PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE
Facultad de Ciencias Biológicas
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Mención Genética Molecular y Microbiología

"Influence of copper on the taxonomic composition of picophytoplankton in the bay of Chañaral"

CARLOS HENRÍQUEZ CASTILLO

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“Influence of copper on the taxonomic composition of picophytoplankton in the bay of Chañaral”

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List of abbreviators

CCA	: Canonical correspondence analysis
CDKA	: Cyclin-dependent kinase A
Cu/ZnSOD	: Copper/Zinc superoxide dismutase
dNTP	: Desoxinucleotide triphosphate
EC_{50}	: Effective concentration 50
EDTA	: Ethylenediaminetetraacetic acid
GPWP	: Polyethersulphone
GSWP	: Mixed cellulose esters
HapT	: High affinity phosphate transporter
HCS	: High copper site
ICP-MS	: Inductively coupled plasm-mass spectrometry
LCS	: Low copper site
LHY-7	: morning-expressed MYB transcription factor
Luc	: Luciferase
MDS	: Multidimensional scalling
NED	: 2′-chloro- 5′-fluoro-7′,8′-phenyl-1,4-dichloro-6-carboxyfluorescein
OTUs	: Operational taxonomic units
PCA	: Principal component analysis
PCR	: Polymerase chain reaction
perMANOVA	: Permutational multivariate analysis of variance
PPE	: Photosynthetic picoeukaryotes
RH	: Homologous recombination
RI	: Random insertion
rmANOVA	: Repeated measures analysis of variance
ROS	: Oxygen reactive species
rRNA	: Ribosomal ribonucleic Acid
SIMPER	: Similarity percentage

Taq	: *Thermus aquaticus*
T-RFLP	: Terminal restriction fragment length polymorphisms
T-RFs	: Terminal restriction fragments

Abstract

Microorganisms are the most abundant biological entities in the biosphere. Microbial communities are now recognized as critical components of marine food webs and nutrient cycles in the ocean. The autotrophic fractions of such communities are responsible for about half of the total CO_2 fixation on earth and the heterotrophic fractions direct a large proportion of total flows of matter and energy. Traditionally, marine picophytoplankton (< 3µm in cell diameter cells) was thought to include only cyanobacteria of the genera *Synechococcus* and *Prochlorococcus*. However it has recently been determined that the eukaryotic microbial component of the picophytoplankton, known as picoeukaryotes, are also abundant and important within marine systems. An unexpectedly high diversity of picoeukaryotes has been revealed in marine environments, displaying a dynamic behavior. Both components of the picophytoplankton (bacteria and eukarya) are crucial players in coastal systems. However, basic questions about how such communities are regulated by environmental factors and disturbances are still poorly resolved. The development of new methodologies of study, based on molecular tools, has improved our understanding of these communities and has introduced new ways of understanding how they respond to and regulate the environment in which they live.

Many marine coastal systems are subjected to disturbances by human activities. Disturbances related to increases in the concentration of heavy metals such as copper are of particular interest, due to their negative effects on photosynthetic microbial communities. Copper is an interesting trace metal because of its dual role. On one hand, it is needed at trace levels for the correct functioning of the cellular machinery, being part of proteins involved in electron transfer and activation and transport of oxygen, but at higher concentrations copper becomes toxic to the cell. Besides that, both copper-excess and cooper-deficiency differentially affects the different components of the picophytoplankton, in terms of cellular requirements and toxicity. This intrinsically means that ecosystem balance would be altered by copper availability whether at levels that cause toxicity or at levels that relieve metal-limitation for some species. In this context, the relationship between elevated concentrations of metals and changes in picophotosynthetic microbial communities has been poorly studied. Currently, processes of copper mining are responsible by far for the greatest pollution observed due to this metal, creating environmental problems in terrestrial and aquatic ecosystems. However, recent studies indicate that copper pollution could become a more spread problem due to deposition of copper-enrich atmospheric dust.

The main objective of this work was to study how communities of picophotosynthetic organisms respond to prolonged exposure to elevated copper levels, with special emphasis on the picoeukaryotic component, through a comparative analysis of

coastal areas with different history of exposure to this type of disturbance. We demonstrate that i) copper is the main factor that explain the physico-chemical differences between site, ii) copper does not exert a negative effect over the cellular abundance of picophytoplankton inhabit the area and iii) the community composition of the study area is different from other coastal environments, where low copper levels has been reported. This differences indicates that, in fact, copper levels are pushing to changes in community composition of photosynthetic picoeukaryotes. A sustained and intensively sampling during years are necessary to conclude if the amount of copper, which is deposited daily on the sea, have an impact on microbial communities, which can alter the energy transfer through the food web.

GENERAL INTRODUCTION

Marine picophytoplankton

Marine phytoplankton are a polyphyletic group of microbial photoautotrophs that evolved in the Archean ocean more than 2800 million years ago. Is a group of crucial importance in regulating aquatic food webs, biogeochemical cycles and Earth's climate (Bidle & Falkowski, 2004). By the use of solar energy and carbon dioxide they generate oxygen, and also important organic compounds that are pumped to higher trophic levels and to the deep ocean through the biological pump (Finnazi *et al*., 2010). Even when they are <1% of Earth's photosynthetic biomass, phytoplankton produce near 50% of global annual carbon-based primary productivity (Field, 1998). Bacterial and eukaryotic microorganisms that colonizes the upper part of the water column, until the limits of light penetration, compose this ecologically relevant group.

Phytoplankton span a wide range of cellular sizes, and often is classified based on equivalent spherical diameter, as a proxy to carbon content. Phytoplankton range in size from < 1µm to 1mm in equivalent spherical diameter, corresponding to > 8 orders of magnitude variation in cell volume (Finkel *et al.*, 2004). According to that, phytoplankton can be divided into microphytoplankton (200-20 µm diameter), nanophytoplankton (20- 3µm diameter) and picophytoplankton (3-0.2 µm diameter) (Sieburth *et al.*, 1978). The size structure of phytoplankton assemblages strongly influences energy transfer through the food web, because cell size influences nutrient uptake kinetics, photosynthesis efficiency, respiration, growth and sinking rates, as well as genome size and rate of evolution (Finkel 2001).

The pico-sized fraction of the phytoplankton has been received increased attention during the last fifteen years (Díez *et al.*, 2001, Moon-van der Staay *et al.*, 2001). The fast progress in the study of this organisms with both field and large-scale sequencing projects approaches, have provide fundamental advances in knowledge insight basic biology, evolution, and molecular machineries that control organism responses to the environment (Worden *et al.*, 2015).

Traditionally, it was thought that marine picophytoplankton only include cyanobacteria of the genera *Synechococcus* (Waterbury *et al.*, 1979) and *Prochlorococcus* (Chisholm *et al.*, 1988). These two monophyletic groups dominates most oceanic waters in terms of numbers (~10^5 cells/mL) and are considered the most abundant photosynthetic organisms on Earth (Scanlan *et al.*, 2009). Due to its metabolic flexibility, an ecological partitioning of the two genera has been observed (Scanlan *et al.*, 2009). In terms of its global distribution, *Prochlorococcus* displays a gradient of abundance with higher cell concentrations in offshore than in coastal areas, and *Synechococcus* present a more ubiquitous distribution found in virtually any ecosystem (Scanlan *et al.*, 2009) being the dominant cyanobacterial group in coastal waters (Zwirglmaier *et al.*, 2008). The use of molecular tools for taxonomic assignation, like the analyses of 16S rRNA gene sequence and photosystem II gene *pet*B, has shown that *Synechococcus* have a larger number of phylogenetically distinct lineages than *Prochlorococcus* (Zwirglmaier *et al.*, 2008; Humily *et al*., 2014). These differences in phylogeny makes the community structure of *Synechococcus* highly complex, however, the physiological and ecological traits associated with this diversity are not completely understood (Stuart *et al*., 2013). Nowadays is clear that eukaryotes are an important component of the picophytoplankton (Díez *et al.*, 2001).

Photosynthetic picoeukaryotes (PPE) are a highly diverse group composed by a number of different taxa with representatives of 4 of the 5 supergroups of eukaryotes (reviewed in Not *et al.*, 2012), including the Stramenopiles, the Haptophyceae and the Mamiellophyceae class as ecologically relevant groups. The Mamiellophyceae class contains the smaller eukaryotic species in the planet, with the genera *Micromonas*, *Bathycoccus* and *Ostreococcus* as part of its major representatives (Derelle *et al.*, 2006; Palenik *et al.*, 2007; Vaulot *et al.*, 2008). Among the unique characteristics of this group, highlights its ubiquity in coastal systems (Collado-Fabri *et al.*, 2011; Vaulot *et al.*, 2012), high genetic diversity (Shi *et al.*, 2011), bloom-forming capacity (Wang *et al.*, 2011) and extremely small cell size (Palenik *et al.*, 2007). That is the case of *Ostreococcus tauri,* the smallest free-living eukaryote known to date, with an average size of 0.8 µm, and a single mitochondrion and chloroplast inside the cell (Chrétiennot-Dinet *et al.*, 1995). Genome analysis of this organism has open new questions about the evolution of the green algae linage and showed new remarks in eukaryotic phytoplankton evolution (Derelle *et al.*, 2006; Worden *et al.*, 2009). In general terms, eukaryotic picophytoplankton has been significantly less studied than the bacterial picophytoplankton (reviewed in Scanlan *et al.*, 2009), although it is critical for the complete understanding of the dynamics of marine microbial food webs and its response to environmental changes (Vaulot *et al.*, 2008).

Picophytoplankton relevance in coastal areas has been recent highlighted. Instead of being a relevant group just during the low-nutrient low-temperature seasons, when bigger photosynthetic cells like diatoms are less abundant, picophytoplankton, particularly PPE constitute a persistent group through the year, conforming a

"background" community with a high contribution to total biomass, in low-nutrient low-temperature but also in rich-nutrient high-temperature seasons (Barber & Hiscock, 2006; Ward *et al*., 2013). Specifically, PPE community composition has shown to vary during the year, with both "types" of communities (i.e. summer and winter community) composed or dominated by different members (Collado-Fabri *et al*., 2011). Seasonality seems to be an important factor that governs community composition, but this pattern is still poorly understood (Massana *et al.*, 2004). This variation in PPE community composition makes seasonal studies essential for a full understanding of the factors that control the performance of communities facing disturbances in coastal areas. Being small will be beneficial in this changing world, and picophytoplankton is expected to be more relevant than is nowadays. (Morán *et al*., 2010; Barton *et al.,* 2013).

Disturbances in coastal marine environments

Human-derived activities have an important impact on marine ecosystems. The pressures exerted are diverse and result from different activities such as coastal engineering, sediment dredging, fishing, aquaculture, urban development, maritime transport, tourism, mining, oil extraction, transport and refining, agricultural and industrial activities (Nogales *et al*., 2011). All those activities have an effect over marine food webs, from microorganisms to top animal predators. A recent report analyzed the ecologic impact of anthropogenic activities in the oceans worldwide by focusing on drivers of stress, which can be evaluated at a global scale (Halpern *et al.*, 2008). According to that study, more than one-third of the world's oceans (41%)

were predicted to be under medium to high impact and certain regions, representing 0.5% of the oceans but a surface of approximately 2.2 million km^2, are predicted to be under high impact. Particularly, coastal zones appears to be the more impacted due to land derived activities. The kind of repercussions that those impacts have on marine microbial communities remain poorly explored.

The effect of disturbances on marine microbial communities is complex. Most often, multiple stressors (natural and anthropogenic) co-exist in a single area, and therefore there is a combination of pollution risks, i.e. eutrophication, hypoxia, chemical pollution, etc. In near-shore waters receiving anthropogenic influence, there are additional stochastic components of temporal variation due to the irregular occurrence, intensity, variety and duration of the stressors. As a consequence, temporal variation of communities in human-impacted coastal environments seems to be more irregular and less predictable than parameters that vary seasonally (Nogales *et al.*, 2007; Cloern & Jassby, 2008). To determine the effects of a particular stressor is important to contrast data from disturbed sites with those from reference areas. Therefore, comparative studies are imperative and sampling must be perform along a gradient of disturbance and by defining reference stations (Nogales *et al*., 2011).

One of the most dramatic disturbances in coastal areas is the release of trace metals as results of mining and industrial activities. Trace metals (like Iron, Cobalt, Zinc and Copper) that are critical for the correct functioning of cellular metabolism are potentially toxic at higher concentrations, due to their high reactivity and the production of Oxygen Reactive Species (ROS) (Harrison *et al.*, 2007). From all these

trace metals, copper is the one that strongly increased its concentration on the continental margins (Paytan *et al*., 2009).

Copper in the environment

Before the advent of atmospheric oxygen, some 2.7 x 10^9 years ago, the Earth was highly reductive (Miller & Orgel 1974). In this environment, biological systems preferentially captured and used soluble Fe (II) and Mn (II) ions for metabolic reactions, rather than copper, which was found mainly as poorly soluble sulphite, oxide or carbonate (Cu_2S, Cu_2O, $Cu_2CO_3(OH)_2$) (Cuillel 2009). When oxygen appeared in the atmosphere, due to the photosynthetic activity of cyanobacteria (Bekker *et al.*, 2004; Kopp *et al*., 2005), the transition from reducing to oxidizing conditions trigger a change in all metal oxidation states. Because of these atmospheric changes, copper was released from the insoluble forms to soluble copper ions and became a prevalent metal in biological systems. In the anaerobic atmosphere, the enzymes worked at low redox potential (Fe–S proteins from -0.8 to -0.4 V), and the presence of oxygen and copper availability induced an evolution towards cupro-enzymes that were able to work at higher potentials (between 0.25 and 0.75 V) (Cuillel 2009; Dupont *et al*., 2011; Festa & Thiele, 2011). While trace amounts of copper serve essential biological functions, including respiration by cytochrome oxidase, oxidative stress protection by Cu/Zn-superoxide dismutase and photosynthesis by plastocyanin, concentrated amounts of copper can be deleterious. Yet, copper is a heavy metal and the redox-cycle between Cu(I)/Cu(II), property that makes copper an essential element, contributes to its inherent toxicity at high concentrations, so a fine control of copper metabolism in the presence of

oxygen became a key factor in the evolution of aerobic organisms. Cu^{+} (the reduced form), is one of the most toxic element because it induces Fenton-like ($Cu^{+} + H_2O_2 \rightarrow HO^{\cdot} + HO^{-} + Cu^{2+}$) and Haber–Weiss ($O_2^{\cdot-} + Cu^{2+} \rightarrow Cu^{+} + O_2$) reactions. Reactive oxygen intermediates produced in these reactions are responsible for lipid peroxidation, oxidation of proteins and damage to nucleic acids (Harrison *et al.*, 2007). Due to the high affinity for thiolates, copper can also destabilize iron-sulfur clusters, and excess Cu (I) would lead to an intracellular sink for iron making increased iron uptake necessary (Christon & Pierre 2001). This would put cells at a serious disadvantage in an environment with increasing concentrations of bioavailable-copper.

Copper - picophytoplankton interaction.

In marine systems, the normal biogeochemical cycle of copper is controlled by natural sources, like submarine volcanoes and, especially in costal zones, by erosion and runoff of soils and degradation of rocks (Correa *et al.*, 1999). However, during the last century, anthropogenic activities such as mining operations, industrial wastes, urban settlements and agricultural reservoirs have modify the natural biogeochemical cycle of this metal (Nogales *et al.*, 2011). Copper from these sources are carried mainly by the wind, to reach the water column due to atmospheric deposition (Paytan *et al.*, 2009) and discharged directly into riverines, which can flow into the sea, transporting the metal to the water column.

Although normal values of copper in the ocean are in the order of 0.1 µg L^{-1}, reported concentration ranges from 0.0025 µg L^{-1} in Australian coasts (Webb & Keough, 2002), 0.05 µg L^{-1} in the Black Sea (Haraldsson & Westerlund, 1988), and up to 48 µg L^{-1} in Chañaral, Chile (Lee & Correa, 2005). The knowledge of copper toxicity

over the community composition of picophytoplankton and its role in their physiology, especially in the eukaryotic component, is still scarce, being imperative understand copper phytoplankton interaction in natural phytoplankton communities (Semeniuk *et al*., 2009).

Studies of the interaction between copper and picophytoplankton have shown that *Synechococcus* abundances are inversely related to copper concentrations in polluted harbors (Moffet *et al.*, 1997) and isolated monoclonal strains have a differential susceptibility to the metal (Mann *et al.*, 2002). Microcosm experiments also suggest that cyanobacteria are more susceptible to copper levels than the eukaryotic picophytoplankton decreasing their abundance dramatically with the addition of 2.5 µg L^{-1} of copper with regards of the taxonomic groups inhabit that area (De La Broise & Palenik, 2007).

Chañaral bay as a natural laboratory for the study of copper disturbance

In the Chañaral Zone, Northern coast off Chile (Figure 1), a long-term coastal copper enrichment event has been well-documented (Castilla, 1983; Paskoff & Petiot, 1990). Studies on this area have shown a variable, but persistently high concentration of total dissolved copper in intertidal seawater, ranging from 8.7 µg L^{-1} to 48 µg L^{-1} (Stauber *et al.*, 2005; Andrade *et al.*, 2006). The Chañaral bay is located in a desert zone where the sources of freshwater, either rivers or rain, are limited. Due to this, human population densities are low, keeping the discharge of domiciliary wastewaters lower than in other coastal zones. There is no important agricultural activity in the area, and mining is the only significant industrial activity in the region. Moreover, the mining discharges to the ocean do not contain significant organic elements present on it (Correa *et al.*, 1999). All the above-mentioned characteristics

make mining activities the only important source of pollution in the area. Chañaral has sadly proven to be a valuable natural laboratory to study the effects of a single stressor, in this case copper, over marine communities (Lee *et al*., 2002; Stauber *et al*., 2005). Recently, the effect of high copper levels on the composition of planktonic and epiphytic bacterial communities on intertidal macroalgae (Morán *et al*., 2008; Hengst *et al.,* 2010) and sulfur-reducing bacteria in marine sediments (Besaury *et al.*, 2012, Besaury *et al.*, 2013) has been addressed. Moreover, copper enrichment microcosm experiments demonstrated rapid changes in bacterial epilithic communities due to copper exposure (De la Iglesia *et al*., 2012). However, the effect that the disturbance is causing over the composition of picophytoplanktonic community in the water column has not been addressed. Understand factors that lead community composition, like under this single chronic disturbance, is imperative and can help to predict the response of an ecosystem to environmental changes based on species traits, allow defining appropriate sentinel species for water quality. In this work, we study the response of picophytoplanktonic communities, in terms of cellular abundance and taxonomic composition, to a chronic copper disturbed environment, the Chañaral bay.

In chapter 1, we elucidate the seasonal dynamics of picophytoplankton and environmental variables in the Chañaral zone, in order to determine the cellular abundance and taxonomic composition of the picophytoplankton that inhabit in two adjacent bays with different copper exposure history. The results presented in this chapter, demonstrate that picophytoplankton can cope with high copper concentrations, displaying a strong seasonality in terms of cellular abundance and taxonomic composition. No clear differences in terms of cellular abundance and

taxonomic composition were found between selected sites. However, differences in taxonomic composition were strong when compared to other unpolluted coastal areas.

Chapter 2 explores the effect of copper addition over eukaryotic picophytoplankton inhabiting the area, using a microcosm approach. We demonstrate that picoeukaryotes can cope with high copper concentrations additions. Moreover, community composition analysis of PPE demonstrate that pico-sized diatoms can cope with higher copper concentrations, opposite to smaller free living eukaryotes like Prasinophyceae class representatives, that seem to be more susceptible to copper amendments, in strong agreement with seasonal field studies.

As a spin-off of this work, annex one was focused on the copper ecotoxicology in the smallest free-living eukaryote *Ostreococcus tauri*. We compare different ecotypes, isolated from different geographic locations, and test 5 different *Ostreococcus tauri* luminescence reporter lines involved in diverse biological functions such as cell division, circadian clock, Iron transport, and phosphate transport using different copper concentrations. We demonstrate that the ecotype isolated from a polluted harbor can sustain their growth rate until 4 µM of Copper addition. Analyzed constructions display a differential response to copper amendment. Iron transporter construction is the most sensible marker to copper amendments. Assays using seawater from heavily polluted harbors demonstrate that opposite to field analysis, in the laboratory, *Ostreococcus* can tolerate the range of copper concentrations that we found in Chañaral.

