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Estructura comunitaria y dinámica estacional del picoplancton eucarionte en un sistema de surgencia costero con presencia de suboxia

Tesis para optar al grado de Doctor en Oceanografía

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La Tesis de "*Doctorado en Oceanografía*" titulada "Estructura comunitaria y dinámica estacional del picoplancton eucarionte en un sistema de surgencia costero con presencia de suboxia", de la Srta. *Silvana Andrea Collado Fabbri* y realizada bajo la Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, ha sido aprobada por la siguiente Comisión de Evaluación:

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A mis padres

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Resumen

Estructura comunitaria y dinámica estacional del picoplancton eucarionte en un sistema de surgencia costero con presencia de suboxia.

Silvana Andrea Collado Fabbri

Programa de Doctorado en Oceanografía
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Dr. Osvaldo Ulloa Quijada, Profesor Guía

En las últimas décadas el estudio de los microorganismos en el ambiente marino ha ido en franco aumento. La gran mayoría de los estudios microbiológicos se han enfocado en bacterias, archaeas y virus, mientras que los organismos eucariontes unicelulares, denominados protistas, han recibido mucha menos atención. Casi todo el árbol filogenético eucarionte está constituído por organismos unicelulares y es probablemente esta gran diversidad genética, construida a lo largo de 1.500 millones de años de evolución, la que explica su diversidad de funciones ecológicas. Además de la fotosíntesis, proceso que los hace principales responsables de la producción primaria en el oceano global, los protistas tienen un amplio rango de comportamientos tróficos relacionados con la heterotrofía (ej. mixotrofía, predación, parasitismo, saprotrofía), lo que los convierte en parte fundamental en el flujo de carbono a través de las tramas tróficas. Sin embargo, la falta de conocimiento acerca de la diversidad biológica (y funcional) de estos organismos ha hecho que no se haya cuantificado su actividad e impacto reales en el ciclo global del carbono. Dentro del estudio de los protistas uno de los mayores avances ha sido el descubrimiento, a través de la secuenciación del gen que codifica el ARN ribosomal 18S, de la enorme diversidad y existencia de grupos previamente desconocidos en la fracción de tamaño más pequeña (< 3 μm), los picoeucariontes. En el desarrollo de esta tesis se estudió la estructura comunitaria de los picoeucariontes que habitan en la columna de agua de la zona de surgencia costera estacional frente a Chile central, particularmente frente a la bahía de Concepción, en una estación de

muestreo mensual ubicada sobre la plataforma continental. El sitio de muestreo tiene una variabilidad estacional en las condiciones hidrográficas: durante primavera-verano hay vientos favorables a la surgencia, el sistema es altamente productivo y se genera una Zona de Mínimo Oxígeno (ZMO) subsuperficial; mientras que en otoño-invierno es menos productivo y la ZMO tiende a disolverse. En un primer capítulo describimos la estructura y la dinámica estacional de la comunidad fotosintética picoeucarionte en aguas superficiales a través de secuenciación de ARNr 18S y conteo directo de células por hibridación fluorescente in situ. En un segundo capítulo estudiamos la diversidad genética de la comunidad, fundamentalmente heterótrofa, que habita la capa profunda de la columna de agua y el efecto de las condiciones variables de oxígeno sobre ella. Nuestros resultados indican que en la capa fótica la abundancia de picofitoeucariontes es alta en otoño-invierno y baja durante la temporada de surgencia, cuando domina el fitoplancton de mayor tamaño (diatomeas). El grupo Chloroplastida representó la mayor parte de la comunidad picofitoeucarionte, y la suma de los géneros *Ostreococcus*, *Micromonas* y *Bathycoccus* representa la totalidad de Chloroplastida. *Ostreococcus* domina numéricamente a lo largo de todo el año, a diferencia de otros sitios costeros, donde se ha observado en altas abundancias solo de manera esporádica. Por otro lado, la comunidad picoeucarionte que habita la zona afótica varía a lo largo del año en diversidad y en funcionalidad. El grupo Syndiniales (parásitos) fue el más representado en las librerías de clones y su composición varió claramente de acuerdo al oxígeno. Se destaca la presencia de grupos saprófitos durante y después del período de surgencia, cuando hay mayor flujo de material orgánica: Fungi en enero y Labyrinthulomycetes en abril, siendo este último representado en su mayoría por secuencias que forman un grupo probablemente nuevo. En invierno, cuando la columna de agua está más oxigenada, se observa una mayor diversidad de grupos taxonómicos. En primavera la fagotrofía parece tener mayor relevancia debido a mayor representación del grupo Ciliophora. La fluctuación en términos no solo de grupos taxonómicos sino también en términos de funcionalidad de la comunidad heterótrofa eucarionte en la ZMO frente a Chile Central sugiere un rol ecológico de estos organismos que no ha sido considerado. Los resultados de esta tesis representan la primera descripción de la comunidad picoeucarionte en el area de estudio y constituyen una base para abordar próximos estudios ecológicos de protistas.

Abstract

Community structure and seasonal dynamics of the eukaryotic picoplancton at a coastal upwelling system with the presence of suboxia

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Dr. Osvaldo Ulloa Quijada, Profesor Guía

In recent decades the study of microorganisms in the marine environment has been on the rise. The vast majority of microbiological studies have focused on bacteria, archaea and viruses, while the unicellular eukaryotic organisms, called protists, have received much less attention. Most of the eukaryote phylogenetic tree is constituted by unicellular organisms and is probably this enormous genetic diversity, developed over 1,500 million years of evolution, that explains its diversity of ecological functions. In addition to photosynthesis, the process that makes them primarily responsible for primary production in the global ocean, protists have a wide range of trophic behaviors related to heterotrophy (e.g. mixotrophy, predation, parasitism, saprotrophic nutrition), that makes them fundamental part of the flow of carbon through the food web. However, there is a lack of knowledge about biological (and functional) diversity of these organisms, and this has resulted in an underestimation of the activity and impact of protists on the global carbon cycle. Within the study of protists one of the major advances has been the discovery, after the sequencing of the gene encoding 18S ribosomal RNA, of the enormous eukaryotic diversity and the existence of previously unknown groups in the smaller size fraction (< 3 um), the so-called picoeukaryotes. In the present thesis we studied the community structure of picoeukaryotes inhabiting the water column of a seasonal coastal upwelling area off central Chile, at a fixed time-series station (monthly sampled) located over the continental shelf, at the Concepción Bay. The sampling site has a seasonal variability in the water column: during spring and summer, when the winds are favorable to

upwelling, the system is highly productive and there is a subsurface Oxygen Minimum Zone (OMZ), while in autumn-winter the system is less productive and the OMZ tends to dissappear. In the first chapter of these thesis we describe the structure and seasonal dynamics of the photosynthetic picoeucaryote community in surface water, using 18S rRNA sequencing and direct counting of cells by fluorescence in situ hybridization. In a second chapter we studied the genetic diversity of the (mainly heterotrophic) community that inhabits the deep layer of the water column and the effect of the variable oxygen conditions on this community. Our results indicate that in the photic layer picophytoeukaryote abundance is high in autumn-winter and low during the upwelling season, when the larger phytoplankton (diatoms) dominates. Chloroplastida accounted for most of the picophytoeukaryotic community and the sum of the genera *Ostreococcus*, *Micromonas* and *Bathycoccus* represents all the Chloroplastida counts. *Ostreococcus* is numerically dominant throughout the year, unlike other coastal sites previously studied, where there have been reports of high abundances only sporadically. On the other hand, the picoeukaryote community inhabiting the aphotic zone varies throughout the year in terms of diversity and functionality. The group Syndiniales (parasites) was the most abundant in the clone libraries and their composition clearly varied according to oxygen. The presence of saprotroph groups was particularly detected during and after the period of upwelling, when there is a larger flux of organic matter: Fungi in January and Labyrinthulomycetes in April, being this group represented mostly by sequences with no affiliation to previously known groups. During the (austral) winter, when the water column is more oxygenated, the picoeukaryotic community is more diverse. During (austral) spring phagotrophy seems to be relevant, because of the prominent presence of Ciliophora. Fluctuations in terms of taxonomic groups and in terms of functionality of the heterotrophic community at the OMZ off Central Chile suggest an ecological role of these organisms that has not been considered. The results of this thesis represent the first description of the picoeucaryote community in this study area and constitute a basis for upcoming environmental studies on protists.

