohamed Yacine Ben Kacem, Fourat Akrouf, Asma Hamza, and Ramon Massana. “Picoeukaryotic diversity in the Gulf of Gabès: Variability patterns and relationships to nutrients and water masses”. In: *Aquatic Microbial Ecology* 81.1 (2018), pp. 37–53.