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CHAPTER 1

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Seasonal dynamics of picophytoplankton in a long-term heavy metal polluted bay

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Abstract

Picophytoplankton (<3 µm in cell diameter) is an essential component of marine ecosystems, both in oligotrophic open Ocean and in highly productive coastal regions. A key goal in picophytoplankton research is to estimate how the abundance and diversity of these organisms is affected by environmental variability linked to anthropogenic influence in coastal environments. In this work, the seasonal dynamics of picophytoplankton was studied at two locations in Chañaral area, a chronic heavy metal disturbed zone, northern Chile, over a three-year period. Cell abundances were determined seasonally by flow cytometry. Taxonomic composition and diversity of the eukaryotic phytoplankton community was monitored seasonally by Terminal Restriction Fragment Length Polymorphism and clone libraries of the 16S plastid RNA gene. In coastal areas, picophytoplanktonic fraction constitutes a background population throughout the year, whit an increase in terms of abundance during summer. Picocyanobacteria from the genera *Synechococcus* can inhabit in this region and can tolerate copper concentrations by far higher than reported as toxic, becoming the dominant picophytoplankter in the area during the warm season. On the other side, eukaryotic picophytoplankton display seasonal changes in community composition without differences in cellular abundance through the year. No differences were observed between study sites, but strong taxonomic signature was found when compared with other non-polluted coastal environments.

Introduction

Picophytoplankton (< 3µm in cell diameter) is an important component of marine ecosystems in terms of biomass and primary production (Grob *et al*., 2011), contributing up to 50% of the primary production in some open ocean regions (Li 1994). It is composed by a extensively studied bacterial component (Scanlan *et al*., 2009, Flombaum *et al.*, 2013), represented by the genera *Prochlorococcus* and *Synechococcus*, and a highly diverse group of small microbial eukaryotes denominated together Photosynthetic Picoeukaryotes (PPE) , with representatives from 4 of the 5 algal super groups (Vaulot *et al.*, 2008; Not *et al.*, 2012).

The abundance of picocyanobacteria in coastal areas has been extensively investigated. While *Phroclorococcus* is virtually absent in these zones, *Synechococcus* is the dominant picophytoplanktonic organism, with a seasonal patterns of abundance, (Scanlan *et al*., 2009; Tai & Palenik 2009, Pittera *et al*., 2014). In contrast, the abundance of the eukaryotic fraction has been less explored (Kirkham *et al.*, 2013). PPE shown a relatively stable cell abundance through the year, constituting a “background” community in coastal areas, however, higher abundances were reported during fall and winter (Massana *et al.*, 2004; Collado-Fabri *et al*., 2011). In terms of composition, Coastal *Synechococcus* populations are mainly composed by the subcluster 5.1a, particularly clades I and IV (Tai *et al.*, 2009). PPE display fast temporal changes in composition. Plastid 16S rRNA gene sequences analysis from two different coastal sites have reveal a high class-level diversity, especially during cold seasons, with a dominance of Chrysophyceae, Cryptophyceae and Prymnesiophyceae classes during summer (McDonald *et al.,*

2007; Lepère *et al.,* 2009). This temporal variability of PPE taxonomic compositions contrasts with the relatively small seasonal changes reported for picocyanobacteria, thus, the mechanisms underlying these different dynamics remains poorly understood. (Massana *et al.*, 2004).

Coastal areas are of considerable interest to study the diversity of these organisms, because directly receive the impact of land-based human activities. The run-off of pollutants and nutrients into coastal waters can alter natural habitats, affecting the ecological conditions of communities (Halpern *et al.*, 2008). However, there is little knowledge about the factors affecting temporal dynamics, spatial distribution and the processes that cause differences in community composition of this phytoplanktonic fraction, especially for the PPE, linked to the anthropogenic influence (Medlin *et al*., 2006; Collado-Fabri *et al*., 2012; Kirkham *et al*., 2013; McKie-Krisberg & Sanders 2014).

Copper is an essential trace metal that has increased its concentration in the ocean, particularly in coastal areas (Paytan *et al*., 2009). The interaction between copper and phytoplankton in coastal waters is of considerable interest because copper concentrations are often significantly elevated in harbors and estuaries due to anthropogenic inputs (Moffet *et al*., 1997) and can be potentially toxic to marine organisms. Because the difficulties inherent to the studies of a single stressor over natural populations inhabit coastal systems (Nogales *et al*., 2010) copper-picophytoplankton interaction has been studied mainly through the use of model organisms or microcosm-based experimental approaches (Mann *et al*., 2002; De la Broise & Palenik 2007; Stuart *et al*., 2009; Debelius *et al.*, 2009, 2010; Henríquez-

Castillo *et al*., 2015). Moffet *et al.* (1997) reported that *Synechococcus* display an abundance pattern inversely related to the copper concentrations in harbors subjected to anthropogenic copper inputs, with a strong decrease in cell densities with total dissolved copper concentration ~ 3 µg L^{-1}. De la Broise report a differential susceptibility between bacterial and eukaryotic picophytoplankton in microcosm experiments.

In a previous work, we demonstrated that PPE inhabiting copper polluted waters can cope with 25 µg L^{-1} of total dissolved copper, tolerating concentrations similar or even higher than previously reported as toxic for phytoplankton species. While *Chysochromulina* and pico-diatoms representatives can resist high copper levels (25 µg L^{-1} and 250 µg L^{-1} respectively), Prasinophyceae representatives shown to be the most sensitive PPE to copper amendments (Henríquez-Castillo *et al.*, 2015).

The Chañaral bay, northern coast off Chile is an excellent coastal site to study the effect of high copper concentrations over the picophytoplanktonic community. In this zone, a long-term metal disturbance event has been well documented (Castilla 1978). 5 x 10^8 metrics tons of copper tailing has been discharged to the marine environment over the last 50 years (Castilla 1983; Paskoff & Petiot 1990). Seawater from the impacted area contains a persistently high total dissolved copper concentration, with levels as high as 281,4 µg L^{-1} at the riverine discharge point (Andrade *et al.*, 2006; Henríquez-Castillo *et al.*, 2015). The Chañaral area is located in a desert zone, with no riverine inputs, low density of populations and no agricultural activity, making the mining activities the only important source of pollution in the area (Castilla &Correa 1997). Due to this, Chañaral area is a valuable

and unique natural laboratory to study the effect of a single stressor, in this case chronic copper exposure, over the picophytoplanktonic community.

The temporal dynamic of the picophytoplanktonic community was examined during a three year period in this long-term copper exposed bay, with special emphasis in the eukaryotic fraction. Analyses were done in a comparative way, contrasting the exposed bay with an adjacent bay, with a different history of copper concentrations.

2. Materials and Methods.

2.1. Sampling

Sampling campaigns were conducted in two adjacent bays, at Chañaral, northern coast off Chile (Figure 1), between August 2010 to February 2013 (N = 10). Based on concentration and chemical speciation of metals in the intertidal zone, Playa La Lancha (26° 13´27,4 " S, 70° 40´2" W) was selected as High Copper Site (HCS) and Playa Blanca (26° 10´58,20"S, 70° 39´45,70" W) as Low Copper Site (LCS) (Figure 1).

2.2 Chemical Determinations

Macronutrients and total Chlorophyll a concentrations were measured using standard methods. Total dissolved metals, and all easily electro-reducible copper species (from here labile copper) were determined as described in Andrade *et al*., 2006.

2.3 Cell abundances

Picophytoplankton (*Synechococcus*, and picoeukaryotes) were detected and enumerated by flow cytometry using a Becton Dickinson (Franklin Lakes, NJ, USA; formerly Cytopeia) jet-in-air, inFlux® flow cytometer, using a combination of 488 nm blue, 532 nm green and 640 nm red excitation lasers and bandpass filters for red (692/40 nm) and orange (580/20 nm) fluorescence. Each sample was run for 5 min, at an average flow rate of 40 µl min^{-1}. 1 or 3 µm fluorescent ultrarainbow beads (Spherotech Inc. Libertyville,IL, USA) were run for alignment. Log-amplified horizontal & vertical Forward angle light scatter, orange & red fluorescence signals were recorded using Spigot software (Cytopeia Inc., Seattle, WA, USA) for data acquisition. FlowJo software (treestar) was used for data analysis.

2.4 DNA Extraction,

DNA was extracted with a Fenol:Chloroform protocol (Fuhrman *et al.*, 1988). DNA integrity was check by 1% agarose gel electrophoresis and DNA concentrations were quantified using the Qubit® 2.0 Fluorometer. (Life technologies).

2.5 Polymerase Chain Reaction (PCR) amplification & Terminal Restriction Fragment Length Polymorphisms (T-RFLP) analyses

Plastid 16S rRNA gene was amplified (in triplicate) using the 5′ end 2′-chloro- 5′-fluoro-7′,8′-phenyl-1,4-dichloro-6-carboxyfluorescein fluorochrome (NED) labeled PLA491F (5'-GAGGAATAAGCATCGGCTAA-3') and the OXY1313R (5'-CTTCAYGYAGGCGAGTTGCAGC-3') primer set (adapted from Fuller *et al.,* 2006).

Amplification and T-RFLP procedure was carried out as described in Henríquez-Castillo *et al.,* 2015.

2.6 Clone libraries and phylogenetic analyses.

Clone libraries were construct from PCR products obtained with unlabeled PLA491F–OXY1313R primer pair using the TOPO TA Cloning® kit (Invitrogen™ Life Technologies). The vector was cloned into *E. coli* competent JM109 cells following the manufacturing specifications (Promega). Clones with the correct size insert were analyzed from each library and sequenced at Macrogen Inc. (Korea) using T7 promoter forward primer and M13 reverse primer (N = 567). To obtain an identification of T-RF detected, we performed an *in silico* analysis of the identified sequences from the clones library, by identifying the first restriction site of each sequence for the restriction enzymes *Hae*III and *Rsa*I, using Vector NTI (INVITROGEN) software.

2.7 Statistical analyses.

Spearman rank-order analysis were performed to test for correlations within environmental parameters and between cell abundances and environmental parameters. Variation on environmental data were evaluated through linear regression for each parameter between sites and residuals values were box plotted. Paired t-test were performed to test for differences of each parameter between sites. Repeated measures analysis of variance (rmANOVAs) were carried out for testing significant differences in metal concentrations and cell abundances seasonally and between sites. Linear regression analyses were performed to test coupling between

copper species at each site. Principal Component Analysis (PCA) of environmental data were performed to ordering samples according to variables that present more variation between sites. For hypothesis test, a posteriori permutational multivariate analysis of variance (perMANOVA) was performed. All test were conducted using vegan package in R software as described in Borcard, Gillet & Legendre 2008. Biological data matrix was standardized by total, and analyzed by clustering based on Bray-Curtis similarities and Multidimensional scaling (MDS) using the Primer v6.1.7 software (Primer-E, Plymouth, UK). Analysis of similarities (ANOSIM) were performed to test significant difference between *a priori* defined groups of samples. Similarity Percentage (SIMPER) values were used to determine Terminal restriction fragments (T-RFs) primarily responsible for the observed differences between groups. To explore the relationship between the two matrices, biological matrix and an explanatory environmental matrix, a partial canonical correspondence analysis (CCA) (Ter Braak 1986) was performed. The statistical significance of the CCA and that of individual canonical axes were tested by permutation analysis using vegan package in R environment (Oksanen *et al*., 2007).

Results

1. – Seasonality of environmental parameters and trace metals

Analysis of environmental data revealed a marked seasonal variation in the water column (table 1) and a disturbance event at HCS in August 2012. Temperature oscillate from ~13°C in winter to 18°C in summer. Nutrients concentrations were higher in winter and lower in summer, without significant differences in nitrate and phosphate between sites. Silicic acid values were lower in LCS (average concentration of 8.411 µM) compared to HCS (average concentration of 11.13 µM) During August a peak in Nitrate, Silicic Acid and Chlorophyll was found at HCS. A high correlation ($P < 0.005$) between nutrients and Chlorophyll *a* were found, negatively correlated with Temperature ($P < 0.01$).

From all trace metals analyzed, total dissolved copper, labile-Copper, Iron, Molybdenum and Zinc concentrations present a strong variation between sites during the study (Figure 2). Iron and Zn varied seasonally. While Iron was positively correlated with Temperature (ρ: 0.685 $P< 0.01$) and negatively correlated with nitrate (r: 0.684, $P< 0.01$), Zn and positively correlated with Chlorophyll a and Silicic Acid. (ρ: 0.515 and ρ: 0.575 respectively, $P < 0.05$) and negatively correlated with temperature (ρ: 0.685 P : 0.01). Copper species (total dissolved and labile) concentrations were highly correlated (ρ : 0.83, $P< 0.001$). From all parameters tested, only total dissolved and labile copper concentrations significantly differs between sites. Copper species concentrations (total dissolved and labile) were persistently higher at HCS compared to LCS (repeated measures ANOVAs, date factor, $p<0.0001$) (Figure 3A-B respectively), with a strong coupling between copper

species at HCS (r^2: 0.8037, p: 0.0011). High total dissolved copper concentrations were accompanied by high labile copper concentrations, that can reach up to 8.7 µg L^{-1} (Figure 3D). In LCS, copper species were uncoupled, and total dissolved copper concentrations does not induced an increment in labile copper concentration beyond 0.8 µg L^{-1} (r^2: 0.091) (Figure 3C).

Principal Component Analysis using *a priori* selected divalent metals demonstrated that copper was the main factor that explained the differences between sites. The two first axes accounted for a 73% of the proportion of the variance of the data with total dissolved copper and ASV-copper as the main variables that make up PC1 (Figure S1). This results were statistically significant (perMANOVA: F= 5.15, $p<0.01$).

2. - Seasonal variation of picophytoplankton cell abundances.

Picophytoplanktonic groups were identified by flow cytometry based on their optical properties (Fig. 4 A-B). According to the definition of picoplankton (< 3 µm in cell in diameter), cyanobacteria (orange fluorescence positive group) and PPE-1 & 2 (that differs in red fluorescence and light scattering properties) were recognize in the cytograms. Abundances ranged from 10^3 to 10^5 cells mL^{-1} (Figure 4 C-D), with no significant differences between sites (repeated measures ANOVAs, site factor, p > 0|.05). Marked differences between seasons were found for picocyanobacteria & PPE-2 (repeated measures ANOVAs, date factor, p <0.05). PPE-1 and picocyanobacteria dominate in colder seasons, contributing up to 50% and 40% respectively to the total picophytoplanktonic cell abundance. In summer 2012, an increase in all populations were observed, with picocyanobacteria as the dominant

picophytoplankter, accounting for 80% of total cell abundance. PPE-2 increase their cell number one order of magnitude, while PPE-1 increased their abundance but remains in the same order of magnitude. In January 2011, lower abundances were found in both components, without clear correlation with environmental forces that were similar to summer conditions of 2012. In terms of community composition during this season *Chrysochromulina* is the main OTU and the responsible of the dissimilarity between that summer and the rest of the data.

3.- Seasonal patterns in picophytoplankton community composition

Sequences retrieved using cyanobacterial biased 16S rRNA gene primers from both sites were related only to *Synechococcus*. All sequences analyzed form a separate branch within the sub-cluster 5.1a, clades I & 4 (figure S2). Seasonal variation of *Synechococcus* populations were monitored only by flow cytometry.

Seasonal variation in the composition of < 3 µm photosynthetic eukaryotes was monitored by T-RFLP and clone libraries of the plastid 16S rRNA gene. NMDS analysis of clustered T-RFLP community data revealed a strong similarity between sites for all samples (65% average similarity) (Figure 5). NMDS analysis of Bray-Curtis similarities calculated from T-RFLP profiles, shown that transition and winter months clustered together (67.51% similarity) while warm samples form a different group (61.58 % dissimilarity, R: 0.631, P : 0.5).

In order to identify the T-RFs responsible for the observed clustering, 16S plastid rRNA gene clone libraries from each season were constructed. A high class-level diversity were found in clones libraries, with sequences related to the main groups

of picophytoplanktonic representatives (Figure 6A). Despite the variety of classes and the number of clones sequenced, 15 OTUs were identified, that represent 40% of total RFs.

SIMPER analysis revealed that a Crytophyceae/Haptophyceae mixed OTU and two *Chrisochromulina* OTUs were the main contributors to similarity in summer samples (67% cumulative contribution). Two T-RFs related to an uncultured Chrysophyte and a Cryptophyceae were the main responsible of the similarity in cold samples (Average similarity 67.51%)

4. PPE distributions: relationship to environmental parameters and metal concentrations

Picophytoplankton cellular abundances were correlated with environmental parameters. *Synechococcus* abundance was positively correlated with Molybdenum (ρ: 0.564 $P < 0.05$) and PPE-1 was negatively correlated with increment in Iron concentration (ρ: -0.745 $P < 0.001$), while PPE-2 negatively correlated with Phosphate concentrations (ρ: 0.539 $P< 0.05$).

Seasonal variation in environmental forces significantly influence PPE community structuring. In order to explore the influence of trace metals over community structure and their relationship with environmental parameters that seasonality influence over the biological data distribution, a partial correspondence analysis was conduct. That allowed us to graphically display the PPE community structuring according to metal concentrations selected as environmental constraints based on the regression analysis. The first two axes explain 71.16 % of samples distribution (Figure 7).

Permutational analysis suggested that from all trace metals analyzed only Iron contribute significantly to the samples distribution (perMANOVA; Fe, F: 3.8049, Pr < 0.009). Summer profiles were highly related to Iron and Molybdenum concentration. OTUs related to a Bacillariophyceae class representatives and Chrysochromulina were highly associated to this environment. Transition samples clustered together, related to copper species concentration and in an opposite direction of Fe constrain. High class-level diversity were found, with Clade IX/*Eutreptiella*, Clade VI/Prasinococcaceae a Cryptophyte OTU and a *Chrysochromulina* related OTU associated to this condition. A Prasinophyceae class OTU related to *Micromonas/Ostreococcus* a Crysophyceae OTU, a Bacillaryophyceae OTU and unidentified OTUs were the main representatives of winter samples, when copper concentrations were lower. HCS winter sample (AUG_12) was relate to Zinc concentration that differentiate it from the LCS sample.

Discussion

In a oceanographic context, the coastal area off Chañaral, near the Atacama Desert, is highly influenced by the South Pacific Anticyclone that condition the wind regimen, predominantly southward, and the Humboldt Current, that made a surface circulation northward. Normally during the year, surface water comes from sub-Antarctic currents, with temperature around 12-13 ºC, that can rise to 14°-15° C in transition months. During summer, the intrusion of subtropical water increase the temperature, that rise up to 18 ºC. Nutrient content is higher during winter (~12 µM of nitrate), and lower in summer (~5 µM of nitrate). We can argue that general characteristics of the water masses are similar between sites and obey to general processes previously described for other coastal sites off northern Chile (Escribano *et al.*, 2004). Exceptionally, in August 2012 strong differences between water masses were found, particularly Nitrate and Silicic Acid at HCS duplicate the concentration at LCS with an associated chlorophyll increment. A bloom of chrysophytes was observed during this season at HCS being responsible of the higher dissimilarity between samples.in winter. This episodic change in water column parameters were reverted in the next season with a concomitant increment in the average similarity between sites in terms of composition.

From all environmental parameters analyzed, copper permanently and seasonally differs significantly between water masses from selected sites, in agreement with previous values obtained from intertidal zone at the same area (Andrade *et al.*, 2006). HCS display high copper levels, exceeding the complexing capacity of the

water in this zone, with labile copper concentrations persistently higher than values reported as toxic for phytoplanktonic species (Duran & Beiras 2013).

According to our results, picophytoplanktonic community display a strong similarity between sites, with group specific differences in terms of cellular abundance during the annual cycle. During winter and transitions months, cellular abundances remains lower. Summer conditions with the exception of 2011, favors the increment of all cell types, however the increase is marked in PPE-2 and particularly in *Synechococcus* in both sites.

The detection of *Synechococcus* representatives from clades I & IV within the zone need to be revisited in detail. A three years study in a nearshore site Off California, South Pacific Ocean, demonstrate an increase in *Synechococcus* abundance specifically from the clades I & IV, when the temperature rise up to 17°C (Tai & Palenik 2009). However, the factors that govern their increment in abundance at that site remain unclear. High copper levels have been reported in the zone, which would lead to strong selection for resistance to high metals levels as described in this work. Recent studies on the coastal strain *Synechococcus sp*. CC9311, highlighted the role of genomic islands in the adaptation process and reiterated the role of iron & copper on this important group of organisms (Eriksson 2013; Stuart *et al*., 2013). We can argue that *Synechococcus* have the ability to respond to key nutrients as copper & iron, and this can determine how *Synechococcus* can compete for and occupy various ecological niches in light of anthropogenic change of the modern ocean.