1.- INTRODUCCION

1.1. *Definición de picoplancton eucarionte*

Con el objetivo de facilitar el estudio de los organismos planctónicos, Sieburth et al. (1978) establecieron una forma de clasificación cuyo criterio se basa en el tamaño de los organismos. De acuerdo a esta clasificación, ampliamente aceptada y utilizada, los seres vivos cuyo diámetro está entre 0.2 y 2 µm conformarían el picoplancton, aunque muchos autores han optado por ampliar el término a organismos capaces de pasar un filtro de poro de 3 µm, debido a que la separación de las fracciones de tamaño por filtración sería inexacta, ya que estaría afectada por la presión de filtración y la forma y flexibilidad celular (Murphy & Haugen 1985; Simon et al. 1994; Moon-van der Staay et al. 2001; Biegala et al. 2003). Por mucho tiempo se consideró que los principales organismos que componían el picoplancton eran sólo bacterias, sin embargo, hoy sabemos que en esta fracción están representadas las 3 líneas mayores de evolución o dominios: Archaea, Bacteria y Eukarya (Woese et al. 1990). Así, los organismos picoplanctónicos que pertenecen al dominio Eukarya han sido denominados picoeucariontes.

Hacia los años 50s se conocía, como caso excepcional, la existencia y relativamente alta abundancia en ciertos ambientes marinos de la picoalga cocoide y flagelada *Micromonas pusilla* (Knight-Jones & Walne 1951). Varias décadas después, algunos estudios demostraron mediante el uso de microscopía la presencia abundante de eucariontes fotótrofos menores a 2 µm tanto en estuarios y aguas costeras (Johnson & Sieburth 1982; Hall & Vincent 1990) como en océano abierto (Murphy & Haugen 1985). Sin embargo, estos estudios fueron escasos, ya que los organismos picoplanctónicos tienen muy pequeño tamaño y su morfología es más bien simple, lo que hace difícil su distinción al observarlos por microscopía óptica. Las observaciones por microscopía electrónica pueden dar más antecedentes acerca de la ultraestructura de las células (Johnson & Sieburth 1982), pero muchas veces las características morfológicas no son suficientes para poder describir una especie o clasificarla dentro de un grupo taxonómico (Potter et al. 1997). En este sentido, el uso de la citometría de flujo en muestras ambientales constituyó el mayor avance en la estimación de abundancia del

picofitoplancton (Yentsch et al. 1983; Olson et al. 1985). Con el citómetro de flujo se pueden obtener abundancias de células de los grupos que constituyen el picofitoplancton: cianobacterias (*Synechococcus* y *Prochlorococcus*) y picoeucariontes; ya que permite separar y contar distintas poblaciones fitoplanctónicas de acuerdo a su tamaño y a la fluorescencia natural de sus pigmentos (Marie et al. 2005). La citometría de flujo también ha sido útil para calcular la contribución en términos de biomasa (carbono) a partir del diámetro promedio de una población picofitoplanctónica natural y factores de conversión, lo que busca explorar y cuantificar la contribución ecológica de estos organismos en un ambiente determinado (Worden et al. 2004; Grob et al. 2007a).

Lo que realmente amplió el conocimiento acerca de las comunidades picoeucariontes fue el análisis de su diversidad genética en muestras ambientales. Los primeros estudios moleculares sobre diversidad microbiana fueron en picoplancton procarionte, lo que llevó al descubrimiento de nuevos linajes de bacterias (Giovannoni et al. 1990) y archaeas (Delong 1992), sin embargo, estudios de este tipo en eucariontes fueron posteriores (López-García et al. 2001, Moon-van der Staay et al. 2001, Díez et al. 2001). Se demostró consistentemente que el componente eucarionte no sólo está constituido por pequeñas algas, sino que por la mayoría de las principales ramas del árbol filogenético eucarionte y que, por tanto, existen muchos taxones heterótrofos en esta fracción (Figura 1.1), lo que generó un nuevo y gran interés en estos organismos. Es quizás esta diversidad filogenética la que le confiere a este grupo una plasticidad funcional que les permite vivir ampliamente distribuidos en el océano, tanto en mar abierto (Díez et al. 2001) como en sistemas costeros (Romari & Vaulot 2004; Worden 2006), a lo largo de toda la columna de agua (Not et al. 2007), desde latitudes altas (López-García et al. 2001; Lovejoy et al. 2006) hasta aguas ecuatoriales (Moon-van der Staay et al. 2000 y 2001) y hasta ambientes considerados "extremos". Se ha encontrado una significativa diversidad eucarionte en zonas anóxicas, como la cuenca del Cariaco (Stoeck et al. 2003), fluidos hidrotermales (López-García et al. 2006) y algunos fiordos anóxicos (Zuendorf et al. 2006; Behnke et al. 2006). Sin embargo, es escasa la información que se posee acerca de sus abundancias, su estructura comunitaria, sus dinámicas poblacionales y, menos aún, de su rol ecológico.

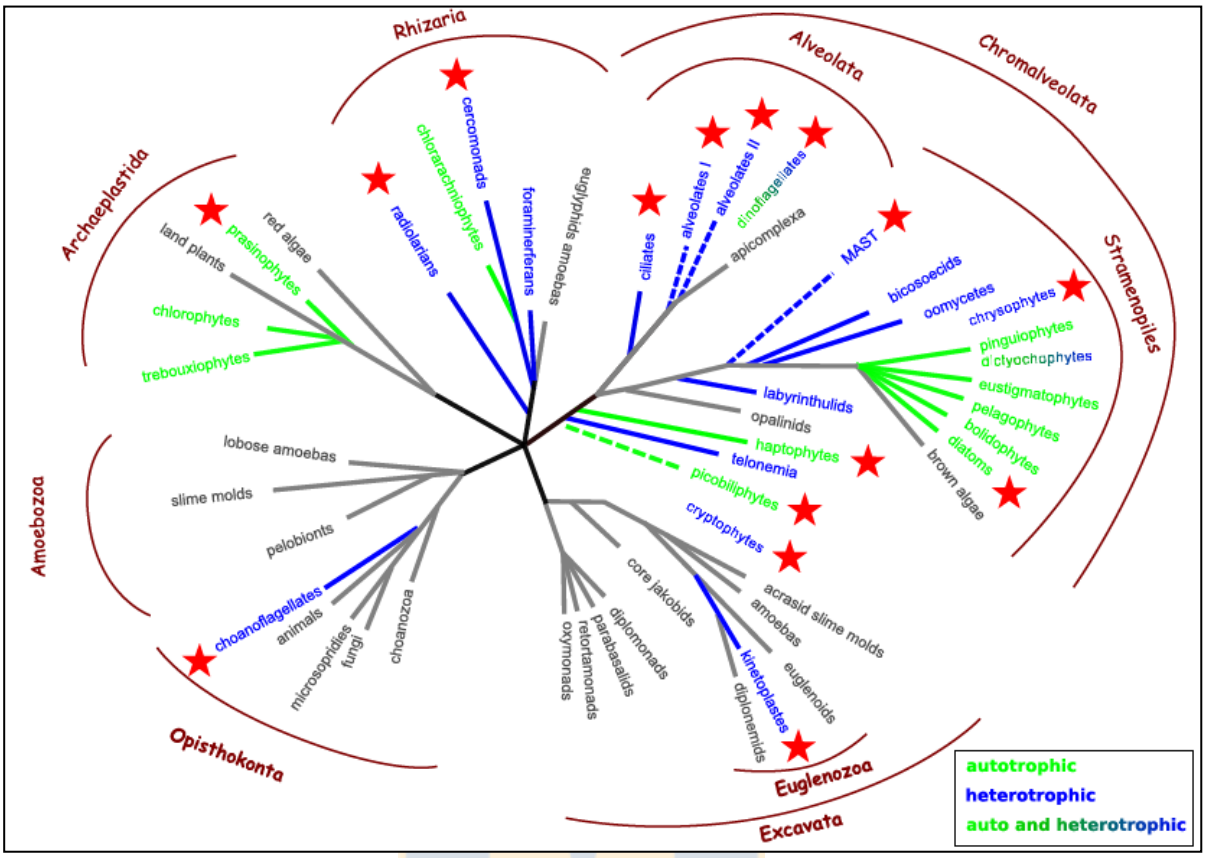


Figura 1.1. Arbol filogenético eucarionte adaptado por D. Vaulot de Baldauf (2003). Se muestran 7 "super grupos": Stramenopila, Alveolata, Rhizaria, Excavata, Opisthokonta, Amoebozoa y Archaeplastida que contienen múltiples linajes (Worden et al. 2015). Las ramas en gris representan los linajes con muy pocos o sin representantes en el ambiente marino, las verdes a linajes fotosintéticos y las azules a linajes heterótrofos. Los linajes que tienen organismos auto y heterótrofos se muestran con un gradiente verde-azul. Las estrellas rojas marcan los grupos más representados en el ambiente pelágico marino.