T-RFLP profiles from PPE community reveal a strong seasonality in the taxonomic composition within this group, with a marked dissimilarity between warm and cold

season samples. In general, OTUs related to Bacillariophyceae, Chryptophyceae and Chrysophyceae were the main contributors to the dissimilarity between summer and cold samples, with a dominance of the Bacillaryophyceae class during warm season. Eukaryotic picophytoplankton respond in terms of composition to fast changes in the environmental conditions, reflected in the dominance of a Crysophyceae representative at HCS and a dominance of a Bacillaryophyceae at LCS when nutrients and chlorophyll differs between sites. Also during the summer in 2011, a dominance of *Chrysochromulina* in the area was found, altering the community structure in the area. These results reinforce the idea that seasonality, but also episodic disturbances shape eukaryotic microbial community structuring as happens for macroorganisms, in the same fashion as reported for other ecosystems (Jones *et al.*, 2013)

Chlorophyte related OTUs, by far the most studied picophytoplanktonic group, were poorly represented by classes previously reported in other coastal ecosystems (Not *et al*., 2012). *Ostreococcus/Micromonas* mixed OTU was retrieved only in winter and spring. Sequences related to *Ostreococcus* clade A typically found in coastal systems were found exclusively in winter (Collado-Fabri *et al.,* 2011), while *Micromonas* related sequences from clade E were represented exclusively spring profiles. This clade of *Micromonas* was detected before from surveys from the Mediterranean sea, the English Channel and the South Pacific (Lovejoy *et al*., 2007), suggesting a wide coastal distribution. Sequences related to *Pyramimonas parkeae* and *Pyramimonas disomata* were present exclusively in winter samples. While *P. disomata* was represented in both sites, *P. parkeae* seems to be restricted to LCS.

This clade I prasinophytes has been recognized as nanoplanktonic, however some pyramimonadales has been reported as picoplankters (Pennick, 1984). An OTU related to the novel and uncultured 16S-clade IX & Prasinococcales was related to transition samples. This 16S-clade IX clade has been related to highly oligotrophic ecosystems as central ocean gyres, without evidence of coastal distribution. These results suggest that Chlorophyte related OTUs were represented almost exclusively in winter and transition seasons.

Pico-haptophytes, recently suggested as the most diverse group of picophytoplankters in the modern Ocean (Liu *et al*., 2009), were highly represented in this study. These families appears to contain a massive undescribed diversity with several branches without cultured representatives (Not *et al*., 2012). The lack of cultured organisms difficult the analysis, however, seems like OTUs related to *Chrysocromulina* were distributed independent of the seasonality. OTUs were mainly affiliate to Chrysochromulinaceae from clades B1 & B2 and Phaeocystaceae.

Stramenopiles related OTUs were represented through the year. Bacillariophyceae class OTUs dominate summer season. An OTU related to *Virgulinella/ Chaetoceros* appear to be the representing of the HCS during summer. There is no clear differences between sites in this group of organism, suggesting a high tolerance to copper inside these diatoms. The microdiversity inside the Bacillariophyceae class need to be revisited due to the absence of pico-diatoms in culture, that difficult the taxonomic affiliation and conclusions in terms of tolerance to high copper levels inside the classes. Recent results demonstrate that in microcosm experiments a Bacillariophyceae related OTU can cope with high copper concentrations, 10 times

higher than the concentrations reported in this work, with no regard of the origin of the sample (Henríquez-Castillo *et al.*, 2015), Reinforcing the idea that Diatoms dominate this coastal area, and display a high tolerance to high metal levels in seawater. Chrysophyceae class (Figure 6B) was highly represented specially in winter samples, with an OTU related to Synurophyceae strongly represented in the HCS. The dominance of silicate scaled chrysophytes during winter at HCS is relate to the increment in silicic acid in this site. This group of organisms is describe as an effective bioindicators of throphic status in aquatic ecosystems, and has been frequently reported in acidic drainages (Wilkinson *et al.*, 1999). Bolidophyceae, Dictyochophyceae & Pelagophyceae, classes were mainly found in winter and transition months. Cryptophytes OTUs related to Pyrenomonadales (6B) without cultured representing organisms were represented throughout the year especially in HCS.

Seasonality is the main force that drive community composition. The increase in temperature and nutrient reduction tend to split summer samples from the rest of the season. A high diversity was found in transition months when Nitrate concentrations were higher, related to copper concentrations, while during summer, when temperature rises, OTUs belonging to diatoms and *Chrysochromulina* were related to Iron. During winter, the marked differences between sites tend to differentiate both sites, with a Synurophyceae OTU highly correlated to Zn when chlorophyll levels were higher.

At date this is the first report of picophytoplankton community structure inhabiting a chronic copper polluted area. We found a high class-level diversity, markedly during

winter and transition months, when total picophytoplanktonic abundances were lower, and a dominance of pico-diatoms in summer. Chrysophyceae, Cryptophyceae and Bacillaryophyceae related OTUs were mainly detected at HCS. In 2012, during winter, local conditions at HCS were highly dissimilar to the rest of the seasons sampled. A Chysophyceae related to Synurophyceae is the dominant OTU at this site, being the main responsible for the dissimilarity in community composition between sites. Mammiellophyceae representing appear to be sensible to copper and Iron concentrations as revealed by the correspondence analysis. This OTU was mainly present in LCS during winter, when lower copper levels were reported. These results suggest a differential susceptibility inside picophytoplanktonic classes to high copper levels. Recently, through a microcosm approach we demonstrate that Primnesiales can tolerate 25 g L^{-1} of copper addition, and the Bacillariophyceae class representing Coscinodiscus can cope with ten times more higher copper concentrations. Both classes were highly represented through the year. While Primnesiales dominate during winter, Bacillariophyceae is the dominant class in the area with higher abundances in warmer months. Although a greater depth is required in the taxonomic assignment, the results of this work are in agreement with experimental fieldwork (Henríquez-Castillo *et al*., 2015).

All these results together confirm that a background population of small sized-cells (*Synechococcus* and PPE) inhabit the area and can cope with metals concentrations that exceed by far the normal concentrations reported for coastal areas (Moffet *et al.*, 1997). The increase in metals supply, weak stratification, the increment in light intensity and quality during summer season can stimulate the growth of bigger cells.

These conditions also stimulate the increase in the abundance of the picocyanobacteria.

The tolerance of *Synechococcus* and some eukaryotic picophytoplankters to heavy metals would confer them an ecological advantage related to other phytoplanktonic organisms. However, there also an important point in which, the continuous increment of this important trace metals can decrease the quotas of Nitrogen, Phosphorous and Silicic acid by consumption, altering normal energy flux through the food web. This item have to be explored by contrasting this data with others polluted bays. A recent study demonstrates that large-scale changes in cellular stoichiometry are possible in marine plankton due to small alterations in in situ chemistry and that these changes can occur over short (1 to 3 days) time scales (Wilhelm *et al*., 2013).

Metagenomics, metatranscriptomics and culturing of sorted population will be helpful to elucidate the mechanisms that underlie the possible genetic/genomic acclimatization/adaptation of the small-sized organisms to chronic disturbed environments.

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Legends to figures

Figure 1. Geographic location of the Chañaral area, northern coast off Chile. Sampling stations were designated as High Copper Site (HCS) and Low Copper Site (LCS) (Black dots). Riverine discharge point is dotted in red.

Figure 2. Residuals boxplots obtained from after linear regression for each environmental parameter between study sites. Metals concentrations were determined for samples taken between august 2010 to march 2014 (N=10) from each site. Nutrients and CTD data were taken from selected seasons (n=5). Linear regressions were performed for each parameter and residuals were box plotted together. X axes correspond to environmental parameters; Cu: Total dissolved copper; Cu_ASV: Labile copper; Fe: Iron; Mo: Molybdenum; Zn: Zinc; Co: Cobalt; Cd: Cadmium; Pb: Plumb; N: Nitrate; P: Phosphate; Si: Silicate; T: Temperature; Sal: Salinity. Y axes correspond to residuals values.

Figure 3. Temporal analysis of copper species concentrations in the study sites. Variation in total (A) and ASV labile (B) copper concentrations between sites across time. Asterisks represent significant differences in concentrations between sites. Repeated measures ANOVAs, time factor, $p<0.0001$ (***), $p< 0.001$ (***). C-D. Linear regression between concentration of copper species at LCS (C) and HCS (D).

Figure 4 - Flow cytometric identification and counting of picophytoplanktonic groups detected at sampling sites. Cytograms (A & B) correspond to Phycoerythrin-orange fluorescence (580/20 nm) vs Chlorophyll-red fluorescence (692/40 nm) using blue excitation laser (488 nm). Main populations were colored in green (*Synechococcus*),

red (PPE-1) and blue (PPE-2). 1 µm reference beads were colored in light green. C-D.-Cell abundances of the main groups detected in LCS (C) & HCS (D) were colored as in figures A & B:

Figure 5. - MDS analysis of T-RFLP$_{RsaI}$ profiles obtained from plastid16S rRNA gene between January 2011 and August 2012 at sampling sites. Similarities between samples were obtained after hierarchical cluster analysis of Bray-Curtis similarity values of T-RFLP data. Green lines grouped samples with 60 % of similarity; Blue lines grouped samples with 65 % of similarity Grey circles: LCS samples; Red circles: HCS samples.

Figure 6. - (A) Maximum likelihood tree based on plastid 16S rRNA gene sequences obtained from HCS and LCS samples. The tree was generated after alignment of 143 partial sequences using 700 informative positions, and constructed using the GTR+T+I model of DNA substitution. Colors in brackets represents phylogenetic groups. Black filled symbols correspond to sequences obtained in this work. Square symbols represent abundant OTUs in the libraries (more than 5 sequences in the OTU). Open symbols represent environmental sequences without cultured representatives. (B) Partial three of Crhysophyceae (upper panel) and Criptophyceae (lower panel) classes

Figure 7. - CCA triplot of the LCS & HCS OTUs constrained by environmental variables and selected divalent metals. Cu: copper, Cu_ASV: Anoding Stripping Voltammetry copper (labile copper), Fe: Iron, Mo: Molybdenum, Nit: Nitrate, Si: Silicic Acid, T: Temperature Zn: Zinc. Arrows pointing in roughly the same direction indicate a high positive correlation, arrows crossing at right angles indicate a near-

zero correlation and arrows pointing in the opposite direction have a high negative correlation. The length of the arrow is proportional to the contribution to the canonical axis.

Figure S1.- PCA biplot merged with cluster analisis of trace metals data from the Chañaral area . Arrows represent the eigenvectors. Red dots: HCS; grey dots: LCS. PC1: Principal Component axes 1; PC2: Principal Component axes 2. Green dotted lines represent a euclidean distance value of 3.

Figure S2. - Maximum likelihood tree based on cyanobacterial biased 16S rRNA gene sequences, indicating the phylogenetic relationships among marine picocyanobacteria. The tree was generated after alignment of 53 partial sequences using 400 informative positions, and constructed using the GTR+T+I model of DNA substitution. Colors in brackets represents subclusters. Bootstrap values of > 50% are shown. Sequences in blue correspond to LCS and sequences in red to HCS

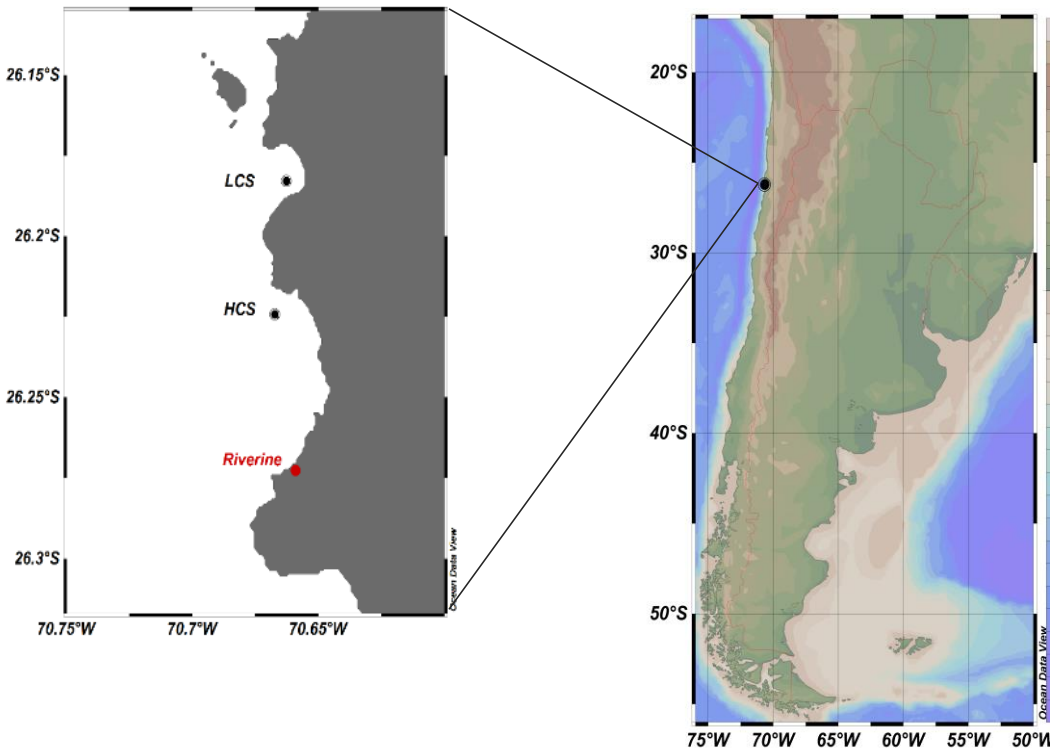


Figure 1 Henríquez-Castillo *et al.*

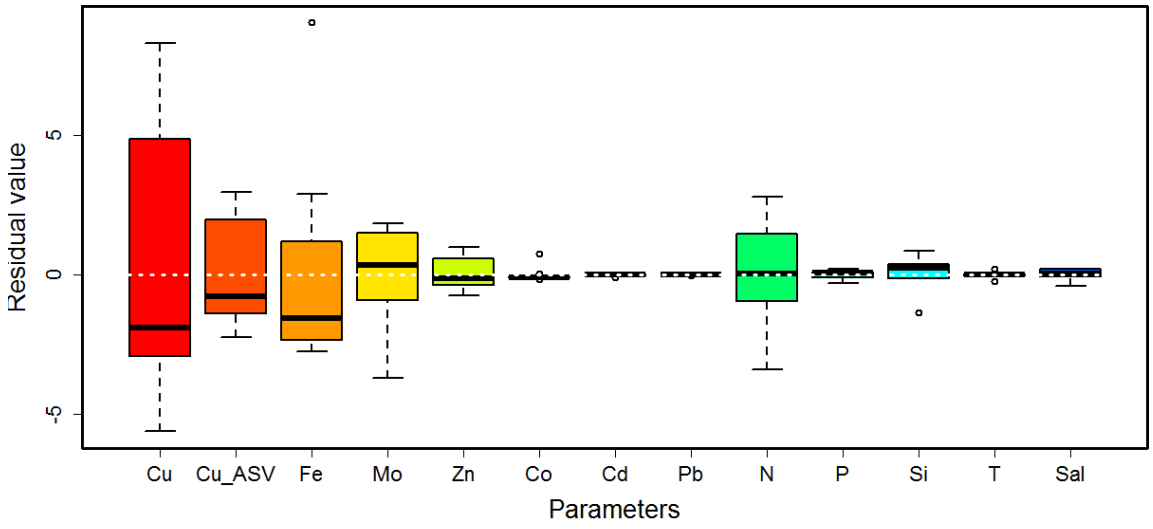


Figure 2 Henríquez-Castillo *et al.*

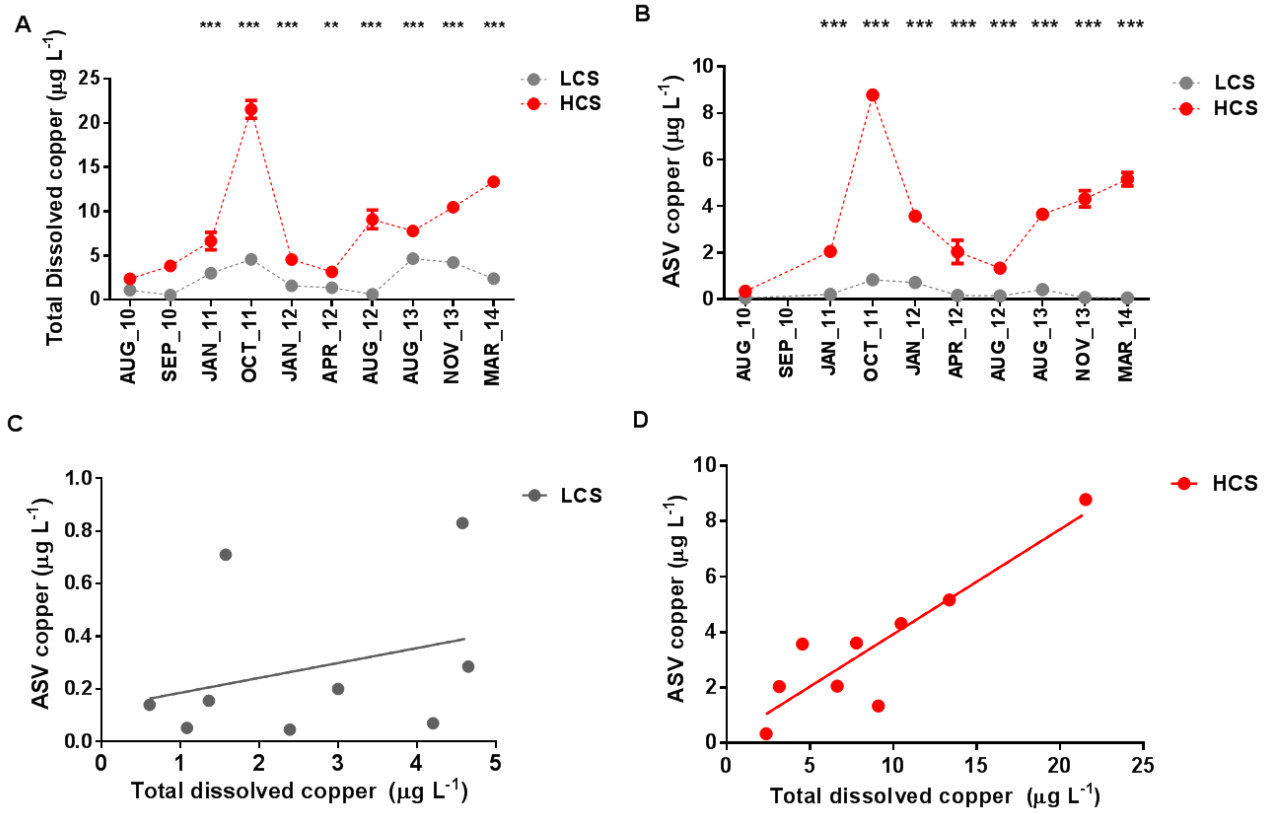
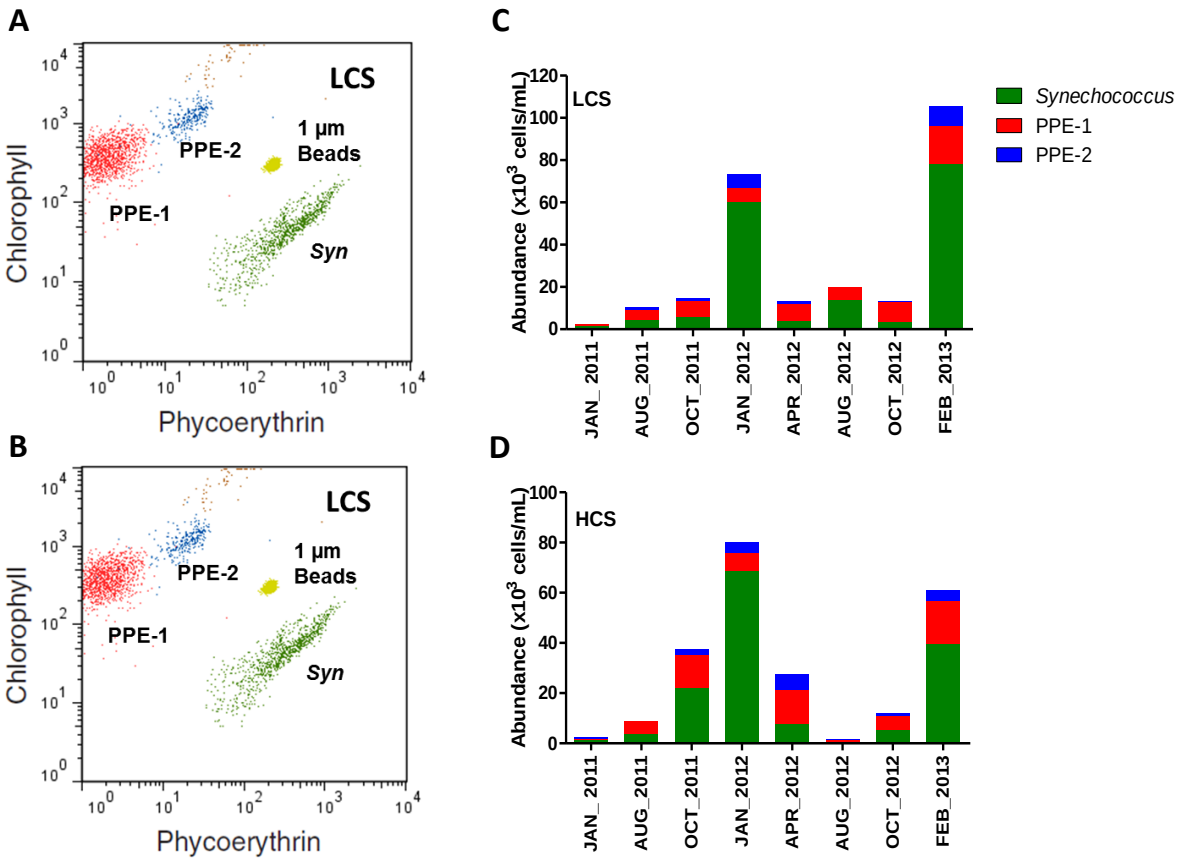


Figure 3 Henríquez-Castillo *et al.*



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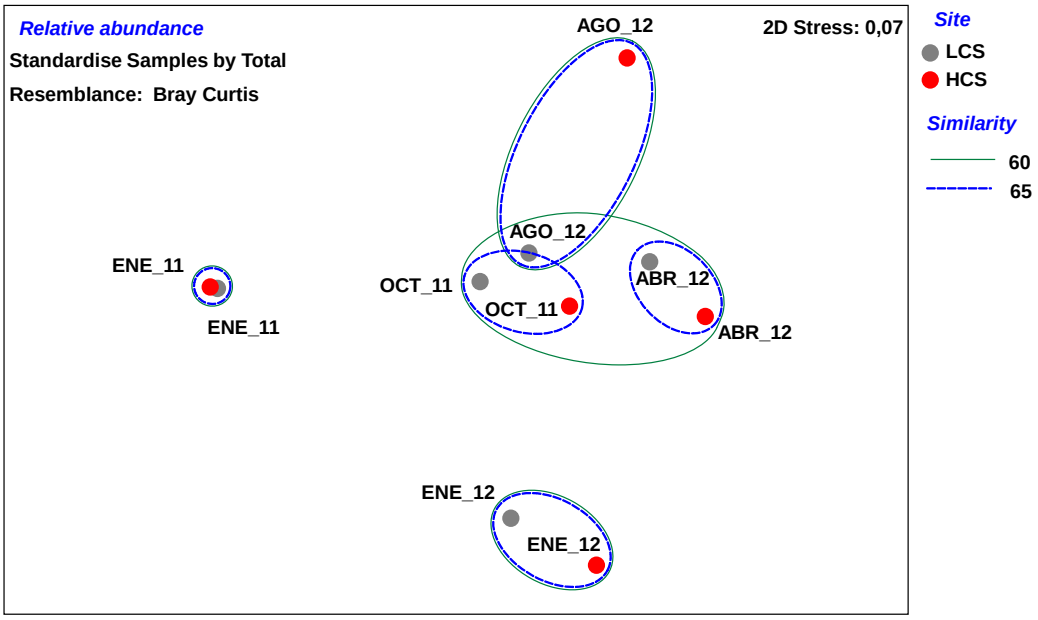

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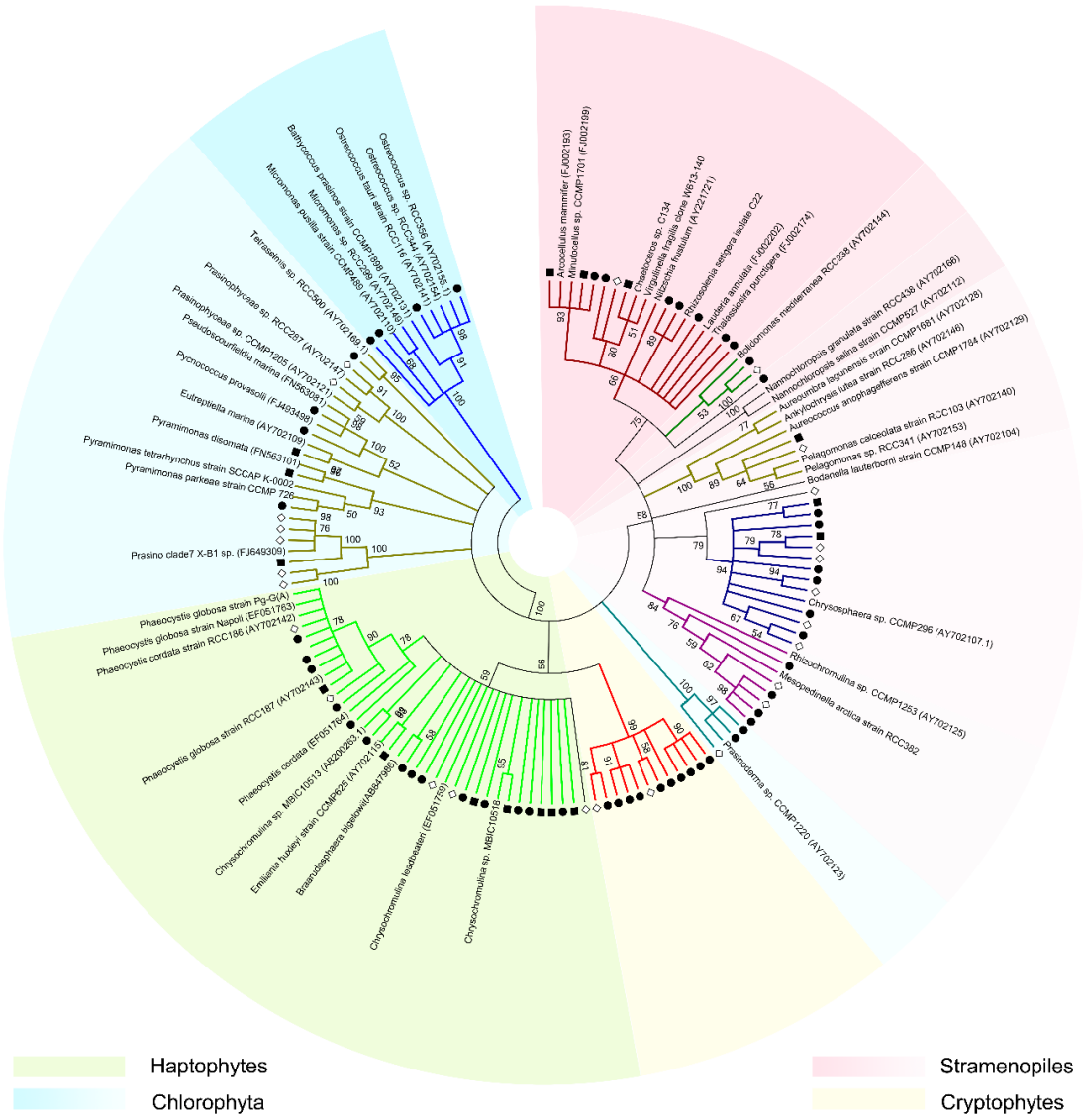

Figure 6A Henríquez-Castillo *et al.*

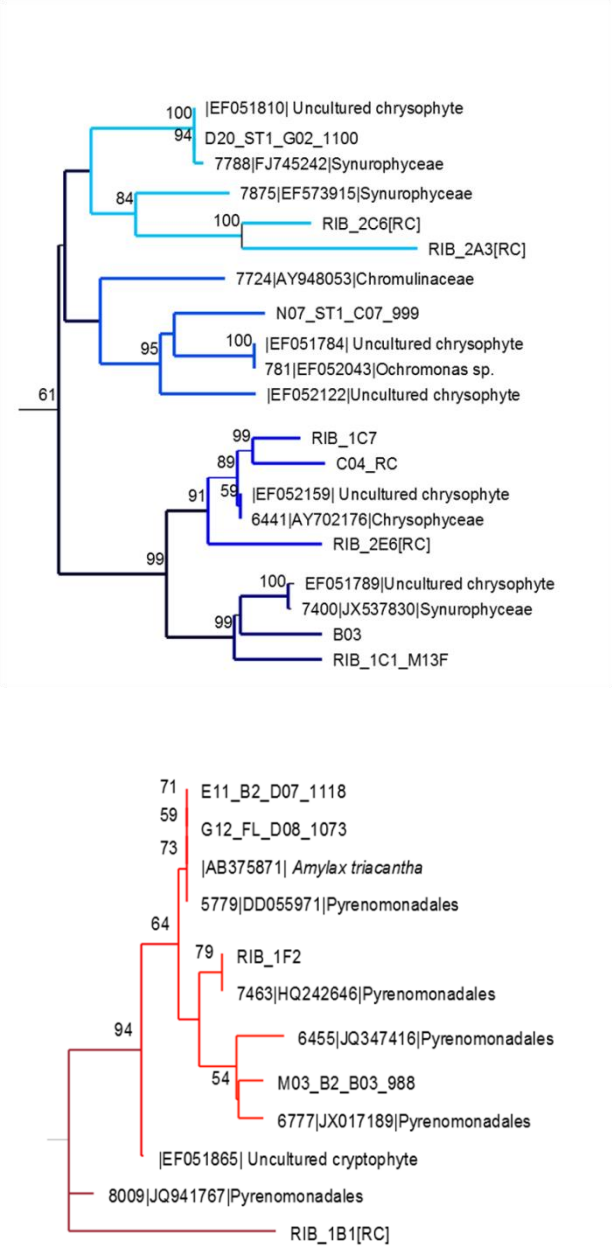

Figure 6B Henríquez-Castillo *et al.*

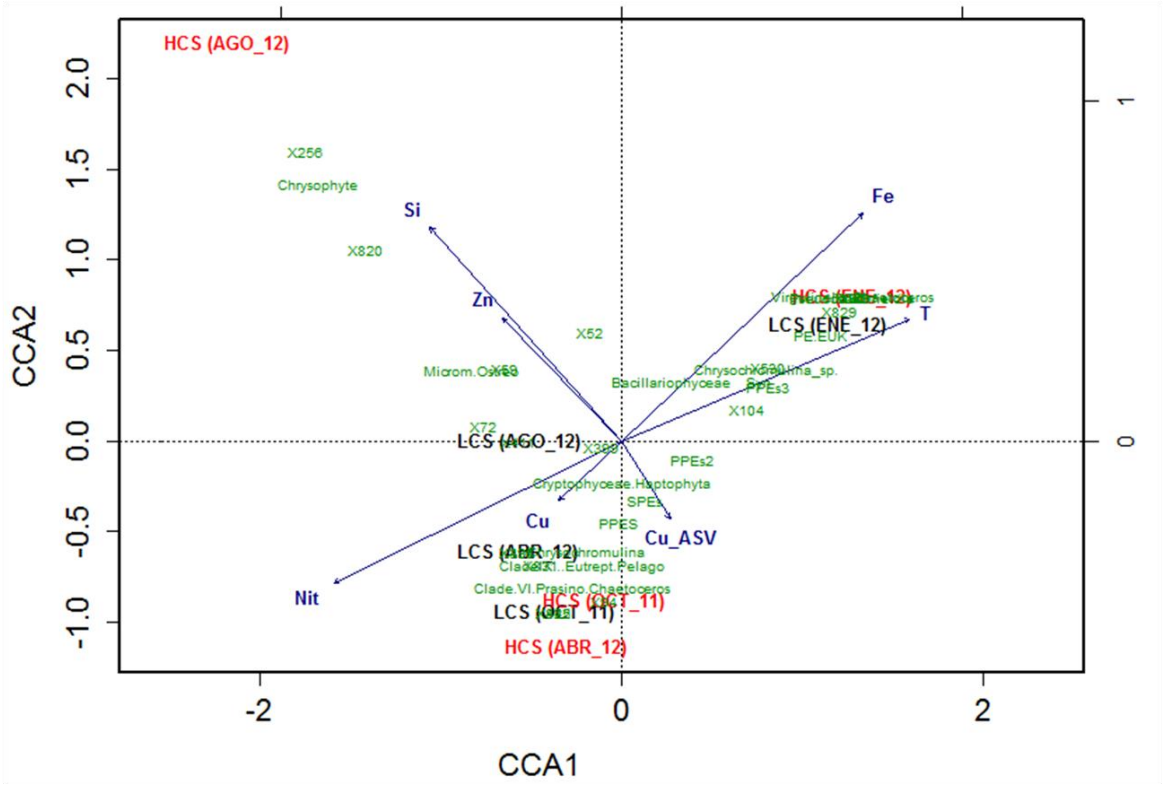

Figure 7 Henríquez-Castillo *et al.*

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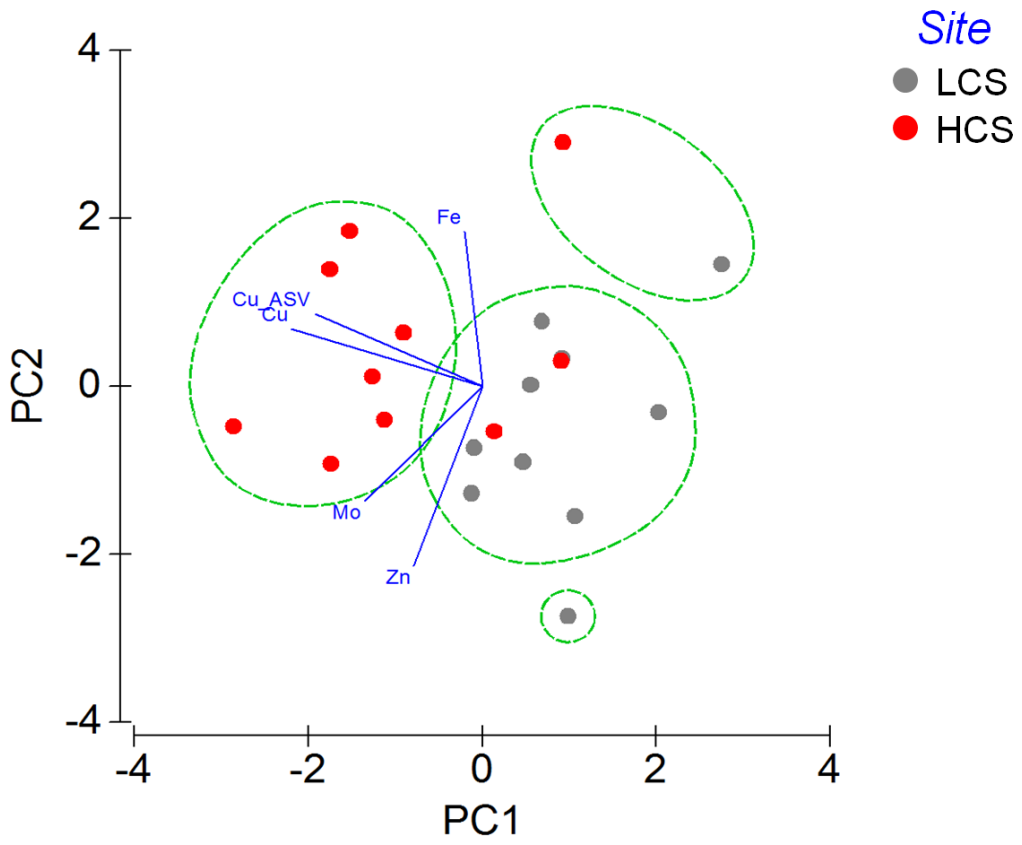

Figure S1 Henríquez-Castillo *et al.*

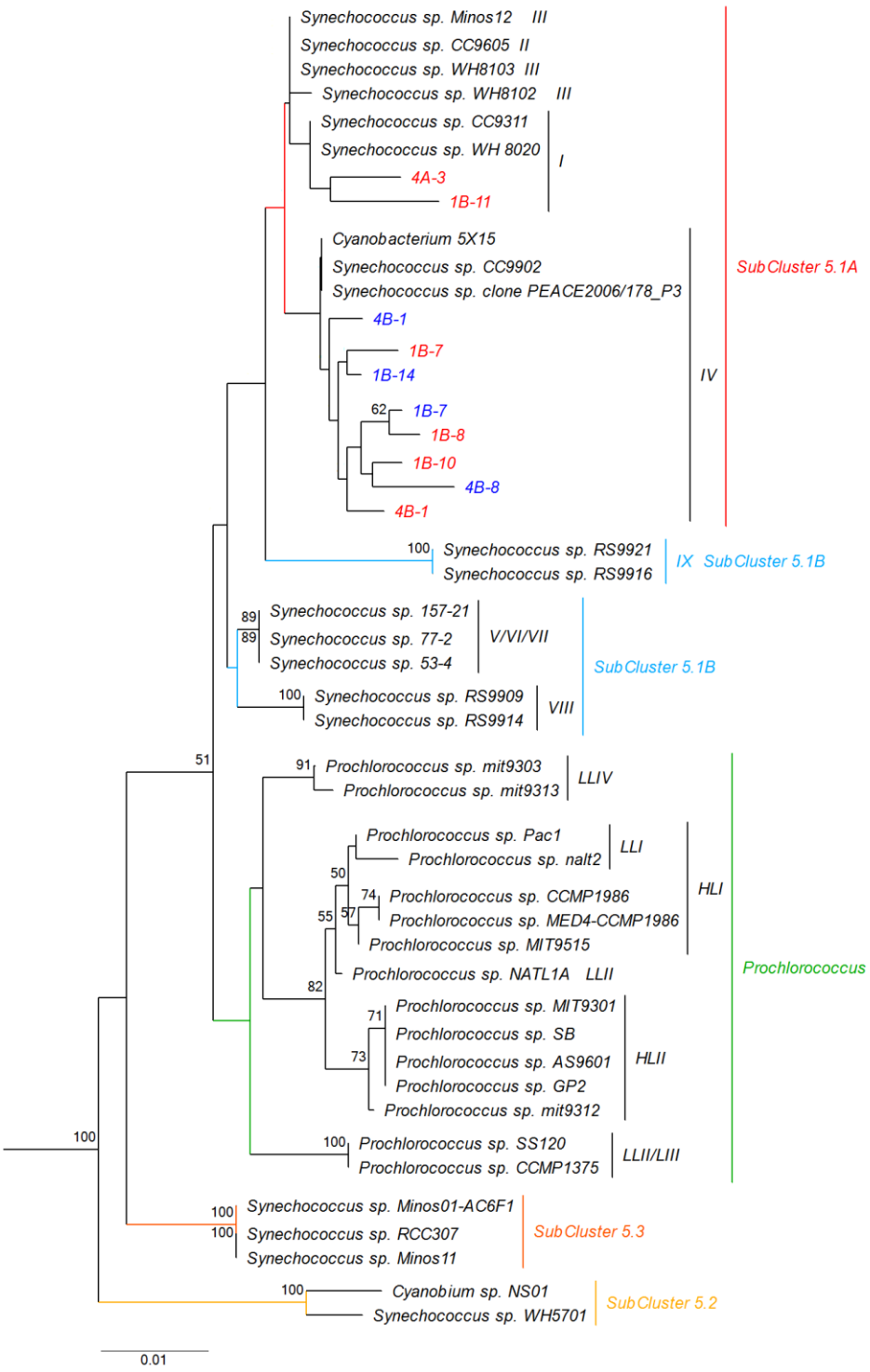

Figure S2 Henríquez-Castillo *et al.*

CHAPTER 2

ACCEPTED IN PHYCOLOGICAL RESEARCH

Eukaryotic picophytoplankton community response to copper enrichment in a metal-perturbed coastal environment

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Keywords: flow cytometry; T-RFLP; disturbances; microcosms experiments

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Abstract

Copper is an essential micronutrient, especially for photosynthetic organisms, but can be toxic at high concentrations. In the past years, coastal waters have been exposed to an increase in copper concentration due to anthropogenic inputs. One well known case is the Chañaral area (Easter South Pacific coast), where a long term coastal copper enrichment event has occurred. That event strongly affected benthic marine diversity, including microbial communities. In this work, microcosm experiments were carried out to address the changes on picophytoplankton community composition of the disturbed area, when was challenged to copper additions. Eukaryotic picophytoplankton communities from two areas were analyzed, one in the most copper-perturbed area and other at the north edge of the perturbed area. Flow cytometry data showed that 25 µg L^{-1} of copper addition exerted a positive effect in the growth kinetics on part of the eukaryotic picophytoplankton communities, independently of the site. 16S-plastid terminal restriction fragment length polymorphisms analysis suggested that eukaryotic picophytoplankton display a short and directional response to high copper levels. Members of the Prasinophyceae class, *Phaeocystis,* as well as a Coscinodiscophyceae diatom respond in a short time to the environmental disturbance, making them excellent candidates for further studies to evaluate phytoplanktonic species as sentinels for copper disturbances in coastal marine ecosystems.