De esta manera, desde los primeros estudios moleculares fue posible identificar grupos desconocidos muy comunes en ambientes marinos y sin representantes en cultivo. A pesar de que muchas veces el único antecedente son secuencias ambientales, ha sido posible acercarse a la ecología (distribución y abundancia principalmente) de estos "nuevos" grupos. Existen técnicas moleculares que permiten cuantificar grupos sin necesidad de que hayan sido aislados ni tengan un fenotipo definido y conocido. Una técnica que se ha utilizado en picoeucariontes es la PCR cuantitativa o QPCR, que consiste en la amplificación de un gen (generalmente 18S rDNA) utilizando partidores específicos para un grupo taxonómico. Esta amplificación es cuantificada en tiempo real por fluorescencia, de esta manera se puede estimar abundancias (Zhu et al. 2005; Marie et al. 2006). Por otro lado, existe la técnica conocida como FISH

(Fluorescent In Situ Hibrydization) (DeLong et al. 1989), que consiste en la hibridización de una sonda de oligonucleótidos marcada con un fluorocromo con el ARNr de células fijadas (Simon et al. 2000), lo que permite visualizarlas por microscopía de epifluorescencia (DeLong et al. 1989; Massana et al. 2002) o por citometría de flujo (Amann et al. 1990; Simon et al. 1995).

Actualmente también se cuenta con datos genómicos de algunas especies picoeucariontes. Tal vez por la mayor frecuencia de estudios en aguas costeras, el objetivo de estos estudios han sido principalmente géneros picofitoplanctónicas típicos de estas áreas: *Ostreococcus* (Derelle et al. 2006), *Micromonas* (Worden et al. 2009) y *Bathycoccus* (Moreau et al. 2012). Estudios de este tipo han permitido entender un poco más la ecología de estos organismos, a través de sus capacidades fisiológicas.

1.2. *Ecología y diversidad*

El término picoplancton incluye un heterogéneo grupo de organismos, tanto eucariontes como procariontes, sin embargo, su pequeño tamaño hace que tengan algunas implicancias ecológicas comunes. El picoplancton vive con bajísimos números de Reynolds (Re), es decir, su movimiento está dominado por fuerzas viscosas muy por sobre fuerzas inerciales o turbulentas (Mann & Lazier 2006). Esto significa que el picoplancton no se hunde por sí solo en la columna de agua a no ser que esté aglomerado formando partículas más grandes (nieve marina, pellet fecales, etc.) y que el transporte de nutrientes y desechos en la cercanía de la célula dependan, ineficientemente, exclusivamente de la difusión molecular. Esto eventualmente tiene consecuencias evolutivas comunes en los distintos organismos picoplanctónicos en términos energéticos, estructurales y/o de comportamiento. Debemos considerar también que el pequeño tamaño celular hace que tengan en común la simplificación de estructuras celulares internas, el empaquetamiento de los pigmentos fotosintéticos y la relación superficie/volúmen celular, teniendo una alta superficie expuesta en relación a su tamaño, lo que también tiene un impacto en la absorción de nutrientes (Raven 1998). Todos estos factores son fundamentales para el éxito de los organismos picoplanctónicos en determinados ambientes marinos, en particular el picofitoplancton en ambientes oligotróficos,

y para comprender su rol en la dinámica ecológica del océano, incluyendo tramas tróficas y ciclos biogeoquímicos.

A pesar de las características comunes que pueda tener el picoplancton marino, se trata de un grupo muy heterogéneo, en particular los picoeucariontes, que probablemente sean tan o más diversos filogenéticamente que su contraparte procarionte (De Vargas et al. 2015). En términos amplios, las células eucariontes pueden clasificarse en fotótrofas y heterótrofas, o dicho de otra manera, productores y consumidores. Sin embargo, existe una mayor diversidad funcional que hace a esta clasificación ecológicamente más compleja. Hay una gran variedad de organismos que son mixótrofos, es decir, que tienen la capacidad inherente de realizar fotosíntesis y predación por fagocitosis (Tittel et al. 2003) e incluso unos pocos pueden fotosintetizar conservando el plastidio de sus presas (cleptoplastía) (Stoecker et al. 2009). La heterotrofía se manifiesta en variados estilos de vida, más allá de la predación por fagocitosis. Si bien la predación es un proceso fundamental que ha sido ampliamente estudiado y se estima que más del 60% de la producción fitoplanctónica es consumida por protistas (Schmoker et al. 2013), existen otras estrategias de nutrición heterótrofa en eucariontes unicelulares que han sido desestimadas. Existen protistas saprófitos (o descomponedores), es decir, que realizan digestión extracelular de materia orgánica para luego alimentarse de monómeros por osmotrofía y, por otro lado, también existen protistas que establecen relaciones simbióticas o parasíticas con otras especies, unicelulares (eucariontes o procariontes) o multicelulares (Worden et al. 2015). Todas estas estrategias de vida implican un rol funcional distinto en el medio ambiente, de ahí la importancia de no tratar a los picoeucariontes como un grupo homogéneo de organismos y poner esfuerzos en dilucidar la diversidad y la abundancia de los distintos grupos taxonómicos presentes en un ambiente determinado.

Aproximadamente el 50% de la producción primaria global es llevada a cabo en los océanos. El fitoplancton unicelular eucarionte es el principal productor primario en el océano y, por tanto, sustenta la vida en este medio y es fundamental en el ciclo del carbono y en la exportación de CO_2 a océano profundo. La estructura de las poblaciones fitoplanctónicas depende principalmente de la disponibilidad de nutrientes, mientras más eutrófico es un sistema, tiende a dominar el fitoplancton de mayor tamaño, representado principalmente por

diatomeas (Vaulot 2001), y las poblaciones picofitoplanctónicas se presentan en bajas concentraciones (Partensky et al. 1996), contribuyendo con un bajo porcentaje a la productividad primaria. Sin embargo, en las vastas áreas representadas por sistemas oceánicos oligotróficos el picofitoplancton es abundante y responsable del grueso de la producción primaria (Campbell et al. 1994; Li 1994). Los picofitoeucariontes, con abundancias que van entre los 10^2-10^4 células/ml en distintas áreas del océano superficial, son usualmente menos abundantes que las cianobacterias hasta en un orden de magnitud, pero contribuyen con una mayor biomasa en términos de carbono y una alta productividad primaria, esto debido a su mayor tamaño celular, su mayor contenido de clorofila a y/o sus mayores tasas de crecimiento relativos (Li 1994; Worden et al. 2004; Grob et al. 2007b). En sistemas más eutróficos, como los las áreas costeras, los picofitoeucariontes constituyen una fuente de carbono importante para organismos que ejercen pastoreo sobre esta fracción de tamaño, como sería la comunidad protista heterótrofa, convirtiéndose en un componente esencial de la red trófica microbiana y contribuyendo al flujo de carbono a niveles tróficos mayores (Worden et al. 2004; Bec et al. 2005). Por otro lado, a pesar que por mucho tiempo se ha asumido que el picoplancton autótrofo no tiene un rol importante en la producción de peces y en el secuestro de carbono, hay estudios que señalan que estos organismos aportarían carbono significativamente al mesozooplancton y al flujo de materia orgánica particulada al océano profundo (Richardson & Jackson 2007; Barber 2007).