Introduction

Copper is an essential trace nutrient for all organisms in the marine environment. Due to its capacity to adopt several ionic forms (Cuillel, 2009) copper has relevant biochemical functions, especially for photosynthetic organisms, including photosynthetic and respiratory electron transport (Barón *et al.*, 1995; Raven *et al.*, 1999; Knauert & Knauer, 2008). However, the high reactivity become this divalent metal potentially toxic at elevated concentrations, and organisms have developed mechanisms to accurately tune its homeostasis (Sunda & Guillard, 1976; Brand *et al.,* 1986; Cuillel, 2009). The knowledge about copper requirements by marine phytoplankton and its role in their physiology and community structuring is still scarce, being imperative understand copper nutrition and acquisition mechanisms of natural phytoplankton populations (Semeniuk *et al.*, 2009). Picophytoplankton (cells less than 3 µm in diameter) are the numerical dominant phytoplankton in the ocean, and one of the major contributors to primary productivity in marine environments, playing a key role in biogeochemical cycles (Senga & Horiuchi, 2004; McDonald *et al.*, 2007; Kostadinov *et al.*, 2010; Lee *et al.*, 2013). It is composed by a bacterial component, dominated by two picocyanobacterial genera, *Synechococcus* (Waterbury *et al.*, 1979) and *Prochlorococcus* (Chisholm *et al.*, 1988), and a eukaryotic component, the photosynthetic picoeukaryotes (PPE), which includes a highly diverse group of microorganisms (Li *et al.*, 1994; Moon-van der Staay *et al.*, 2001; Díez *et al.*, 2001; Lopez-Garcia *et al.*, 2001; Massana, 2011). PPE are extremely relevant in open oceans (Shi *et al.,* 2009), and recently, it has been demonstrated that are also dominant in coastal zones (Collado-Fabri *et al.,* 2011; Vaulot *et al.,* 2012). The effect of copper over PPE has being received

increasing attention (Le Jeune *et al.*, 2007; De la Broise & Palenik, 2007; Debelius *et al.*, 2009; Debelius *et al.*, 2010). These studies have been focused in natural assemblages of PPE in terms of abundance, without detailed information about the taxonomic identity of copper resistant organisms.

The knowledge about the ecological impacts of elevated copper levels on PPE communities is particularly relevant in coastal systems, where an intense disturbance associated to human activities occurs, that increases the run-off of pollutants and nutrients into coastal waters (Halpern *et al*., 2008). Specifically in the Chañaral area, Northern coast off Chile, a long-term coastal copper enrichment event has been well documented (Castilla, 1983; Paskoff & Petiot, 1990). Studies on this area have shown a variable, but persistently high concentration of total dissolved copper in intertidal seawater, ranging from 1 μg L^{-1} to 48 μg L^{-1} (Stauber *et al.*, 2005; Andrade *et al.*, 2006). Chañaral area has been considered an excellent natural laboratory to study copper effects on marine communities, furthermore, it has been demonstrated that mining activities are the only significant source of pollution in the area (Lee *et al.*, 2002; Stauber *et al.*, 2005; Andrade *et al.*, 2006). Recently, the effect of high copper levels on the composition of planktonic and epiphytic bacterial communities on intertidal macroalgae (Morán *et al*., 2008; Hengst *et al.,* 2010) and sulfur-reducing bacteria in marine sediments (Besaury *et al.*, 2012; Besaury *et al.*, 2013) has been addressed. Moreover, copper enrichment microcosm experiments demonstrated rapid changes in bacterial epilithic communities due to copper exposure (De la Iglesia *et al*., 2012). The effect that the disturbance is causing over the community composition of PPE inhabiting the water column has not been addressed. Understanding the factors that lead community composition,

like this single chronic disturbance, can help to predict the response of an ecosystem to environmental changes, based on species traits. This understanding can permit define appropriate sentinel species for water quality monitoring.

In order to explore the response to copper exposure of PPE communities inhabiting the disturbed area of Chañaral, an *in situ* copper enrichment microcosm experiment was carried out. Abundance of PPE was monitored by flow cytometry. The variation of PPE community composition and the identity of copper tolerant/resistant PPE species were determined using terminal restriction fragment length polymorphisms (T-RFLP) and clone libraries analyses of the 16S rRNA plastid gene.

Materials and Methods

Study site

The experiment was conducted in October 2011, in the Chañaral area, Chile. Two adjacent bays inside the Chañaral area were selected based on 10 years description of biological data, concentration and chemical speciation of metals in the intertidal zone (reviewed in Andrade *et al.,* 2006). Playa La Lancha (26° 13´27,4 " S, 70° 40´2" W) was selected as high copper site (HCS) and Playa Blanca (26° 10´58,20"S, 70° 39´45,70" W) as low copper site (LCS). Playa La Lancha is adjacent to a mining waste discharge riverine, and has been long documented as a persistent high copper area, with total dissolved copper levels reaching 32 µg L^{-1}, always exceeding the seawater complexing capacities (Stauber *et al.,* 2005). On the other hand, copper levels at Playa Blanca have been consistently lower than the HCS, but higher than values reported for non-perturbed coastal areas (Moffet *et al.*, 1997).

Sampling

Surface coastal seawater for the experiment was collected using 5 L Niskin bottles at 1 m depth, 1 km offshore the two locations. Water samples were pre-filtered on-board through a 150 µm cut-off nylon net, to exclude big particles and zooplankton, and stored in the dark in acid-washed 5 L carboys until experiment set up (less than 2 h). Physicochemical parameters of seawater were registered *in situ* using a Conductivity, Temperature, Depth and Oxygen profiler, SBE19, (Seabird electronics, Bellevue, WA).

For metal and nutrients analyses, seawater samples were collected with metal free Kemmerer bottles at 1 m depth, filtered through acid-washed 0.45 µm cellulose acetate membrane filters (Millipore, Darmstadt, Germany) using a polycarbonate

filter unit. For nutrient analysis, filtered water was collected in 15 mL polypropylene tubes and stored at -20°C until analysis. For metal determination, filtered water was fixed with 2 mL of concentrated bi-distillate nitric acid (pH < 2.0) per liter of sample. Filtered seawater samples were stored in acid-washed polypropylene bottles and left in double acid-washed plastic bags at 4°C until analysis.

For flow cytometry analysis, 1.35 mL of seawater was fixed with 1% paraformaldehyde/0.5% glutaraldehyde in borate buffer, pH 8.4 solution, deep frozen and stored in cryovials at -80°C until analysis.

For nucleic acid analysis, 1 L sample from each site was pre-filtered through 3 µm pore size SSWP filter and the biomass was collected onto a 0.22 µm pore size polyethersulphone (GPWP) filter using a peristaltic pump (Cole-Palmer, Vernon Hills, lL) with MasterFlex C-flex tunings. Filters were stored in 2 mL sterile cryovials and immediately frozen and stored at -80°C until analysis.

Microcosm experiment

1.5 L acid-washed transparent polypropylene bottles were filled up to 1 L of 150 µm filtered seawater, containing natural phytoplankton communities. Six bottles were filled up with water from HCS and six from LCS. Bottles were mounted in a platform with seawater to keep a stable temperature (14 ºC), covered with a mesh at 1 m, in order to prevent the direct sun exposure and kept the sample approximately at 75 µmol photon m^{-2} s^{-1}. Two bottles from each site were kept without copper addition as control conditions. For copper additions, cupric chloride standard solution (Merck-Millipore, Darmstadt, Germany) were added in duplicates to final concentrations of 25 µg L^{-1} (in the range of copper concentration detected at HCS) and 250 µg L^{-1} (copper concentration detected at the discharge point), respectively.

Every twelve hours (starting at time 0) and until 60 hours after copper addition, 2 mL samples were taken for *in vivo* fluorescence measurement and for flow cytometry determination of picophytoplankton abundance. Total fluorescence was determined *in situ* using a handheld Aquafluor fluorometer (Turner designs, Sunnyvale, CA). At the end of the experiment, the remnant volume from each microcosm was pre-filtered through 3 µm filter, and the biomass collected into 0.22 µm filters for DNA extraction (see below).

Analytical Determination

Nutrient concentrations (NO_3^-, NO_2^-, PO_4^{-3} and SiO_2^{-4}) were determined as described previously (Hansen & Grasshoff, 1983). Total copper concentrations were determined by inductively coupled plasma mass spectrometry (Thermo Fisher Scientific, Waltham, MA) after pyrrolidinedithiocarbamate/diethylammonium diethyldithiocarbamate organic extraction (Bruland *et al.*, 1985). The efficiency of the extraction was established using a certified seawater reference material (CASS-5, NRC-CNRC, Otawa, Canada) with recovery of 97% for copper. Dissolved labile copper concentrations (all easily electro-reducible copper species) were determined by square-wave anodic stripping voltammetry (ASV) using a Metrohm 797 VA computrace system (Herisau, Switzerland) with a thin mercury film deposited in a rotating glassy-carbon disk as working electrode, according to the methodology described previously for the same area (Andrade *et al*., 2006).

Measurement of cell abundances

Cell abundances were determined using a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) flow cytometer equipped with an air-cooled argon laser (15 mW power, 488 nm excitation), and a set of photomultipliers with bandpass filters for

fluorescence emission. 488/10 bands pass for side scatter, and 670 long pass for red fluorescence. Signal detection was triggered on the chlorophyll fluorescence. Samples were run for 5 min at a flow rate of 30 mL min^{-1} to estimate cell abundance. Flow cytometry data analysis of the main groups was done as described previously, based on the optical properties of the cells (Marie *et al.*, 2000), using the FlowJo software (Tree Star, Ashland OR).

DNA Extraction and PCR amplification

At the end of the experiments (60 h), samples were pre-filtered through 3 µm pore size SSWP filter and the biomass collected onto a 0.22 µm pore size GPWP filter. Filters were stored in 2 mL sterile cryovials and immediately frozen in liquid nitrogen until nucleic acid extraction.

Cryovials containing the filters were filled with lysis buffer (0.75 M sucrose, 400 mM NaCl, 20 mM EDTA, and 50 mM Tris-HCl, pH 8.4) and DNA was extracted with a Phenol:Chloroform protocol modified from Fuhrman *et al.* (1988). DNA integrity was visualized by 1% agarose gel electrophoresis and DNA concentrations were quantified using Qubit®2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Plastid 16S rRNA gene was amplified (in triplicate) using the PLA491F forward primer (Fuller *et al.,* 2006) and reverse primer OXY1313R (West & Scanlan, 1999). PCR amplifications were carried out in a total reaction volume of 25 µL per sample, containing 1.25 mM dNTP, 1.25 mM $MgCl_2$, 1.2 µM primers, 2.5 U of Taq polymerase, 1× kappa buffer (Kappa Biosystems, Wilmington MA.), with 0.1 mg mL^{-1} bovine serum albumin (Promega, Madison WI.). Amplification conditions comprised 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s, with a final extension at 72°C for 6 min.

T-RFLP analyses

Plastid 16S rRNA gene was amplified (in triplicate) using the 5′ end 2′-chloro- 5′-fluoro-7′,8′-phenyl-1,4-dichloro-6-carboxyfluorescein fluorochrome labeled PLA491F under the same conditions previously described. Labeled 16S rRNA gene PCR products were digested by overnight incubation at 37°C with 20 U of *Hae*III or *Rsa*I endonucleases, in a final volume of 20 µL. Complete digestion of the PCR products was checked by 2% agarose gel electrophoresis. 10 µL of each digested PCR product was analyzed in an ABI 3730 Capillary Sequencer using Liz1200 as internal standard (Thermo Fisher Scientific, Waltham, MA), which contained discrete size fragments ranging from 50 to 1200 bp at Macrogen Inc. (Seoul, Korea). The ABI files obtained were imported using the PeakScanner Software v1.0 (Thermo Fisher Scientific, Waltham, MA) and consisted on the size (nucleotides) of each terminal restriction fragment (T-RF) and the peak area, measured in relative fluorescence units. The analysis considered all the peaks between 50 and 835 bp, and fragments less than 0.5% of the total area were discarded. Each T-RF was standardized against the sum of total peak areas for each profile. The obtained relative abundance data matrix was analyzed by cluster analysis based on Bray-Curtis resemblances, using the software Primer 6 (Primer-E, Plymouth, UK). Analysis of similarities was used to test significant difference between *a priori* groups of samples, and Similarity Percentage (SIMPER) analysis was used to assess which Operational Taxonomic Unit (OTU) were primarily responsible for the observed differences between groups (Rees, 2004; Ramette, 2007). As both T-RFLP$_{Hae\text{III}}$ and T-RFLP$_{Rsa\text{I}}$ clustering profiles indicated the same trends (Spearman correlation $\rho = 0.989$, $p < 0.0001$), only the *Hae*III profiles are reported.

Clone libraries and phylogenetic analyses

For the taxonomical identification of the T-RFs detected, the plastid 16S rRNA gene was amplified by PCR with unlabeled PLA491F–OXY1313R primer pairs. Purified products were cloned into vector PCR® 2.1 TOPO (Invitrogen™ Life Technologies) and transformed into *E. coli* JM109 competent cells (Promega, Madison, WI), following the manufacturing specifications. Fifty clones with the correct size insert were analyzed from each library and sequenced at Macrogen Inc. (Seoul, Korea), using T7 promoter forward primer and M13 reverse primer. All sequences were edited using VectorNTI software (Thermo Fisher Scientific, Waltham, MA) and chimera presence was discarded using Bellerophon (Huber *et al*, 2004). Sequences were compared against published NCBI GenBank database (release October 2013) using Blastn (Altschul *et al.*, 1990). Identification of the T-RF was performed by identifying the first restriction site for the enzymes *Hae*III and *Rsa*I, using the software Vector NTI, in the sequences from the clone libraries.

Results and discussion

Copper concentration and environmental parameters

Both HCS and LCS showed similar temperature, salinity, fluorescence and dissolved oxygen values, as well as concentrations of inorganic nutrients (nitrate, nitrite, phosphate and silicate) (Table 1). In contrast, total dissolved (paired t test, $p < 0.001$) and ASV-labile copper (paired t test $p < 0.0001$) were significantly higher at HCS compared to LCS (Table 1), with values by far higher than reported for other highly polluted bays (Hall & Anderson, 1999; Wang *et al.*, 2012). These results are the first data of copper concentrations in the water column at the coastal area of Chañaral, since all the previous reports have focused on the intertidal zone (Stauber *et al.*, 2005; Andrade *et al.*, 2006). Based on these results, it is possible to emphasize that copper concentration is the environmental variable measured that differs between sampling sites.

Recently, the Acute Water Quality Criteria for the protection of coastal ecosystems (Duran & Beiras, 2013) has been published. These criteria are a probabilistic approach based on species sensitivity distribution curves and indicate copper concentration of 1.39 µg L^{-1} as the limit to protect 95% of the species present in a specific zone. This value is significantly lower than the copper concentrations present in both sites of the study area. Altogether, these elements reinforce the hypothesis that the Chañaral area is a natural laboratory to address the effect on marine microbial communities of copper as a single stressor.

PPE abundance and composition in the Chañaral area

Initial abundance and taxonomic identity of PPE were analyzed for HCS and LCS at the sampling time. Based on their optical properties, two different PPE groups were identified (Fig. 10a), with PPE-1 abundances one order of magnitude higher than PPE-2 (Table 1). Taxonomic composition at each site was determined based on clone libraries for chloroplastidal 16S rRNA gene to avoid detection of small heterotrophic eukaryotes. The most abundant taxa identified were *Prasinoderma* (97% nucleotide similarity), *Pyramimonas* (97% nucleotide similarity), *Chrysocromulina* (97% nucleotide similarity) and an unidentified Coscinodiscophyceae representative, which were present in both sites. Altogether, these results indicate that the initial abundance and community composition of PPE were similar between sites, and suggest that in the coastal area of Chañaral, natural PPE communities were homogenous at the sampling time.

Copper enrichments effect on PPE fluorescence and abundance

A comparison of the optical properties of the PPE communities at the end of the experiment demonstrated that, independently of the treatment, populations from both sites displayed a similar and well-defined optical pattern in terms of red fluorescence and side scatter, which differs from the initial pattern observed (Fig. 10). The addition of 25 µg L^{-1} of copper exerted a red fluorescence increment of ~20% in LCS samples and 30% for HCS (Fig. 11c, g).

During the experiment, *in vivo* <150 µm total chlorophyll measurement showed similar patterns of fluorescence for HCS and LCS samples, between control and 25 µg L^{-1} treatment (dotted and grey lines in Fig. 11 a,d). In contrast, the 250 µg L^{-1} microcosms did not show any fluorescence increment (black lines in Fig. 11 a, d), indicating a strong effect of this treatment over the whole community. Flow

cytometric counts of both PPE groups over time indicated that the addition of 25 µg L^{-1} of copper did not exert a negative effect neither for PPE-1 (grey lines in Fig. 11b, e) nor PPE-2 (grey lines in Fig. 11c, f) during the microcosm experiment, in terms of abundance. Specifically, PPE-1 had an increase in cell abundance similar in magnitude in both sites tested. In contrast, the addition of 250 µg L^{-1} of copper had a strong effect over both PPE groups after 12 h post copper addition, with the same trend observed for HCS and LCS samples (black lines in Fig. 11b, c, e, f). As PPE communities from both sites displayed similar fluorescence and abundance patterns, we can argue that PPE inhabiting the area can cope with 25 µg L^{-1} of copper without a negative effect in cell fluorescence nor abundance.

Copper enrichment effect on PPE community composition

To gain a more detailed insight of the changes on PPE community composition during this short-time microcosm experiment, T-RFLP analysis of < 3 µm community based on plastid 16S rRNA gene was assessed. T-RFLP profiles of the PPE community at 0 h (before copper addition) and 60 h for all treatments were compared (Fig. 12). Cluster analysis based on the T-RFLP profiles revealed an 83% of similarity in PPE community composition for both HCS and LCS samples, at the beginning of the experiment. After 60 h post copper addition, a differential response between HCS and LCS samples was observed. LCS control samples remained similar to time zero (75% similarity), while HCS control samples formed a different cluster together with 25 µg L^{-1} copper LCS samples. This cluster was associated to an increase in the abundance of T-RF 431 and 433, and a decrease of T-RF 250. The fact that control HCS samples have shown the same pattern as the 25 µg L^{-1} LCS samples, is probably related to the background copper concentration at HCS

(Table 1). Clone library sequence analysis identified T-RF 431 as *Chrysochromulina* (97% nucleotide similarity) and 433 as *Phaeocystis* (97% nucleotide similarity). T-RF 250 was assigned to the Prasinophyceae class, with no possible differentiation between *Prasinoderma* and *Pyramimonas* spp. due to sequence similarities in restriction sites for both enzymes tested.

In the 250 µg L^{-1} copper treatment, even when cellular abundance levels determined by flow cytometry were very low (Fig. 10 d, h), DNA extraction and PCR amplification were feasible, indicating the presence of a low abundance highly copper tolerant PPE community in this treatment. Community composition at 60 h post copper addition showed a strong differentiation from the control community, with less than 40% similarity between groups. A marked decrease in the relative abundance of T-RF 433 and 250, and the dominance of T-RF 437 is responsible of the dissimilitude detected by SIMPER analysis. T-RF 437 was identified as a member of the Bacillariophyceae class (radial centric diatom), but no deeper taxonomic assignation was possible. Interestingly, 25 µg L^{-1} HCS samples clustered with this group, indicating that even when these samples had the same cell abundances than LCS, the 25 µg L^{-1}, the PPE community changed to a copper-like composition. The changes observed indicate that copper caused a directional shift on the PPE community composition, with an initial increase of Haptophyta representatives, followed by a dominance of diatoms when copper levels increased. Cluster analysis strongly suggests that PPE belonging to the Prasinophyceae class (Chlorophyta) are sensitive to additions of copper, decreasing its abundance across all treatments, whereas Haptophytes and diatoms are more tolerant to copper. Mann *et al*. (2002) suggest that the small sized cells, due to the high surface to volume ratio, can be

the most affected by high copper concentrations, and larger cells tend to be more resistant.

By following its variation in abundance, a member of the Prasinophyceae class, *Phaeocystis,* and an unidentified Coscinodiscophyceae, can be valuable candidate as environmental sentinels of copper disturbance. A more detailed taxonomic analysis of the representatives of these OTUs is in progress to fine-tune the taxonomic affiliation of the different groups detected.

The results obtained in this work indicate that PPE inhabiting the Chañaral area can cope with high copper concentrations, tolerating copper levels similar or even higher than previously reported (De la Broise & Palenik, 2007; Le Jeune *et al.*, 2007). Moreover, copper addition causes a directional shift over PPE community composition, leading to a copper-exposed-like community pattern in short times, independent of the history of copper exposure of the samples. Similar results were recently described for ephilitic bacteria from this area (De la Iglesia *et al.*, 2012), indicating that eukaryote microbial communities have a short and directional response to high copper levels, as reported also for other environmental disturbances. The directional response observed will contribute to the definition of environmental sentinels for water quality determinations in coastal environments where high copper levels are present.

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Table 1: Copper speciation, Conductivity, Temperature, Depth, Oxygen, inorganic nutrients and Photosynthetic Picoeukaryotes abundances data from the sampling sites at the sampling time. Total dissolved copper , anodic stripping voltammetry labile copper and inorganic nutrients concentrations are averages from three measures of duplicate samples. Conductivity, Temperature, Depth and Oxygen values are from 1 meter depth at each sampling site.

Parameters	Units	low copper site	high copper site
Total Copper	µg L^{-1}	4.60	21.53
ASV-Labile copper	µg L^{-1}	0.83	8.78
Fluorescence	mg m^{-3}	5.71	5.85
Salinity	PSU	35.09	34.59
Dissolved Oxygen	mg mL^{-1}	1.15	1.01
Temperature	°C	13.70	12.26
NO_3^- + NO_2^-	µmol L^{-1}	13.66	12.60
NO_2^-	µmol L^{-1}	0.44	0.41
PO_4^{-3}	µmol L^{-1}	2.19	2.20
SiO_4^{-4}	µmol L^{-1}	8.63	7.92
PPE-1 Abundance	X 10^4 cells mL^{-1}	2.65	3.01
PPE-2 Abundance	X 10^4 cells mL^{-1}	0.45	0.46

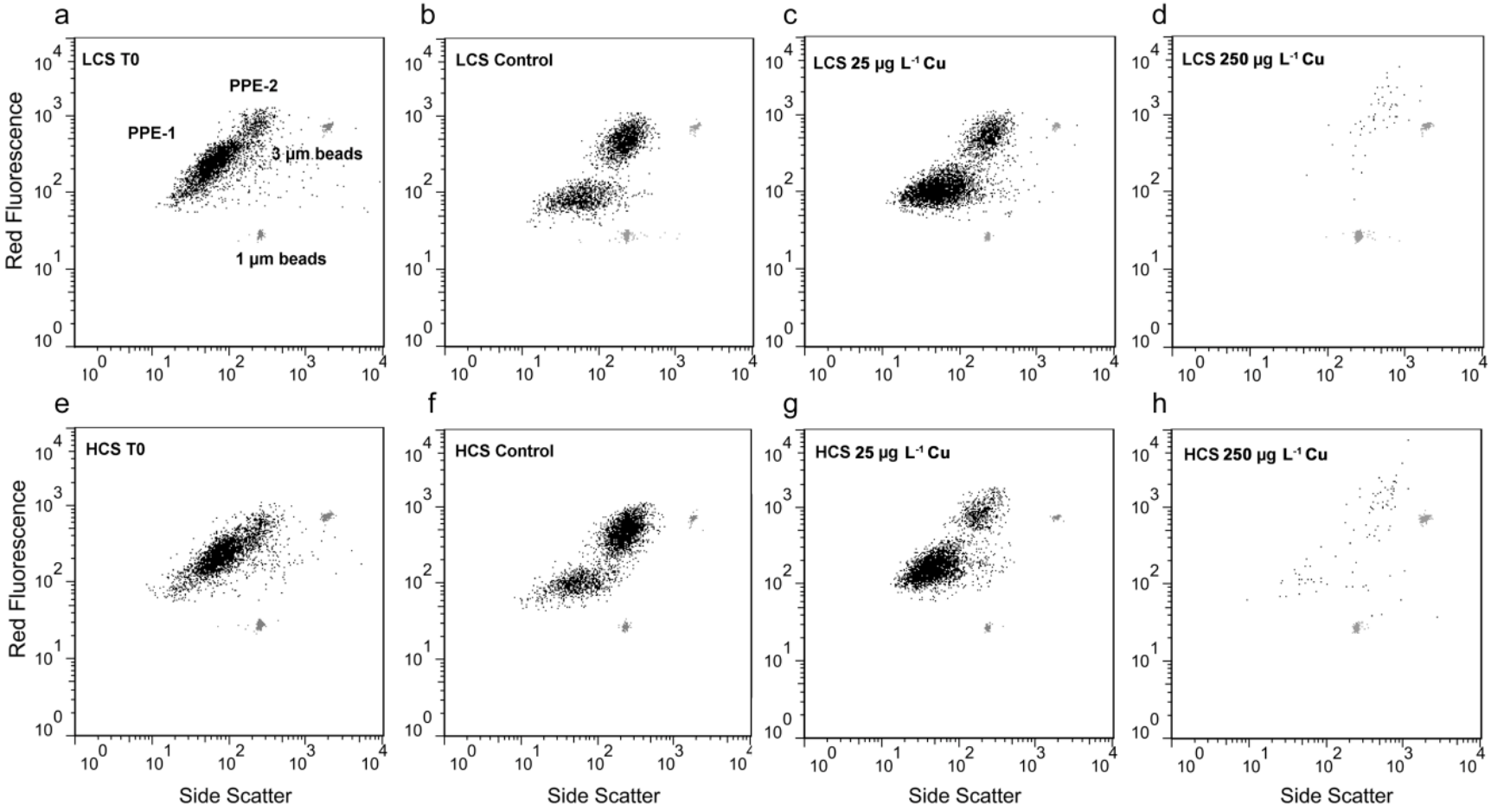


Fig. 1. Flow cytometric plots from the low copper site (LCS; upper panel) and high copper site (HCS; lower panel) at time 0 (a & e) and 60 h post incubation (b-d & f-h) from the microcosm experiment. Cytograms correspond to Side Scatter vs Red Fluorescence. Only populations defined as PPE-1 & PPE-2, 1 µm and 3 µm reference beads are shown in the cytograms.

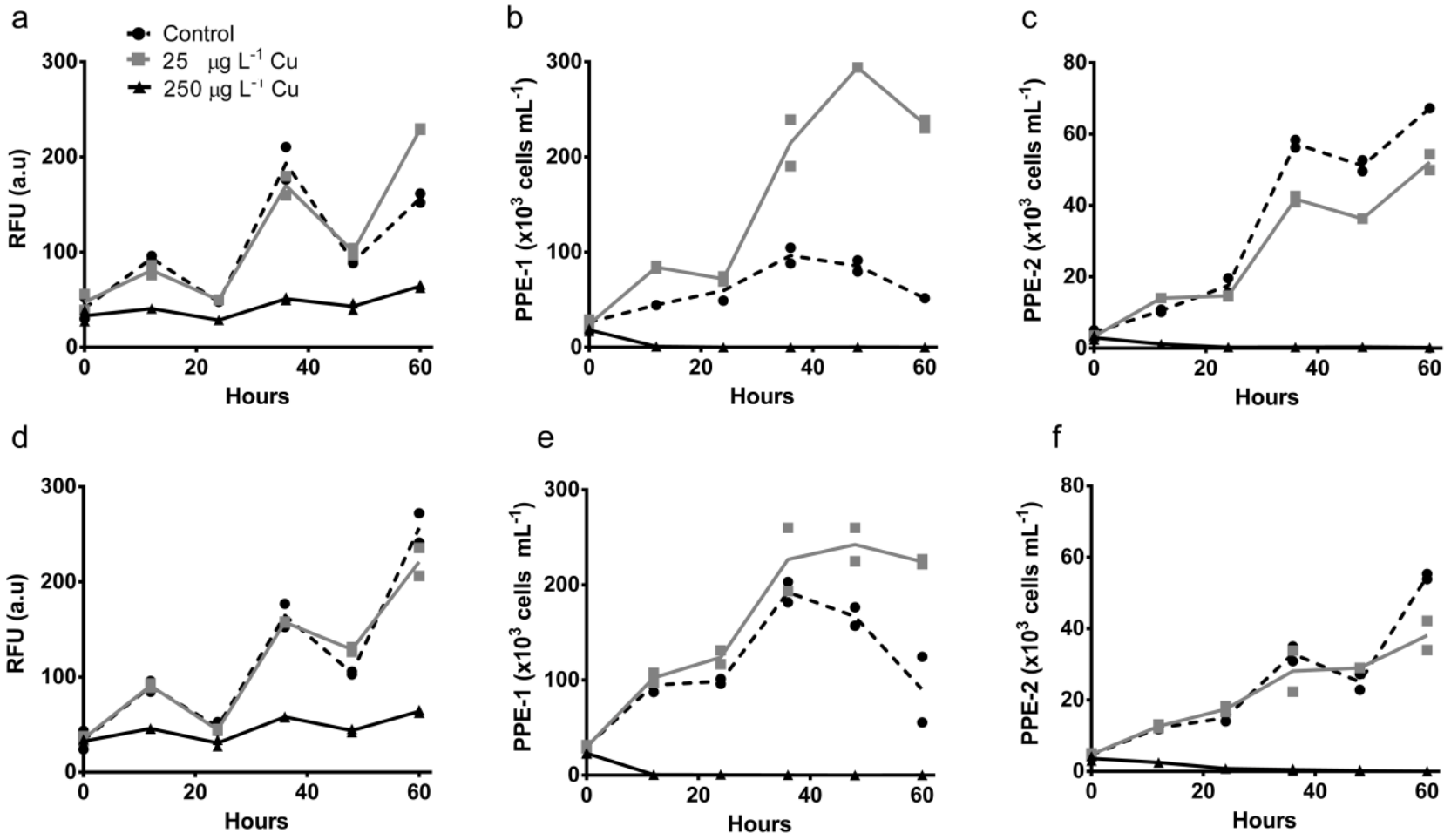


Fig. 2. Effect of copper addition on "*in vivo*" chlorophyll fluorescence based on Relative Fluorescence Units (RFU) of <150 µm community (a,d) & photosynthetic picoeukaryote growth kinetics during the microcosm experiment (b,c,e,f). Upper panel: LCS microcosms, lower panel: HCS microcosms. Each duplicate and tendency line were

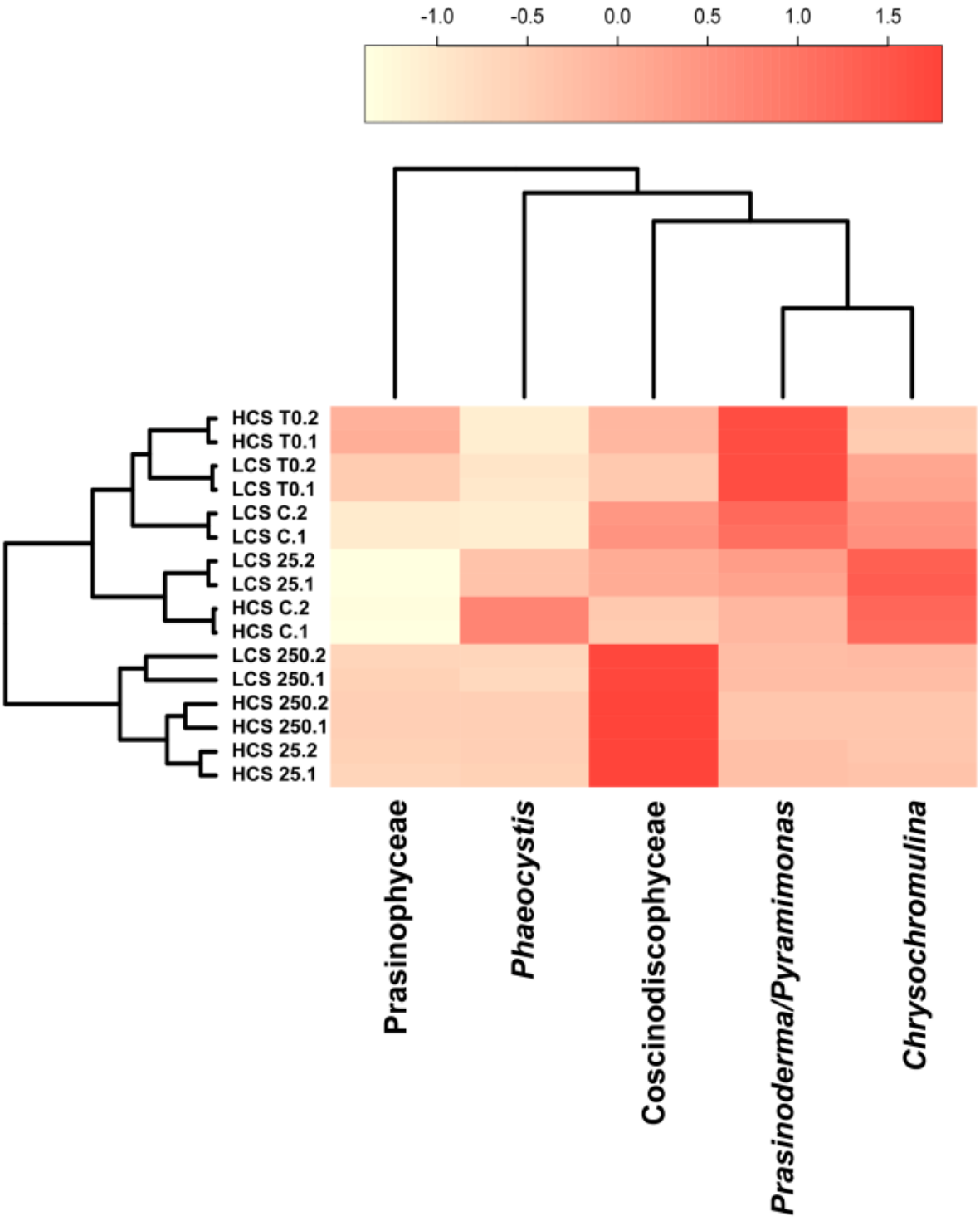


Fig. 3. Cluster analysis of terminal restriction fragment length polymorphisms profiles obtained after digestion of the plastid 16S rRNA PCR product with the endonuclease *Hae*III. Clustering reported were statistically supported by analysis of similarities, with an R value of 0.859 ($p < 0001$) for treatment comparisons. Only the five more abundant Operational Taxonomic Unit are shown. Colored Bar represent relative abundance in logarithmic scale.

General discussions

Marine phytoplankton are key contributors to major global processes such as oxygen production, nutrient recycling, carbon fixation and CO_2 sequestration, sustaining the life on the planet. Accounting for only ~1% of the world's photosynthetic biomass (Bryant, 2003), they are nevertheless responsible for nearly half of the oxygen that we breathe (Field *et al.*, 1998). Cell size plays a key role in determining the diversity and relative abundance of competing phytoplankton species, as well as the transfer of elements between the surface and deeper layers in the ocean and from phytoplankton to higher trophic levels (Smetacek 1985; Cushing 1989). Compilations of *in situ* observations (e.g. Irigoien *et al*., 2004) and global satellite measurements (Uitz *et al*., 2006) indicate that larger cells become more abundant under higher nutrient supply, common in upwelling regions, and smaller phytoplankton cells are generally present in a range of nutrient concentrations.

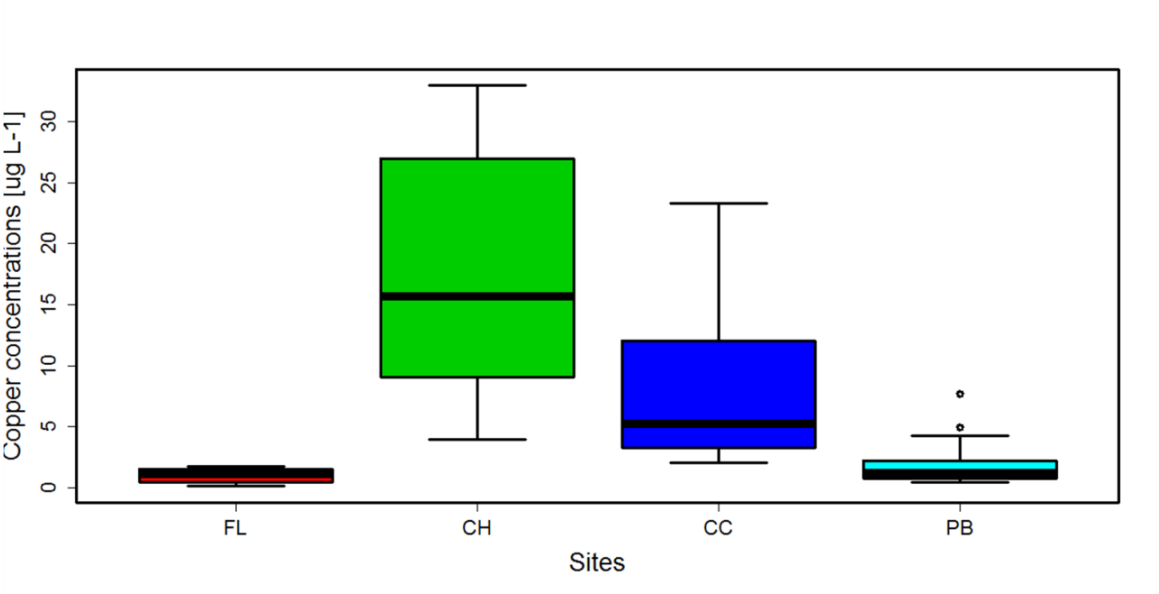
Recently has been recognized that picophytoplanktonic organisms are not exclusively restricted to oceanic pelagic environments. In many coastal regions, they are present throughout the year and constitute a background population, onto which episodic phenomena such as the spring bloom develop. The broke of the paradigm of phytoplankton taxa replacement in diatom bloom formation (Barber & Hiscock 2006; Ward *et al*., 2014) corroborate that the livelihood of the food web in coastal areas does not depend exclusively on large diatoms. Therefore, the small fraction of phytoplankton is essential in terms of productivity in coastal ecosystems. Moreover, in the world ocean, warmed by climate changes, the expected gradual shift towards smaller primary producers could render the role of picophytoplankton even more

important than they are today (Fernández *et al.*, 2003; Grob *et al*., 2011, Barton *et al.,* 2013).

In this work, we studied the cellular abundance and taxonomic composition of picophytoplankton present at Chañaral Bay, a permanently disturbed coastal system due to anthropogenic activity. Two sites were select according to previous reports on copper concentration and diversity at the intertidal zone. It has been clearly stated that high copper levels in the area are associated with a dramatic decrease in the richness and abundance of macroorganisms at the intertidal zone. In the case of bacteria, there is a differential susceptibility between compartments in the intertidal zone, suggesting a differential susceptibility to high copper levels between macro and microorganisms (Morán *et al*., *2008*). We hypothesized that copper is the factor that explain in greater proportion the differences in terms of abundance and taxonomic composition of picophytoplankton between selected sites.

Because no systematic sampling for trace metals and nutrients concentrations analysis have been done in the area (with the solely exception of copper), the first objective of this work was to seasonally monitor the copper concentration and nutrients in the water column. Previously, copper concentration values consistently higher at the HCS compared to LCS has been reported at the intertidal zone (Andrade *et al*., 2006). The results reported in our work demonstrate that differences in copper concentration in the water column are similar to those reported at the intertidal zone. Additionally, copper concentrations found in HCS are also above normal values reported for coastal systems, exceeding the limit values estimated to protect coastal ecosystems (Duran & Beiras 2012).

Copper concentration values also exceed the values reported as toxic to picophytoplanktonic organisms (Mann *et al*., 2002; De la Broise & Palenik 2007; Debelius *et al*., 2011). Complexing capacity at HCS is saturated, so the continuous riverine discharge will increase persistently the bioavailable copper and the potential toxicity of this metal. Copper levels reported in LCS are similar to those reported in other sites located south of the impacted area (Discussion Figure 1). However, temporal increment in concentrations were found. LCS follow the same pattern of fluctuations in copper concentration of HCS indicating that increments in copper concentrations at HCS induce increments in concentrations in the vicinity. We can argue that northward circulation can induce the mobilization of this metal to the adjacent bay persistently, increasing the concentration in LCS to the limit of values reported as toxic for phytoplanktonic taxa. Water masses in the Chañaral zone obey to general process described for coastal sites influenced by the Humboldt Current and the South Pacific Anticyclone (Escribano *et al*., 2004). No significant differences in trace metals, nutrients and physicochemical parameters was found between sites. These characteristics reinforce the idea about the Chañaral area as an excellent coastal laboratory to study the effect of a single stressor over marine communities.



Discussion figure 1: Copper concentrations of selected sites determined by ICP-MS. Flamenco (FL) and Playa Blanca (PB) were reported as low copper sites (LCS). Chañaral was reported as chronically copper polluted, and Cerro Castilla (CC) was reported as High Copper Site (HCS). Flamenco & Chañaral n=5, Cerro Castilla & Playa Blanca n=10.