En el estudio de picofitoeucariontes tanto las aproximaciones moleculares como los análisis de pigmentos fotosintéticos coinciden en que estas comunidades están representadas principalmente por Estramenópilos (particularmente Chrysophyceae y Pelagophyceae), Haptófitos (particularmente Prymnesiophyceae) y Clorófitos (particularmente Mamiellophyceae y otros prasinófitos) (Andersen et al. 1993; Letelier et al. 1993; Shi et al. 2009 y 2011). Pico-primnesiofíceos dominan las comunidades en sistemas oceánicos (Cuvelier et al. 2010; Jardillier et al. 2010), mientras que la clase Mamiellophyceae y sus 3 géneros: *Micromonas*, *Ostreococcus* y *Bathycoccus*, son dominantes en sistemas costeros (Biegala et al. 2003; Not et al. 2004; Zhu et al. 2005). Se ha observado que un género en particular puede dominar la comunidad picoeucarionte, como es el caso de *Micromonas* (Not et al. 2004), también se ha visto que éstos géneros no coexisten (Viprey et al. 2008), lo que

además podría sugerir que sus nichos ecológicos son diferentes. El género *Micromonas* parece estar mejor adaptado a ambientes más ricos en nutrientes, a diferencia de *Ostreococcus*, que parece ser más importante en condiciones intermedias de nutrientes (Viprey et al. 2008). Sin embargo, los ambientes costeros son muy variables entre sí, ya sea por su ubicación (ej., latitud, borde oriental u occidental del océano), por las características de circulación o por la influencia de ríos, entre otros; y son limitados las áreas costeras donde se ha estudiado la comunidad picoeucarionte autótrofa. Se han establecido distintos linajes y ecotipos dentro de los principales géneros mencionados (Guillou et al. 2004, Foulon et al. 2008), por lo tanto, es necesario cubrir mayor cantidad de ambientes para conocer su distribución, su dinámica y su rol en los ecosistemas.

La mayoría de las secuencias aisladas, tanto de la zona fótica como de la zona afótica, se asocia filogenéticamente a grupos heterótrofos, cuya enorme diversidad es cada vez más evidente (De Vargas et al. 2015). Esta insospechada diversidad abre también una amplia gama de posibilidades de comportamiento trófico, haciendo más complejas las conecciones en las redes tróficas pelágicas. Existen muy pocas especies heterótrofas en cultivo en el rango de tamaño del picoplancton (Guillou et al. 1999; Vaulot et al. 2004), por lo que ha sido muy difícil entender el rol de estos organismos en los ecosistemas marinos. La mayor cantidad de secuencias ambientales heterótrofas corresponden a Alveolata (Adl et al. 2012), en particular a MALV (MArine ALveolates), organismos parásitos de diferentes especies marinas (Dolven et al. 2007, Guillou et al. 2008, Not et al. 2009). Otro grupo que parece ser importante pertenece a Stramenopila (Adl et al. 2012) y se trata de los denominados MAST (Marine STramenopiles) (Not et al. 2009, Massana 2011), cuya amplia distribución y activa bacterivoría ha sido comprobada mediante experimentos combinados con FISH (Massana et al. 2006 y 2009). Sin embargo, existen muchos linajes heterótrofos cuyo rol ecológico es aun más desconocido, sobre todo en aguas bajo la capa fótica, donde los esfuerzos de muestreo han sido limitados.

1.3. *Picoeucariontes en la zona de surgencia costera estacional frente a Chile central*

En el Pacífico Sur Oriental (PSO) se desarrolla un sistema de surgencia costera asociado al Sistema de Corriente de Humboldt (SCH) que hace de las costas de Perú y Chile una de las áreas más productivas y donde se desarrolla una de las pesquerías pelágicas más importantes a nivel global. Vientos favorables a la surgencia (vientos S-SW) generan que aguas ecuatoriales subsuperficiales ricas en nutrientes y pobres en oxígeno alcancen la capa fótica, incrementando la producción primaria. En las costas de Chile Central (30º-40ºS) estos vientos son altamente estacionales y se intensifican durante el período de primavera-verano australes, cuando el Anticiclón del Pacífico se desplaza hacia el sur (Arcos & Navarro 1986; Shaffer et al. 1999; Sobarzo et al. 2007). La estacionalidad de los vientos favorables a surgencia tiene como consecuencia que el sistema pueda ser caracterizado por dos situaciones típicas: periodo de surgencia activa (primavera-verano) y periodo de relajación o no-surgencia (otoño-invierno). Cuando la surgencia está activa existe ingreso de nutrientes (ej. NO_3) a la capa fótica, lo que, sumado a la mayor cantidad de luz entre septiembre y marzo, genera una alta producción primaria (clorofila sobre 20 mg Chl m^{-3}). En contraste, aproximadamente entre abril y agosto el sistema está en un período de no-surgencia menos productivo (clorofila menor a 5 mg Chl m^{-3}) (Daneri et al. 2000; Yuras et al. 2005; Vargas et al. 2007) (Fig. 1.2). En periodo de surgencia la producción y la biomasa fitoplanctónica está dominada por la fracción > 20 μm, representada principalmente por diatomeas de los géneros *Skeletonema*, *Chaetoceros* y *Thalassiosira* (González et al. 2007); mientras que el resto del año el responsable de la producción es el fitoplancton de menor tamaño (< 20 μm) (Vargas et al. 2007). A nivel anual, el picofitoplancton (< 3 μm) puede llegar a representar hasta un 25% del carbono orgánico particulado y más del 50% de esta biomasa está representada por eucariontes, a pesar de ser menos abundantes que *Synechococcus* (en general, un orden de magnitud menor). El picofitoplancton eucarionte presenta máximas abundancias en otoño-invierno (10^4 células ml^{-1}) y mínimas en primavera-verano (10^2 células ml^{-1}) (Tesis Gadiel Alarcón) (Fig. 1.2), sin embargo, la estructura comunitaria de este grupo y su variación en composición a lo largo del ciclo anual previa a esta tesis doctoral no ha sido establecida para esta zona.

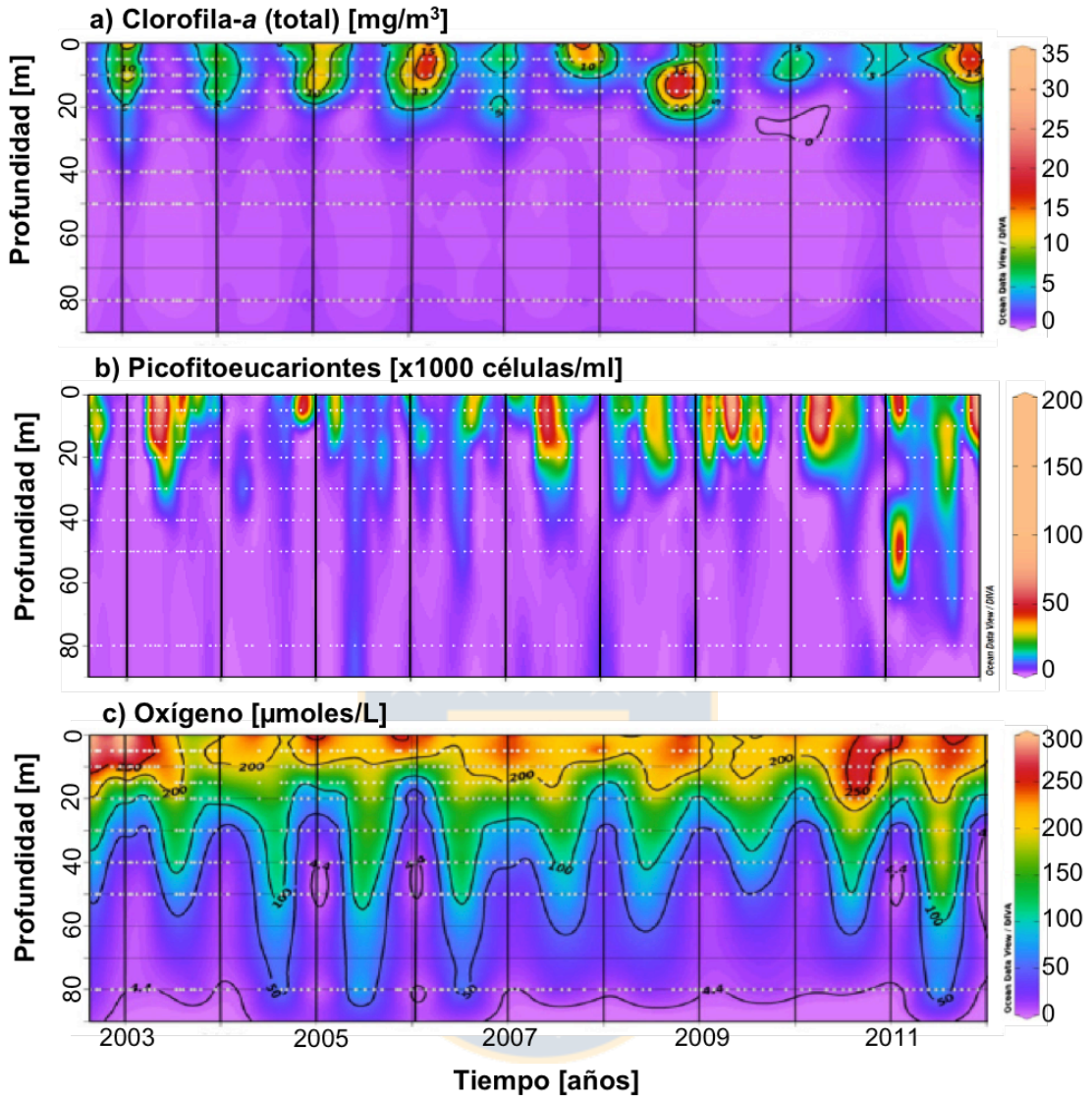


Figura 1.2. Variabilidad temporal de parámetros en la columna de agua de la estación 18, frente a la bahía de Concepción, Chile Central (serie de tiempo COPAS). Los parámetros que se muestran son: concentración de clorofila-*a* (total) (a), abundancia de picofitoeucariontes (b) y concentración de oxígeno (c). Los paneles a) y c) son modificaciones de figuras de Farías et al. (2015) y el panel b) es gentileza del Dr. Carlos Henríquez (no publicado).

Por otro lado, comúnmente relacionadas con sistemas de surgencia de borde oriental se presentan aguas con bajas concentraciones de oxígeno disuelto a nivel subsuperficial (< 20 µmoles L^{-1}) (Zonas de Mínimo Oxígeno, ZMOs), originadas en general por la respiración de la gran cantidad de material orgánico que se genera y la poca ventilación, dejando las aguas de profundidad media muy deficientes en oxígeno (Helly & Levin 2004). Frente a Chile Central, estas condiciones de hipoxia se relacionan directamente con los eventos estacionales de

surgencia, cuando el flujo de materia orgánica a la capa más profunda aumenta (Fig. 1.2). El desarrollo de una ZMO tiene implicancias biogeoquímicas muy importantes a nivel global, ya que cuando estas condiciones se generan, se presentan procesos anaeróbicos como desnitrificación, que conlleva a la producción de N_2O (óxido nitroso) (Cornejo et al. 2006), un poderoso gas invernadero. Esto se suma a la implicancia de estos ambientes en el ciclo del carbono, ya que las áreas de surgencia representan en general lugares donde el océano puede estar liberando dióxido de carbono (CO_2) debido al afloramiento de aguas más profundas enriquecidas en este compuesto, considerado también un poderoso gas invernadero. En general, los procesos biogeoquímicos clave que existen en las ZMO son llevados a cabo por bacterias y archaeas, sin embargo, los eucariontes microbianos heterótrofos controlan a su contraparte procarionte a través de la predación. Existen escasos reportes de picoeucariontes en ZMOs (Ganesh et al. 2014, Parris et al. 2014, Rocke et al. 2013), y lo particular de la ZMO que se desarrolla frente a Chile Central es que podría albergar una comunidad única, dadas las condiciones variables de oxígeno disuelto en un ciclo anual, desde condiciones bien oxigenadas durante los meses invernales a condiciones hipóxicas durante los meses estivales. El análisis de la variación en la estructura comunitaria entre ambas condiciones contrastantes puede entregar mucha información acerca del efecto que tiene el oxígeno sobre algunos grupos eucariontes. El conocimiento de la estructura y función de las comunidades microbianas que habitan el sistema de surgencia asociado al PSO es fundamental para inferir y predecir la dinámica del ecosistema en este ambiente tan importante y único a nivel global en términos de productividad y de ciclos biogeoquímicos. La comunidad picoeucarionte en las costas frente a Chile Central puede ser tan variable como las condiciones del sistema, por lo tanto, el estudio de la estructura comunitaria de estos organismos y su variación en el tiempo puede ayudarnos a comprender la ecología pelágica y los flujos de carbono en esta área. Hasta hoy, no existen reportes acerca de la diversidad, distribución y abundancia de organismos picoeucariontes en esta zona de estudio.

2.- HIPÓTESIS Y OBJETIVOS ESPECÍFICOS

- Hipótesis

Hipótesis 1. En el estrato superficial de la columna de agua (zona fótica), la comunidad eucarionte está dominada por miembros de Chloroplastida, específicamente Mamiellophyceae, y existe un cambio estacional en su estructura, tanto en abundancias como en especies dominantes.

Hipótesis 2. En el estrato profundo de la columna de agua (zona afótica), la comunidad picoeucarionte está dominada por grupos heterótrofos y varía estacionalmente en relación a los niveles de oxígeno disuelto.

- Objetivos específicos

Objetivo 1. Determinar la diversidad genética de la comunidad picoeucarionte autótrofa que habita en la plataforma continental frente a Chile Central y su variación estacional en la zona fótica.

Objetivo 2. Estimar abundancias absolutas de los principales grupos filogenéticos que conforman el picofitoplancton eucarionte en la zona de estudio, identificando grupos dominantes.

Objetivo 3. Determinar la diversidad genética de la comunidad picoeucarionte heterótrofa que habita en la plataforma continental frente a Chile Central y su variación estacional en la zona afótica.

Objetivo 3. Determinar las variables oceanográficas y biológicas que mejor explican la variación estacional de la estructura comunitaria de los grupos picoeucariontes.

3.- MATERIALES Y MÉTODOS

3.1. *Obtención de muestras*

Las hipótesis planteadas para la realización de esta tesis serán contrastadas en una zona de surgencia estacional frente a Chile Central, específicamente frente a la Bahía de Concepción. El punto de muestreo corresponde a la Estación 18 (36º30,8'S y 73º07,7'W), un punto fijo de muestreo mensual en el marco de una serie de tiempo que lleva a cabo el centro COPAS, Universidad de Concepción. La Estación 18 se encuentra sobre la plataforma continental, aproximadamente a 10 km costa afuera desde la localidad de Dichato, teniendo una columna de agua de aproximadamente 94 m de profundidad.

El muestreo de la columna de agua se realizó a bordo de las lanchas científicas "Kay-Kay" y "Kay-Kay II" (Universidad de Concepción) mediante la utilización de botellas Niskin de 10 L asociadas a una roseta, el agua obtenida fue filtrada por una malla de 20 µm. Para el estudio de la capa fótica (objetivos 1 y 2 específicamente) se utilizaron muestras de agua superficial (0 y 5 m) con una frecuencia casi mensual desde junio del 2006 hasta octubre del 2008. Para el estudio de la capa afótica (objetivo 3 específicamente) se utilizaron muestras de agua de 50 m de profundidad de los meses de enero, abril, julio y octubre del año 2010. Siempre asociado a los muestreos se obtuvieron datos hidrográficos (temperatura, salinidad, fluorescencia y oxígeno) con un equipo CTDO Seabird. Datos de concentración de macronutrientes y clorofila-a también están considerados en la serie de tiempo, fueron obtenidos a través de métodos standard y fueron utilizados en esta tesis.