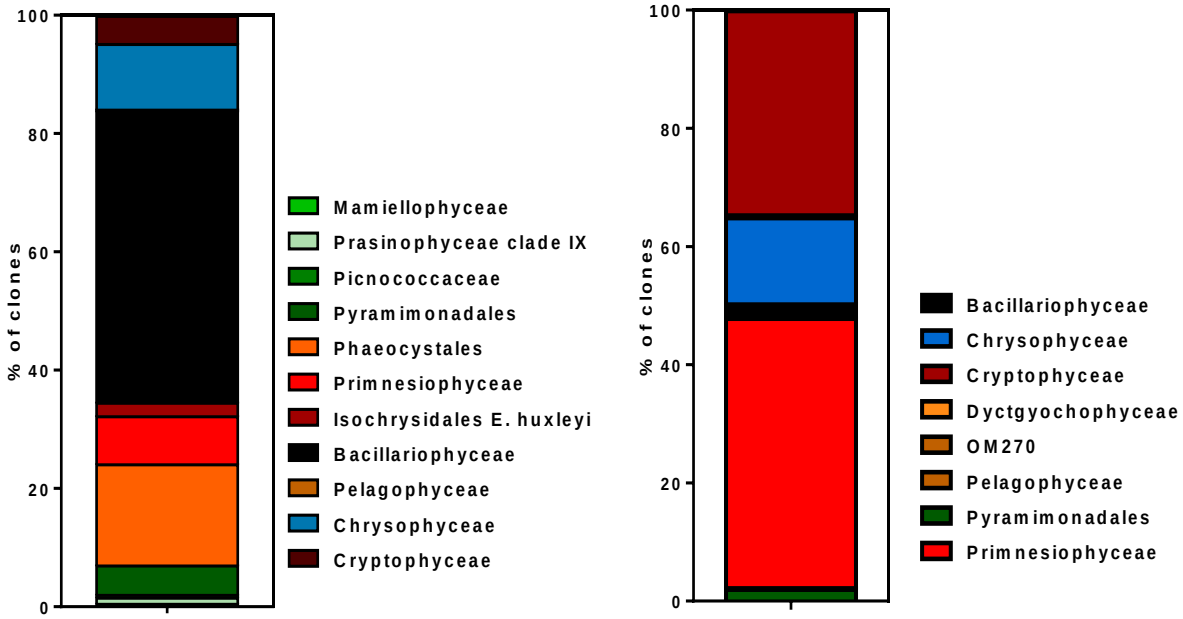
The second objective of this work was to understand the seasonal dynamics of picophytoplankton inhabit the zone. The cellular abundances of picocyanobacteria and PPE were monitored seasonally by flow cytometry. No significant differences between sites were observed, with cellular abundance values similar to that reported for other coastal systems (Massana 2011; Collado *et al*., 2011). Abundance results are in agreement with the results obtained with the taxonomic fingerprinting analysis. A strong class-level alpha diversity was found during the annual cycle, with regards of species composition. During winter all classes were represented, during transition months multiples taxa were represented, while during summer Bacillariophyceae

and Primnesiophyceae representatives dominate the zone. Because the abundances of PPE remains constant during the year and PPE-2 have a strong increase during summer we can argue that PPE-2 is mainly composed of Bacillariophyceae-diatoms that dominate in summer. Methodologies based in sorting of specific groups will be helpful to validate this hypothesis.

No overall differences in composition were found between sites, as correspondence analysis tend to split samples mainly between seasons. However, there are some OTUs that clearly dominate HCS seasonally. During winter, a Crysophyceae OTU without culturing representing and two unidentified OTUs were the main responsible for the differences between sites. This Crysophyceae is related to Synurophyceae and was highly represented at HCS during winter, being the dominant OTU during this season. The lack of reference strains difficult the analysis to find the possible genomic capabilities of these organisms. Crysophyceae appears to be widely distributed and is a relevant picoplanckter in the modern Ocean (McDonald *et al.*, 2006, Lepere *et al*., 2009). Lombardi & Vieira (1998) demonstrate that *Synura* sp., a freshwater Crysophyceae, produce high molecular weight compounds that can complex copper and lead. These capabilities can impose an ecological advantage to this Crysophyceae in disturbed ecosystems. During transition months, a Cryptophyceae related OTU appears to be the representing of HCS, in agreement with the results of clone libraries. This OTU was affiliated to an uncultured Cryptomonas, facultative heterotroph that present green-orange absorbing phycobiliproteins that distinguish them from other eukaryotic microalgae. High abundances of the Chrysophyceae *Ochromonas* and Cryptophytes related to

Cyptomonas were found in artificial lakes created by mining activities, reinforcing the idea of the tolerance of these organisms to heavy metals. However the acclimation modes of these organisms to high metal concentrations is still unknown (Sciandra *et al*., 2000).

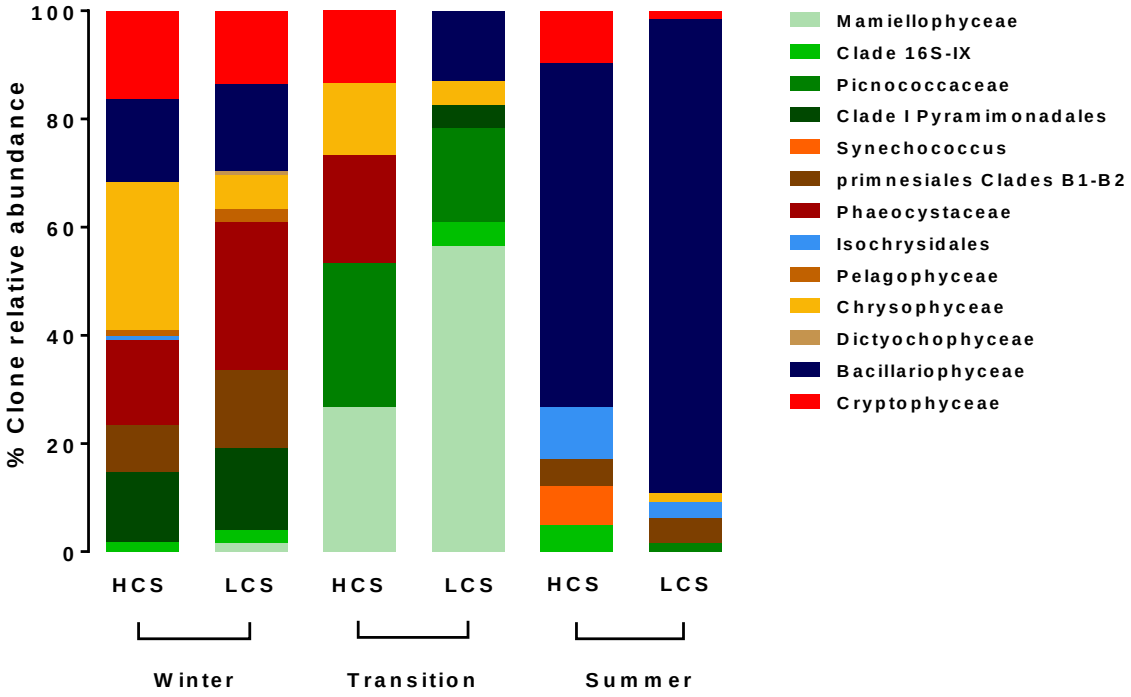
During summer, OTUs related to *Virgulinella* & *Chaetoceros* were mainly present at HCS. The increment in temperature and Iron concentrations can explain the high representation of this organisms during summer, since Iron enrichment can induce the bloom of diatoms in marine ecosystems (Barber & Hiscock 2006).



Discussion figure 2: Comparison of the proportion of each taxonomic class found in the libraries obtained during this work (left) and in the gulf of Naples, Italy (right) using the same methodologies.

The taxonomic composition of picophytoplankton determined at Chañaral area differs to the reported in other coastal ecosystem (Discussion figure 2). Even when there is a concordance in the presence of a high-class level diversity during winter

and a dominance of a few classes during summer (McDonald 2006), specific classes appears to be more relevant and dominant in the Chañaral zone, like Primnesiales, mainly *Chrysocromulina*-Phaeocystales and Crysophyceae in winter; Mamiellophyceae during transition months and Bacillariopyceae during summer. (Discussion figure 3)



Discussion figure 3: Proportion of each taxonomic class found in the 10 libraries obtained from the study sites. two libraries for each season were constructed for both sites.

For the bacterial fraction, *Synechococcu*s is present through the year without differences in terms of abundances between sites. The composition is restricted to clades I & IV, both reported as coastal. The increase of these cells during summer have been previously reported for other coastal system in the north pacific, however

the mechanisms that drive this increment during summer are still unknown. Recent studies on *Synechococcus sp*. CC9311, a coastal strain, highlighted the role of genomic islands in adaptation processes and reiterated the role of iron & copper on this important group of organisms in light of anthropogenic change of the modern ocean (Eriksson 2013; Stuart *et al*., 2013). *Synechococcus* have the ability to respond to key nutrients as copper & iron, and this can determine how *Synechococcus* can compete for and occupy various ecological niches.

With this two objectives we demonstrate that copper is the main factor that difference both sites, however, differences in copper concentrations does not exert a clear effect in terms of cellular abundance nor community composition.

In order to explore the response of picophytoplanktonic communities to high copper concentrations, microcosm experiments were carried out. Our results suggest that picocyanobacteria are most sensitive to pulses of high copper concentrations, with a fast response between 12 hours post copper addition (data not shown). Opposite, PPE were able to cope with 25 µg L^{-1} without a negative effect in cell abundances. In terms of PPE composition, Primnesiales appear to be highly represented with the addition of 25 µg L^{-1} of copper; while diatoms appear to be the dominant picoplankters with higher copper concentrations, in agreement with the environmental data.

One important conclusion obtained from this experiment relies on the fact that copper addition causes a directional shift over PPE community composition, leading to a copper-exposed-like community pattern in short times, independent of the history of copper exposure of the samples. As well, these copper concentrations are

higher than reported in this work for the selected sites. The continuous discharge of mining activities and the complexing capacity overwhelmed at HCS, copper release will impose eventually a strong pressure to the organisms inhabit this zone. This pressure will affect the abundance and composition of the picophytoplankton, in a direction as we observe in the microcosm experiments.

Cerro Castilla (HCS) has been received copper discharges during the last 20 years, and a new sandy beach is appearing because of this discharges. The effect of this disturbance over organisms inhabit the zone will expand progressively. Recently we measure the copper levels not only in the sites reported here, but also in the Chañaral port, historically reported as heavy copper polluted and in pristine waters north and south of the polluted zone. Our results suggest that Chañaral port is by far the heaviest polluted site, and interestingly, copper concentrations reported at HCS (Cerro Castilla) follow the same pattern of concentration of the Chañaral port (Discussion figure 1). This led us to reinforce the idea that HCS is an excellent site to study the effect of copper increase over marine communities temporally.

The results of this work represent a baseline for the studies of picophytoplankton in the zone. We demonstrate that copper is the main factor that explain the physico-chemical differences between sites and picophytoplankton is a relevant component in this coastal disturbed ecosystem. A sustained and intensively sampling during years are necessary to conclude if the amount of copper, which is deposited daily on the sea, have an impact on microbial communities, which can alter the energy transfer through the food web.

One important conclusion we can extract relies on the capacity of *Synechococcus* to cope with the concentrations of copper that we report here. Many laboratory and field observations approaches suggest a strong sensibility of this species to copper (Mann *et al*., 2002; Moffet 1997; De la Broise & Palenik 2007). The unique characteristics of this study site allow to determine that naturally occurring *Synechococcus* can cope with concentrations of copper higher than reported as toxic, and the interaction of Iron-copper in these genus can impose an ecological advantage that need to be explored deeply through metagenomics and metatranscriptomics.

Respect to the eukaryotic component, there are more difficulties in the study of this fraction. The seasonal variability in terms of composition as reported for other unpolluted coastal areas is a marker of the health of the community inhabit the area but also difficult the study over particular populations. It is probably that the range of concentrations reported in the area does not exert a marked effect over the composition, so the seasonally study over time for this component is priority to understand the mechanisms underlying the capacity to inhabit the water column with high metal concentrations. Through the microcosm experimental approach we reinforce the seasonal results, by the fact that Primnesiales and diatoms representing that dominate the area seasonally were the most resistant organisms to copper challenge in both sites tested, and the Prasinophyceae class that tend to be sensible to copper, was poorly represented during the study. The combination of seasonal sampling and microcosms approaches is useful to determine sentinels of anthropogenic disturbances in marine ecosystems.

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Annex 1

To be submitted to Environmental Toxicology and Chemistry

Copper ecotoxicology in the smallest free-living eukaryote *Ostreococcus tauri*.

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Abstract

Copper pollution was increased during the last century, especially in coastal environments. The study of copper effect over ecologically relevant organisms is a priority topic to understand the mechanisms underlying toxicity in the environment. Mamiellophyceae class has been recently described as a dominant picophytoplankter in coastal areas. During the last years, the Mammiellophyceae representing *Ostreococcus* emerged as new eukaryotic model organism. In this work, the copper ecotoxicology in the smallest free-living eukaryote *Ostreococcus* was assessed. Growth kinetics of different ecotypes suggest that the coastal strain RCC745, isolated from a copper polluted harbor, can cope with higher copper concentrations than other ecotypes isolated from non-polluted locations. Bioluminescence reporter lines screening demonstrate that Ferritin construction have a strong dose dependence response against copper amendments and against heavily copper polluted seawater. Our results suggest that *Ostreococcus* can cope with copper concentrations, and can be an important component of copper polluted environments.

Keywords: copper, ecotoxicology, *Ostreococcus* ecotypes, luminescence

Introduction

Copper is an essential metal at trace levels, especially for photosynthetic organisms (Barón *et al*., 2005), but become toxic at high concentrations. During the last century, an increase in copper concentration in marine environments has been reported, particularly in coastal areas, more subjected to disturbances associated to human settlement (Mann *et al*., 2002; Paytan *et al*., 2009). The main sources of copper increase is related to a number of anthropogenic activities including leaching from antifouling paint of ships, aerosol deposition, re-suspension from contaminated sediments and discharge riverine from mining activities (Blake *et al*., 2004; Nogales *et al*., 2012). The increase in copper levels in coastal environments strongly impact the diversity of meiofaunal assemblages (Sardá *et al*., 1997; Lee & Correa, 2007), with few information about its effect over the base of the trophic network, the phytoplankton (Moffet *et al*., 1997; Henríquez-Castillo *et al*., 2015). In near-shore waters receiving anthropogenic influence, multiple stressors can coexist in a single area (Halpern *et al*., 2008). Also, a stochastic component of temporal variation make difficult the study of the effect of a single stressor over natural communities (Nogales *et al*., 2012). Laboratory approaches emerged as a valid alternative to study the effect of a single stressor over particular populations.

Phytoplanktonic species are sensitive indicators of environmental changes, (Guilloux *et al*., 2013). Isolation, culturing and physiology studies has improved the knowledge about copper physiology mechanisms in the ocean (Davis *et al*. 2006; Wang *et al*., 2011). A differential susceptibility has been found between bacterial and eukaryal component of the phytoplankton. Picocyanobacteria (*Synechococcus* &

Prochlorococcus) are known for their sensibility to copper additions. Inside the *Synechococcus* genus, coastal strains are more tolerant to this metal than open ocean strains (Stuart *et al*., 2013). Respect to the eukaryotic component, the wide distributed diatom *Thalassiosira pseudonana* and the brown-tide forming algae *Aureococcus anophagefferens* were characterize as copper resistant organisms. (Davis *et al*., 2006; Wang *et al*., 2012).

The *Ostreococcus* genus*,* Mamiellophyceae class, contains the smallest free-living eukaryotes described to date (0.8-1.3 µm diameter). Due to its unique characteristics (small size, compact genome, sexual reproduction) has emerged as a new model photosynthetic organism (Courties *et al*., 1994; Derelle *et al*., 2006, Palenik *et al*., 2007; Marin B. 2014). Originally isolated at the Thau lagoon, *Ostreocccus* is a cosmopolitan marine primary producer, with different ecotypes adapted to different light intensities (Demir-Hilton *et al*., 2011). Recently has been described as a relevant organism in coastal systems, where can bloom and dominate picophytoplanktonic community (Countway and Caron 2006; Collado *et al*., 2012). The suitability of genetic manipulation made *Ostreococcus* a new reliable organism to performing short-term toxicity tests in the laboratory. Bioluminescence reporter lines were tested against biocides and could potentially be used to screen other toxic compounds in the marine environment (Sanchez-Ferandin *et al*., 2013).

Genomics analysis suggest that *Ostreoccocus* have an obligate use of copper in essential cellular process as photosynthesis (plastocyanin), Iron Adquisition (Multicopper oxidase), respiration (cytochrome c oxidase), and oxidative defense (Cu/ZnSOD), imposing an absolute requirement for this metal. (Palenik *et al*., 2007;

Piganeau *et al*., 2011). These characteristics and the availability of sequenced genomes from different ecotypes make *Ostreococcus* an excellent relevant model organism for the study of copper toxicity in the laboratory.

In this work, we study the copper ecotoxicology in the smallest free-living organism *Ostreococcus.* We analyze the sensibility of ecotypes isolated from different geographic locations, in terms of abundance and growth kinetics, and the response at cellular level to copper amendments through the monitoring of the expression of bioluminescence constructions of genes involved in a variety of cellular process as circadian rhythm, nutrient storage and transport and cell division. We also test the effect of natural seawater heavily copper polluted over bioluminescence constructions.

MATERIAL AND METHODS

Ostreococcus growth inhibition test conditions.

For cell growth curves assays, wild-type *O. tauri* strain RCC745 (clade C), RCC789 (Clade D), RCC802 (Clade A), RCC809 (Clade B) and *Ostreococcus lucimarinus* (Clade A) were grown in T25 aerated flasks (Sarstedt) with Keller media (Keller *et al*., 1987). Cells were cultured with continuous blue light (20 µmol quanta/cm^2 s^{-1}), until reach exponential phase (~10^7 cells per mL) and then transferred into Artificial Seawater supplemented with Keller nutrients. Trace metals were added without EDTA. Copper was added as copper sulfate 20 nM, and iron was added as iron-citrate 0.1 µM final concentration, as described in Botebol *et al*., 2014. Late exponential phase cultures of strains RCC745, RCC789, RCC802 & RCC809 were transferred into 24 well plates at a cell density of 1 Million cells mL^{-1}, and incubated with 20 nM, 40 nM, 0.2 µM, 0.4 µM, 2 µM, 4 µM, 40 µM of copper Sulfate**.** 20 µL subsamples were taken every 24 hours, diluted in 180 µl of Keller media with glutaraldehyde (0.25%, vol/vol, final concentration) and stored at -20°C. For exponential phase copper amendments, RCC745, RCC789 & *O. lucimarinus* were grown until exponential phase (20 Mcells mL^{-1}) in T75 aerated flasks, and then transferred into 24 well plates containing 0.4 µM, 1 µM, 2 µM, 4 µM & 10 µM of copper sulphate. Subsamples (20 µL) were taken at 1, 4 and every 12 hours, diluted in 180 µl of Keller medium with glutaraldehyde (0.25%, vol/vol, final concentration) and stored at -20 °C. Cells were counted by flow cytometry (Accuri C6 flow cytometer, BD) using 488 nm excitation laser and detecting by side scatter and red fluorescence, using log scale amplifiers.

For luminescence assays, RCC745 genetically modified lines with a luciferase gene reporter system were grown until exponential phase as described in Sanchez-Ferrandin 2014. Cells were refreshed into a 96-well microplate, at final densities of 5 Mcells mL^{-1}. Luciferin (10 µM final concentration) was added to the microplates containing the genetically modified lines. Copper was added at different concentrations: 0.4 µM, 1 µM, 2 µM, 3 µM, 4µM, 6.5 µM, 10 µM, 20 µM, 40 µM. Each concentration was tested in triplicate using independent wells. Control samples containing 20 nM of copper (minimal concentration of copper that does not affect growth kinetics) were also included in triplicate. Luminescence linked to the expression of Ferritin Iron transporter Random Insertion (RI), Homologous Recombination (RH), Cyclin- Dependent Kinase A (CDKA), High Affinity Phosphate Transporter (HapT) and morning-expressed MYB transcription factors (LHY-7) reporter lines was monitored using a luminometer (Berthold Technologies), every hour during 30 hours.

Ecotoxicological test using natural seawater from a polluted harbor.