3.2. *Diversidad genética*

El estudio de la diversidad genética picoeucarionte en la zona de estudio se llevó a cabo mediante el análisis de las secuencias que codifican la subunidad pequeña (SSU) de los ribosomas. Esta molécula tiene regiones altamente conservadas, es decir, que pueden ser encontradas en todos los organismos vivos, y regiones más variables, que se encontrarían sólo en organismos relacionados filogenéticamente (Moyer 2001). Aplicado a muestras

ambientales, el estudio del gen que codifica el ARNr permite tener una aproximación a la biodiversidad. En el caso de las células eucariontes, el ARNr que se utiliza es el 18S. La obtención de la secuencia que codifica el ARNr 18S y su posterior alineación y comparación con una base de datos permite dilucidar la estructura y la diversidad de las comunidades naturales. La metodología utilizada para el análisis del ARNr 18S implica extracción del ADN de una muestra ambiental y la posterior amplificación del gen de interés mediante una reacción en cadena de la polimerasa (PCR) (Moyer 2001). Una vez que se obtiene el gen amplificado, es posible clonarlo y obtener unidades taxonómicas operacionales (OTU, Operacional Taxonomic Unit), que son secuencias ambientales que por cierto porcentaje de similitud pueden ser asignadas a un mismo grupo filogenético (López-García et al. 2001; Moon-van der Staay et al. 2001). En la Figura 3.1 se muestra el procedimiento con el que se logró el análisis de la diversidad genética de las muestras, los detalles de las condiciones y protocolos están descritos en las publicaciones correspondientes.

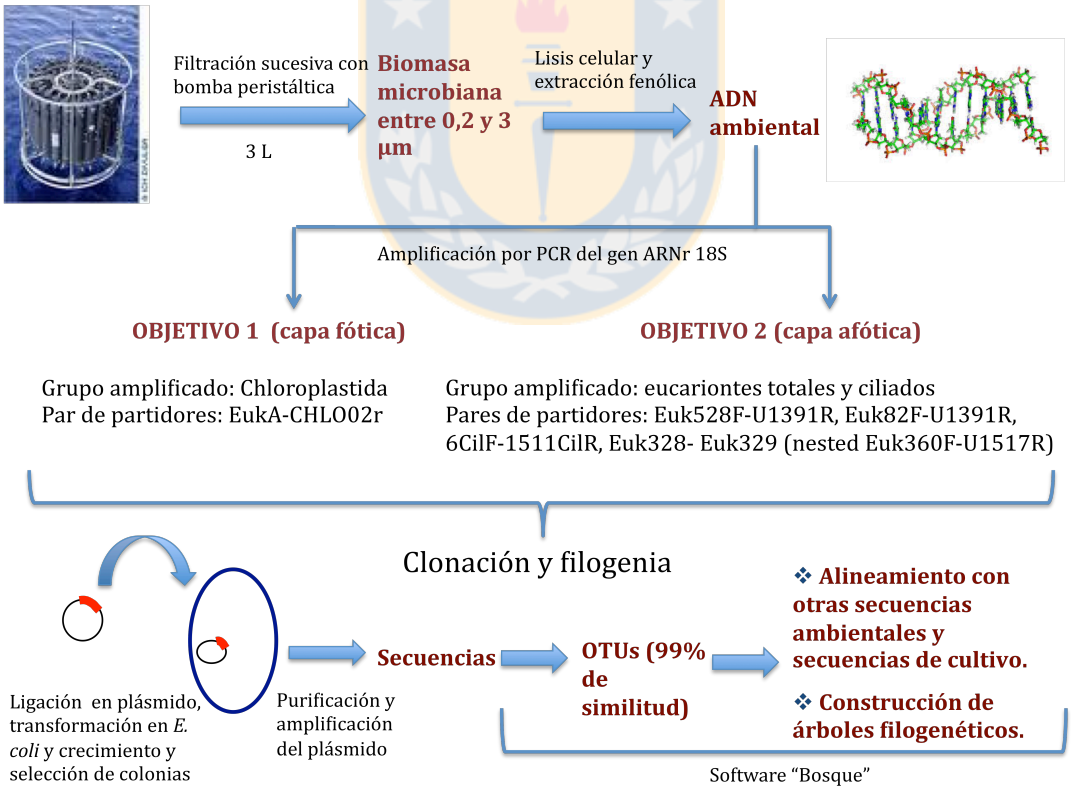


Figura 3.1. Esquema que describe la obtención de ácidos nucléicos, la amplificación del gen ARNr 18S y la clonación y análisis filogenético de las muestras obtenidas en esta tesis. Fuente: Elaboración propia.

3.3. *Hibridación fluorescente in situ (FISH)*

Para llevar a cabo el objetivo 3 de esta tesis se estimaron abundancias absolutas a través del uso de la técnica FISH, acoplada con la amplificación de la señal por tiramida (TSA-FISH). La técnica se llevó a cabo según lo descrito por Not et al. 2002 y consiste básicamente en la utilización de sondas de oligonucleótidos que puedan hibridar o unirse a ARNr de ciertos grupos filogenéticos. La secuencia de la sonda le confiere la especificidad, de esta manera, de acuerdo a la sonda utilizada, se puede marcar desde especies hasta clases, ordenes o incluso dominios (Amann & Fuchs 2008). Lo interesante de esta técnica es que permite contar directamente (*in situ*) de células de organismos que usualmente no son distinguibles bajo microscopía tradicional. En la figura 3.2 se muestra el procedimiento y las sondas utilizadas en esta tesis, detalles del protocolo y de las condiciones están descritos en la publicación correspondiente.

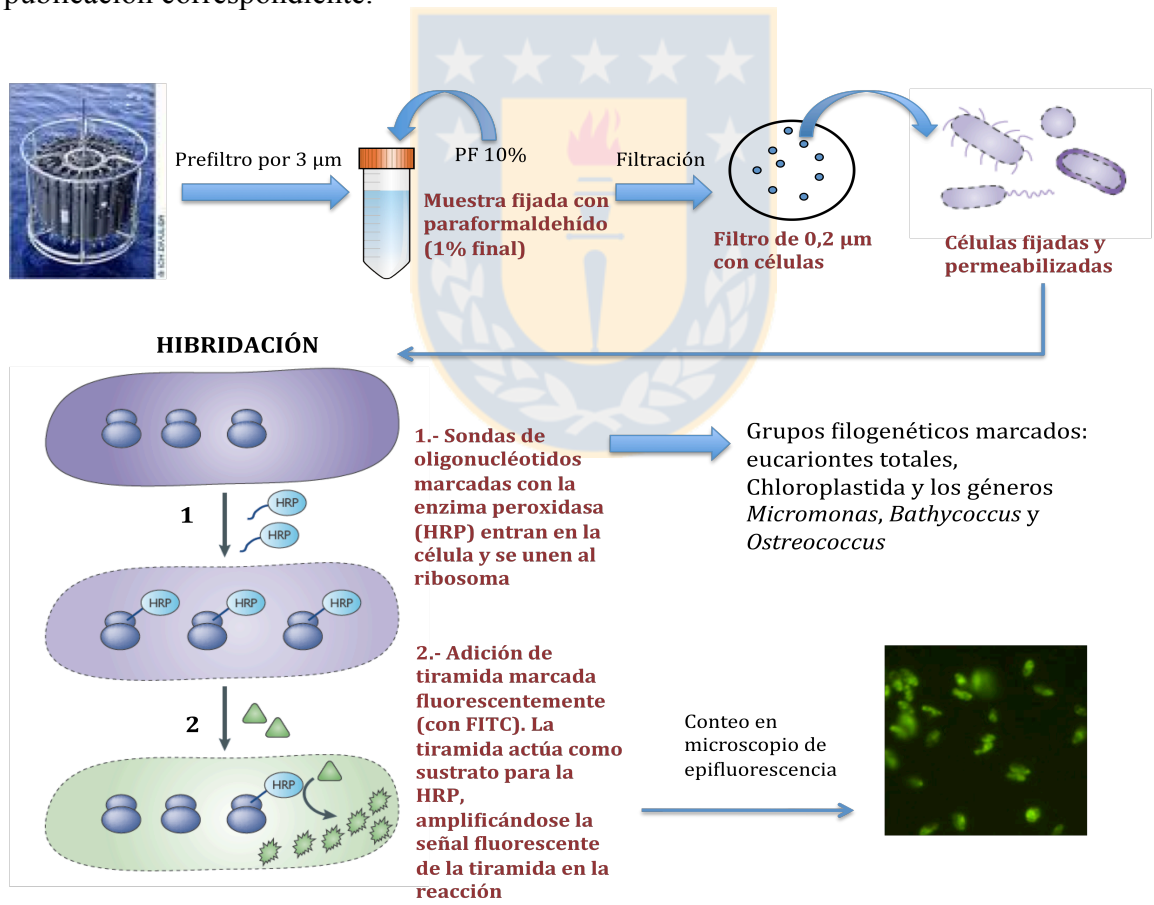


Figura 3.2. Esquema que describe la técnica de hibridación fluorescente *in situ* utilizada en esta tesis. Algunas imágenes fueron extraídas de Amann & Fuch (2008). Fuente: Elaboración propia.

3.4. *Citometría de flujo*

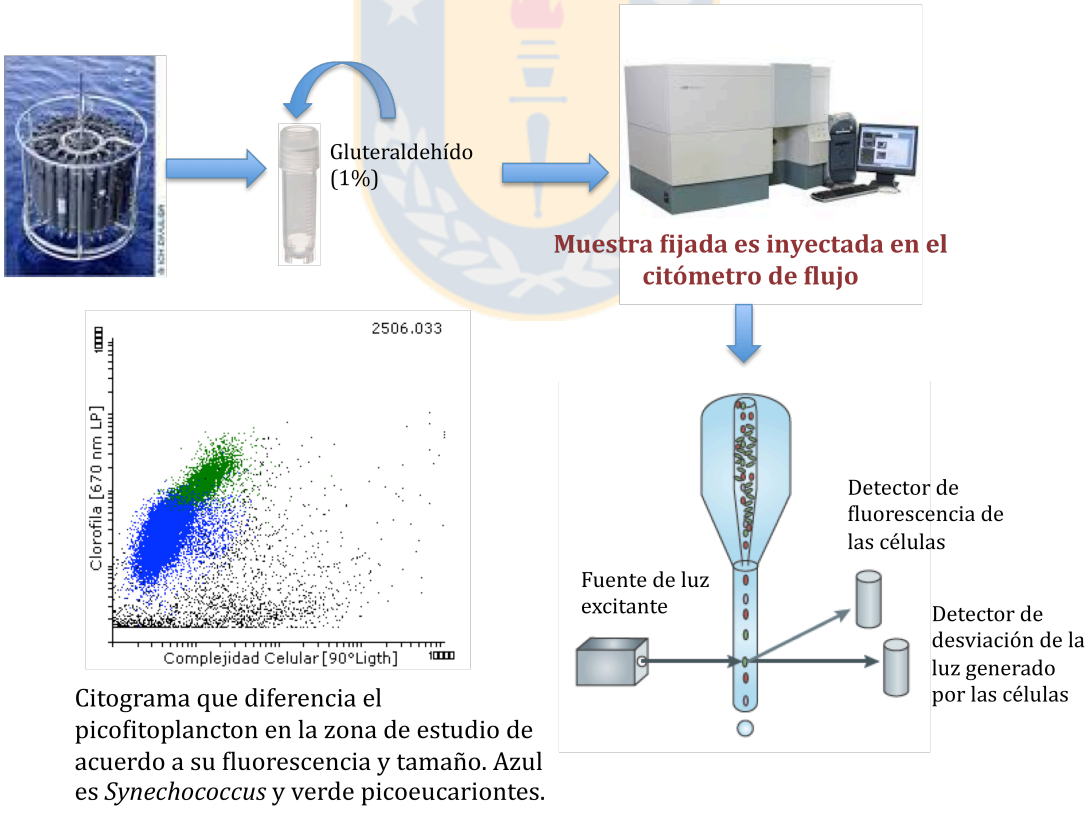
Un citómetro de flujo FACSCalibur equipado con un laser de Argón de 15 mW de potencia y excitación a 488 nm (Becton Dickinson) fue utilizado para estimar abundancias de picofitoplancton, en este caso picofitoeucariontes y *Synechococcus* (*Prochlorococcus* no ha sido detectado en el area de estudio). En la figura 3.3 se observa el tratamiento básico de la muestra antes de ser inyectada en el citómetro y el procesamiento de la misma por el equipo. El citómetro de flujo mide células alineadas en una corriente de fluido muy delgada a la que se enfocan haces de luz, la forma en que esta luz es desviada entrega información acerca del tamaño y la forma de la célula, y la fluorescencia que emite la célula es detectada y convertida en una señal procesada por un computador (Figura 3.3). De esta manera, la citometría de flujo permite contar células y clasificarlas de acuerdo a su fluorescencia (natural o adquirida). En este caso, la concentración de células picofitoplanctónicas por ml se calculó utilizando el número de eventos (conteos) estimados con el programa CytoWin. La diferenciación de las células se realizó por la fluorescencia de sus pigmentos fotosintéticos (propiedades ópticas) (figura 3.3). Picofitoeucariontes fueron discriminados de las cianobacterias porque los primeros emiten solo autofluorescencia roja (clorofila), mientras que las segundas también emiten fluorescencia naranja (ficoeritrina) y tienen un tamaño aproximado de 1 μm (Marie et al. 2005). Por otro lado, se estimó la abundancia de bacterioplancton total, que no es autofluorescente, por lo que se utilizó una tinción molecular (SYBRR Green I), que permitió separarlo de los otros grupos por su emisión en el color verde (Li 1995). La desviación de la luz y la fluorescencia fueron normalizadas agregando beads fluorescentes de 1 μm.

3.5. *Conversión de carbono*

Para estimar la importancia en términos de biomasa de carbono de los picofitoeucariontes en comparación a su contraparte cianobacteriana (*Synechococcus*), se calculó el contenido promedio intracelular de los 2 grupos a partir de los datos de citometría de flujo. Para esto, se usó una relación log-log establecida por Grob et al. (2007a) entre la señal “forward scatter” (FSC, relacionada con el tamaño de la célula y normalizada a beads de referencia) y el contenido de carbono intracelular (C) a través de la siguiente ecuación:

$C=2.65x(FSC)^2+3.27xFSC+2.58$. El contenido intracelular de carbono para cada grupo picofitoplanctónico fue multiplicado por el número de células estimadas por citometría de flujo.

Para estimar la contribución relativa de cada uno de los géneros detectados por TSA-FISH (*Ostreococcus*, *Bathycoccus* y *Micromonas*) a la biomasa picofitoeucarinte se utilizó un factor de conversion de 237 fg C μm^{-3}, que fue propuesto por Worden et al. (2004) basado en mediciones de carbono-hidrógeno-nitrógeno (CHN) de fitoplancton de cultivo, incluyendo a especies como *Micromonas pusilla* y *Ostreococcus lucimarinus*. Este factor de conversion fue multiplicado por la abundancia obtenido por TSA-FISH y el volumen de especies (o cepas) en cultivo de cada género, calculados a partir de las longitudes celulares obtenidas de literatura: 0.95 µm para *Ostreococcus lucimarinus* (cepa CCE 9901, Worden et al. 2004), 2 µm para *Bathycoccus prasinos* y 2 µm para *Micromonas pusilla* (Vaulot et al. 2004).



Citograma que diferencia el picofitoplancton en la zona de estudio de acuerdo a su fluorescencia y tamaño. Azul es *Synechococcus* y verde picoeucariontes.

Figura 3.3. Esquema que muestra el procesamiento de una muestra para análisis citométrico y el funcionamiento básico del equipo que la analiza. Fuente: Elaboración propia.