Seawater samples were collected with metal free kemmerer bottles at 1 meter depth, in August 2013, from 3 different harbors and a copper discharge riverine, near Chañaral bay, northern coast of Chile. Based on previous data on copper concentrations (Andrade *et al*., 2006; Henríquez-Castillo *et al.*, 2015), Playa Blanca (26° 10´58,20"S, 70° 39´45,70" W) was selected as a low copper site (LCS). Playa La Lancha (26° 13´27,4 " S, 70° 40´2" W) was selected as a high copper site (HCS). Chañaral (26° 20´58,46"S, 70° 37´58.97” W) was selected as a chronic copper

disturbed bay, and the riverine (26° 16´29.42"S, 70° 39´39.37" W) as a heavily copper polluted seawater.

For copper toxicity assays, *Ostreococcus tauri* luminescent reporter lines CDKA- Luc and RI-Luc were selected. Cells were pre-acclimated in Seawater Keller media until reach late exponential phase. Cells were then refreshed into 96-well microplates at final densities of $5x10^6$ cells ml^{-1} in seawater from the different sites tested, or dilutions of the tested water in LCS water. In the case of the riverine, salinity was adjusted until values from reference seawater using MiliQ water. Seawater was enriched with Keller nutrients (Nitrate 8.8×10^{-4} M, Phosphate 1×10^{-5} M and f/2 vitamins). Copper sulfate (20 nM), iron-citrate (0.1 µM) and luciferin (10 µM final concentration) were added to the plates. Luminescence of the reporter lines was measured with a luminometer every hour during 30 hours. (Berthold Technologies).

Determination of half-maximal effective concentration (EC_{50}).

Copper toxicity after 24 hours was expressed as the concentration of copper inducing a reduction of 50% in growth or luminescence relative to control (50 % effective concentration [EC_{50}]). Cell abundances or luminescence curves of the treatments were normalized against control samples. The percentages were then plotted against the logarithm of the copper concentrations. The equation of the regression was used to determine the EC_{50}. (Prism software)

Copper concentration in seawater from polluted harbors

Total dissolved copper concentration in seawater was determined by ICP-MS (Thermo Fisher Scientific X series 2) as described in Andrade *et al*., 2006.

Results

Response of *Ostreococcus* Ecotypes to copper amendments.

Growth rates as function of copper concentration were obtained for the different *Ostreococcus* ecotypes (table 1). All the strains tested sustained the growth rate until 0.4 µM of copper. RCC789 displayed the same growth rate with copper concentrations up to 4 µM of copper, one order of magnitude higher than the other ecotypes. To further investigate the differential susceptibility of the ecotypes to copper addition, growth inhibition assay was performed. RCC745, RCC789, and *Ostreococcus lucimarinus* exponential phase cultures were challenged with increased copper concentrations, and the EC_{50} 24h estimated by growth inhibition was calculated (Table 2). After 24h, RCC745 & *O. lucimarinus* displayed a 50% of growth inhibition, while RCC789 displayed a 40% of growth inhibition at 1 µM of copper with an EC_{50} 24h higher than the other two ecotypes tested.

Responses of different *Ostreococcus* luminescence constructions to copper amendments.

To gain inside the response of *Ostreococcus* in terms of gene expression, bioluminescence curves were obtained after copper addition for each construction. Each line was characterized by different luminescence levels during the experiment. The ferritin constructions (RH, RI) gave the weakest fluorescence (maximal relative luminescence units of 4000), while the Phosphate Transporter (HapT-Luc) construction reached the maximal RLU (100000). For a better comparison of the results, the luminescence values obtained with the different copper amendments

were normalized against the luminescence measured in control conditions. (Figure 1). The addition of copper resulted in a differential response of the lines tested. All luminescence lines but HapT-Luc displayed a strong increment in the luminescence, in a dose dependent manner at the first hours post copper addition. Only the two highest copper concentrations (20 & 40 µM of copper) induced a fast and strong increase in the luminescence in the RI-Luc and CDKA-Luc constructions at the beginning of the experiment. RH-Luc and LHY-Luc lines had an increase in the luminescence, starting at copper concentration of 6.5 µM. While RH-Luc reached highest expression in the first 10 hours, LHY-Luc had a time-delayed expression in a copper dependent manner. In the case of copper concentrations below 10 µM RI-Luc, CDKA-Luc and HapT-Luc displayed a dose response effect after 6 hours (figure 1).

If we consider a 20% of decrease in luminescence is sufficient to define a toxic effect, all lines but LHY-Luc were significantly inhibited with 4 µM of copper after 24 hours post copper amendment. EC_{50} values for copper were calculated for each luminescent line at 24h (table 2). The EC_{50} value varied among the different lines tested. All lines tested but LHY-7, displayed a clear dose response kinetics, with a maximal decrease in luminescence after 24h. RI-Luc was to be the most sensitive luminescent line to copper toxicity. Opposite to the RI, the Homologous recombination line RH gave the highest EC_{50} value (Table 2). Taken all these results and previous data (Sanchez-Ferandin), we select the RI and CDKA for subsequent experiments and comparisons

Responses of different *Ostreococcus* luminescence constructions to seawater from different copper disturbed bays.

In order to explore the sensibility of the lines to seawater with different copper levels, the RI-Luc and CDKA-Luc lines were challenged with seawater from copper polluted harbors. Incubation of the RI-Luc and CDKA-Luc lines with water from Playa La Lancha (data not shown) & Chañaral bay (figure 2 A-B) did not result in a decrease in luminescence significantly. Seawater from the mining riverine discharge exerted a negative effect over both constructions (figure 2 C-D). The effect of this water was similar to the result obtained in the copper addition experiments (compare figure 1A-C with figure 2C-D). Especially the RI-Luc construction displayed a concentration dependent kinetic (Figure 2D). EC_{50} values at 24 hours obtained for RI-Luc & CDKA-Luc constructions were in the same range of concentrations obtained in the experiments of copper addition (table 2).

Discussion

Ostreococcus, a relevant and widely distributed phytoplanktonic organism, has received increasing attention in terms of its abundance and distribution in the global ocean, especially in coastal systems. Sequencing of the different ecotypes and the possibility to generate luminescence reporter lines open the possibility to perform short-term toxicity tests, which can helpful to understand how anthropogenic activities affect marine relevant phytoplanktonic organisms. Sanchez-Ferandin (2013) demonstrate the suitability of *Ostreococcus* luminescence reporter lines to test the toxicity of biofouling pesticides. In this work, we study the effect of copper over different *Ostreococcus ecotypes* and the cellular response in terms of expression of genes related with different cellular processes. This methodology can help not only to explore copper ecotoxicology of this model organism but also to define possible biosensors, in this case, to sense copper toxicity in the marine environment.

Ostreococcus RCC789, a copper tolerant strain

Growth kinetic monitored by flow cytometry indicate that all ecotypes tested sustained the growth rate until copper concentration of 0.4 µM, values higher than reported as toxic for other phytoplanktonic species. The RCC789 ecotype, isolated from surface water at Blanes bay, Barcelona sustained the growth rate until 4 µM of copper concentration, one order of magnitude higher than the other ecotypes. Exponential growth inhibition test also demonstrate that RCC789 can cope with higher copper concentrations than the other ecotypes, highlighting a better tolerance to copper in this strain. The Blanes bay is a closed port, which has been described

as highly contaminated by copper. In this port, have been reported copper levels in sediments of 97 μg g^{-1}, mainly due to copper-based antifouling paint of ships. (Pinedo, 1998). Genomic and transcriptomic analysis of this strain will be conducted to elucidate the mechanisms that allow to this strain to cope with higher copper concentrations than the other ecotypes tested.

Differential response of the bioluminescence lines to copper addition

Normalized luminescence data of the translational reporter proteins fused in frame with luciferase, demonstrate that all lines tested respond in terms of luminescence in the range of copper concentrations used in this work. We found a differential response of the constructions, due to the variety of cellular processes analyzed in this screening. The Ferritin transporter RI-Luc line was the most sensitive, in a dose response manner, to copper additions in terms of toxicity. RI-Luc display a 20% of decrease in luminescence with 1.5 μM of copper concentration at 24h, while the rest of the constructions were insensible to copper concentrations below 4 μm. CDKA-Luc & HapT-Luc constructions display the same kinetic with an induction of toxicity at higher copper concentration. The response of the Ferritin constructions can be relate to the tight coupling between copper and Iron Homeostasis; copper can exert a direct effect over ferritin constructions that can response in a dose dependent manner, opposite to the indirect effect of copper over other cellular processes. Interestingly, the response of the two ferritin constructions differed during the course of the experiment. Homologous recombination Ferritin construction (RH-Luc) displayed the higher EC_{50} 24h. This construction have a non-functional ferritin protein, while the Random insertion line retain the original ferritin protein plus the

luminescence construction. More experiments focused in these constructions are necessary to understand the mechanisms underlying the differences between the lines, and the tight relationship between Copper and Iron. We can hypothesize this differential susceptibility could be a side effect of ferritin mutation in RH; there is no more iron scavenging in RH lines. Cells should deal with much more oxidative stress and, they have to express much more protein for detoxification and/or antioxidant pathway than the RI strain. RI still have ferritin, so it deal with iron easily but might not be ready to face high oxidative stress. In mammalian cells, under oxidative stress, there is a strong induction of ferritin synthesis (Orino et al 2001), as we observe in the first hours of the challenge. We can argue that accumulation of this protein, in the case of the two copies of the Ferritin (RI) can be also potentially toxic for the cell. Thus, the balancing of deleterious and beneficial effects of iron thus emerges as an essential aspect of cell survival.

RI-Luc & CDKA-Luc response to copper polluted seawater.

The addition of seawater with dissolved copper concentration of 1 µM did not affect the luminescence of the lines tested. However, riverine discharge water having 6 µM of copper exerted a toxic effect similar to that observed in the experiments of copper toxicity on both lines. These results suggest that the picoalgae *Ostreocccus* can cope with concentrations of total dissolved copper that we can find in polluted harbors, as occur in Chañaral bay, Chile. Recently through microcosms experiment we demonstrate that picoeukaryotic populations can cope with ~ 0.4 µM of copper, without a decrease in the fluorescence nor cell abundance. These results reinforce the idea that eukaryotic picophytoplankton can cope with higher concentrations of

copper than its bacterial counterpart, and can sustain the trophic chain in disturbed environments.

Concluding remarks

At date, this is the first attempt to study the copper ecotoxicology in the smallest free-living photosynthetic eukaryote *Ostreococcus*, through the analysis of the response of different ecotypes and luminescence reporters. Although the results of studies in monoculture cannot always be correlated with results obtained in natural communities, the use of model organisms that are relevant in coastal ecosystems, as in the case of *Ostreococcus* can be helpful to elucidate the mechanisms involved in copper homeostasis in the smallest eukaryotic fraction. The study of the interaction Copper-*Ostreococcus* through RNA sequencing of copper challenged cells will be helpful to design new biosensors that can be able to sense copper polluted environments.

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Table 1. - Growth kinetics of the different *Ostreococcus* ecotypes in response to copper additions

Strain	RCC745	RCC809	RCC802	RCC789
Cu^{+2} (µM)	(k d^{-1})	(k d^{-1})	(k d^{-1})	(k d^{-1})
control	1.219 ± 0.09	1.123 ± 0.15	0.995 ± 0.06	1.233 ± 0.03
0.02	1.306 ± 0.08	1.345 ± 0.09	1.039 ± 0.08	1.146 ± 0.03
0.04	1.195 ± 0.08	1.248 ± 0.12	1.029 ± 0.07	1.153 ± 0.06
0.20	1.236 ± 0.06	1.218 ± 0.10	0.900 ± 0.06	1.257 ± 0.04
0.40	1.287 ± 0.09	1.610 ± 0.19	0.979 ± 0.08	1.169 ± 0.04
2.00	-0.091 ± 0.17	-0.206 ± 0.19	-0.369 ± 0.25	1.279 ± 0.04
4.00	-0.475 ± 0.18	-0.539 ± 0.27	-0.117 ± 0.37	1.284 ± 0.04

Data represent the means ± SEM of experimental triplicates.

Table 2. - Comparison of the EC_{50}s of the different *Ostreococcus* ecotypes and luminescent lines tested

Toxicant	Cell line (Assay)		EC_{50}24H (µM)
$CuSO_4$	RH	(LI)	10.13 ± 0.44
	LHY-7	(LI)	9.80 ± 0.44
	CDKA-Luc	(LI)	6.06 ± 0.34
	HapT-Luc	(LI)	5.01 ± 0.05
	RI-Luc	(LI)	3.83 ± 0.28
	RCC745	(GI)	2.90 ± 0.61
	RCC745	(EGI)	1.22 ± 0.48
	lucimarinus	(EGI)	1.29 ± 0.32
	RCC789	(EGI)	1.63 ± 0.24
Riverine	RI-Luc	(LI)	2.58 ± 0.05
	CDKA-Luc	(LI)	5.23 ± 0.16

LI, luminescence inhibition test; GI, growth inhibition test; EGI, exponential growth inhibition test. Data represent the mean ± standard error media of experimental triplicates.

Table 3. - Copper species concentrations and salinity of seawater samples selected for luminescence assay

Location	TD-Cu (uM)	ASV-Cu (uM)	Salinity (PSU)
Riverine	6.25	**n.d**	80.2
Chañaral Bay	1.01	0.0029	35.0
La lancha	0.12	0.0009	34.8
Playa Blanca	0.07	0.0001	34.8

TD-Cu, Total Dissolved Copper; ASV-Cu, Anodic Stripping Voltammetry Copper (labile copper) in micromolar concentrations; PSU, Practical Salinity Unit. Data for copper species represent the mean ± standard error media of three replicates.

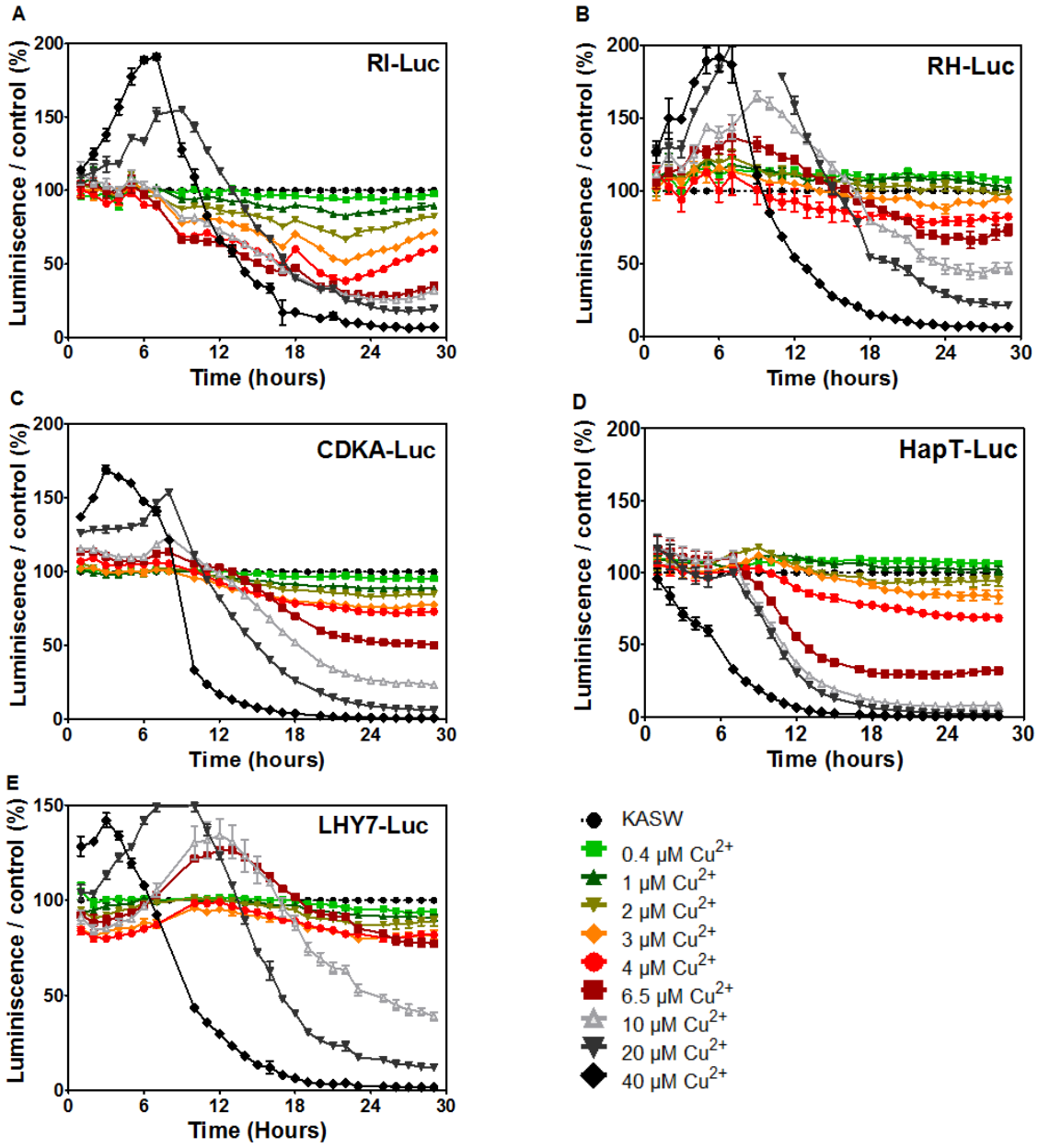

Figure 1. - Comparison of the different *O. tauri* luminescent lines, as indicated, after exposure to different copper concentrations. Graphs represent normalized data compared to the control lines (20 nM of copper). Values correspond to the means and SEM of triplicate wells on the microplate.

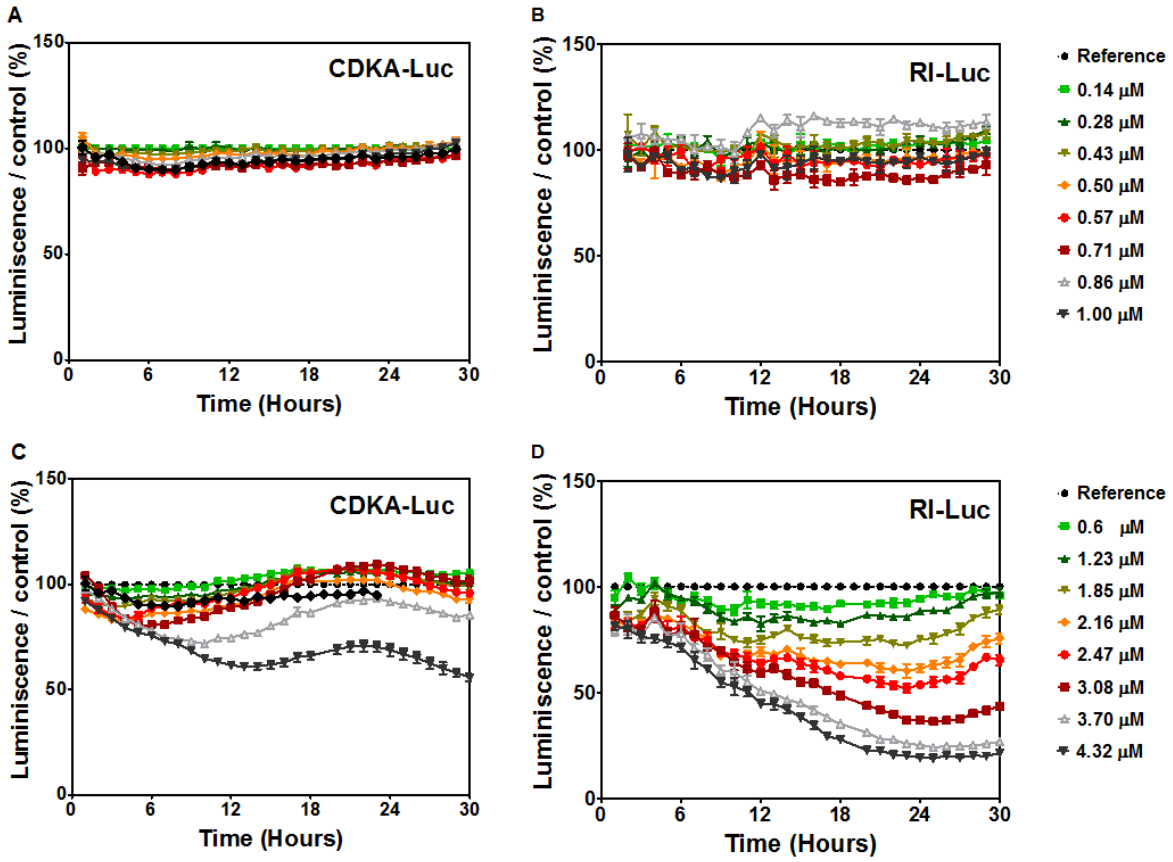

Figure 2. - Comparison of the different *O. tauri* luminescent lines, as indicated, after exposure to seawater with different copper concentrations. Graphs represent normalized data compared to the control lines (20 nM of copper). Values correspond to the means and SEM of triplicate wells on the microplate.