4. RESULTADOS

4.1. Estructura y dinámica estacional de la comunidad de picofitoplancton eucarionte en un ecosistema de surgencia costera

Limnology and Oceanography, 56(6): 2334-2346, 2011.

Resumen

Estudiamos la estructura y la dinámica estacional de la comunidad fotosintética picoeucarionte en una zona costera frente a Chile Central fuertemente influenciada por una surgencia estacional impulsada por viento. Determinamos la diversidad filogenética eucarionte mediante clonación y secuenciación del gen de ácido ribonucléico ribosomal (ARNr) 18S, amplificado con partidores para Chloroplastida. La representatividad de Chloroplastida en las librerías de clones fue 100% en otoño, 61% en primavera y 20% en verano. La mayoría de las secuencias fueron de Mamiellophyceae, agrupándose con *Micromonas* (clados A.BC.1, B.E.3, C.D.5), *Ostreococcus* (clado A) y *Crustomastix* (clado A). Hibridación fluorescente in situ acoplada con amplificación de la señal con tiramida (TSA-FISH) se usó para determinar la variación de la abundancia en el tiempo de la comunidad total picoeucarionte, de Chloroplastida, y de los géneros *Micromonas*, *Ostreococcus* y *Bathycoccus* durante un periodo de 2 años y una frecuencia casi mensual. La abundancia de picoeucariontes fotosintéticos fue baja durante la temporada de surgencia (primavera y verano) y alta durante la temporada de no-surgencia (fines del otoño e invierno). Chloroplastida representó, en promedio, el 74% de la comunidad picoeucarionte, y la suma de los 3 géneros estudiados representó el 100% de la abundancia de Chloroplastida. *Ostreococcus* dominó numéricamente la comunidad picofitoplanctónica a lo largo del año y aparece como un componente clave la comunidad picoplanctónica del área de surgencia del Pacífico Sur oriental.

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Structure and seasonal dynamics of the eukaryotic picophytoplankton community in a wind-driven coastal upwelling ecosystem

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Abstract

We studied the structure and seasonal dynamics of the photosynthetic picoeukaryote community at a coastal site off central Chile that is strongly affected by seasonal wind-driven upwelling. We determined the picoeukaryote phylogenetic diversity by cloning and sequencing the 18S ribosomal ribonucleic acid (rRNA) gene amplified with Chloroplastida-biased primers. Chloroplastida made up 100% (autumn), 61% (spring), and 20% (summer) of the clone libraries. Most sequences belonged to Mamiellophyceae and clustered with *Micromonas* (clades A.BC.1, B.E.3, C.D.5), *Ostreococcus* (clade A), and *Crustomastix* (clade A). Fluorescent in situ hybridization coupled with tyramide signal amplification (FISH–TSA) was used to determine the time variability in abundance of the total picoeukaryotic community, the Chloroplastida, and the genera *Micromonas*, *Ostreococcus*, and *Bathycoccus* over a period of 2 yr with nearly monthly sampling. The abundance of photosynthetic picoeukaryotes was low during the upwelling season (spring and summer) and high during the nonupwelling season (late fall and winter). Chloroplastida made up, on average, 74% of the total picoeukaryotic community, and the sum of the three genera probed accounted for 100% of the Chloroplastida abundances. *Ostreococcus* dominated the picophytoplanktonic community numerically throughout the year and, thus, appears to be a key component of the upwelling picoplanktonic community in the eastern South Pacific.

Size is often used to classify the organisms that inhabit the water column. Picoplankton ($\leq$ 2–3 μm in diameter) comprises the three major domains of life: Archaea, Bacteria, and Eukarya. Photosynthetic marine picoplankton includes both cyanobacteria, represented mainly by two genera (*Synechococcus* and *Prochlorococcus*), and photosynthetic picoeukaryotes (PPEs), that can belong to a wide range of algal classes (Vaulot et al. 2008). Elucidating the diversity of PPEs started just over a decade ago with the application of molecular techniques such as 18S ribosomal ribonucleic acid (rRNA) gene cloning and sequencing (Moon-van der Staay et al. 2001).

Despite the evidence that PPEs can contribute significantly to carbon biomass in the picoplanktonic fraction (up to 75%, Worden et al. 2004), PPEs have been much less extensively studied than their cyanobacterial counterparts, and their ecological and biogeochemical roles are still poorly understood. This is particularly true in productive coastal waters, where large phytoplankton dominates in terms of biomass and productivity, but where PPEs can represent an important carbon source for the heterotrophic protist community that graze on the small size fractions (Bec et al. 2005). The large carbon biomass of PPE is due to their large cell size in comparison with the numerically abundant cyanobacteria (Worden et al. 2004). Thus, PPEs may contribute significantly to the carbon flux through the microbial web in coastal systems (Worden et al. 2004; Bec et al. 2005), especially when larger phytoplankton (nanoplankton and microplankton) is not blooming (Vargas et al. 2007). Furthermore, Richardson and Jackson (2007) have proposed that photosynthetic picoplankton can also play a major role in the carbon export to the deeper ocean, even in eutrophic areas.

Pigment and molecular analyses are important tools to study the presence and abundance of certain groups in the eukaryotic fraction of the picoplankton. So far, studies have found that prasinophytes (Chloroplastida) dominate in coastal waters (Not et al. 2005; Worden 2006), mainly because of the presence of three genera from the class Mamiellophyceae: *Micromonas*, *Bathycoccus*, and *Ostreococcus* (Not et al. 2004; Zhu et al. 2005; Worden 2006). However, little is known about the ecology of these organisms (e.g., abundance and distribution), but it has been observed that a single genus can, in some cases, dominate the picoeukaryotic community (Not et al. 2004). *Micromonas*, despite being widely distributed (Slapeta et al. 2006), seems to be better adapted to nutrient-rich conditions (Not et al. 2004, 2005; Viprey et al. 2008), unlike *Ostreococcus*, which seems to be adapted to mesotrophic conditions (Viprey et al. 2008). Although *Ostreococcus* has been detected generally at low abundances in marine waters (Not et al. 2004; Zhu et al. 2005), high abundances have been reported at some coastal sites during short bloom periods (O'Kelly et al. 2003; Countway and Caron 2006). *Ostreococcus* dominates the picophytoplankton community throughout the year in the Thau Lagoon, where it was originally discovered (Courties et al. 1994). On the other hand, while several intrageneric clades have been identified for *Micromonas* (Guillou et al. 2004; Slapeta et al. 2006; Worden 2006), *Ostreococcus* (Guillou et al. 2004),

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and *Crustomastix* (Viprey et al. 2008), *Bathycoccus* seems to be quite homogeneous genetically (Guillou et al. 2004; Viprey et al. 2008). However, coastal environments can be quite distinct with respect to factors such as coastal circulation, land and river influence, or availability of trace metals, and PPEs have been surveyed in only a few coastal systems, mostly in the Northern Hemisphere. Moreover, microbial time-series studies are still scarce, and, with a few exceptions (Not et al. 2004; Countway and Caron 2006), they mostly report on the PPE community as a whole, without taxonomic resolution (Worden et al. 2004; Sherr et al. 2005).

The role of eukaryotic picoplankton in coastal upwelling ecosystems of the eastern South Pacific, one of the most productive marine systems of the world, has not been extensively studied. In the coastal area off central Chile (30°–40°S), wind-driven upwelling of nutrient-rich subsurface waters is highly seasonal. The system shifts from very productive in spring–summer, because of the presence of large chain-forming diatoms (González et al. 2007), to less productive in autumn–winter, when smaller phytoplankton is responsible for the productivity (Vargas et al. 2007). Similar results have been obtained off the Oregon coast, another eastern boundary upwelling ecosystem (Sherr et al. 2005). There, observations over a period of about 2 yr showed that the abundance of PPEs was lower in summer, coinciding with blooms of large diatoms.

The aim of the present work was to determine the diversity and seasonal abundance of the dominant PPE taxa in the coastal upwelling zone off central Chile. To evaluate the genetic diversity of the community, Chloroplastida-biased primers were used to amplify environmental deoxyribonucleic acid (DNA) in order to clone and sequence the 18S rRNA gene, used to identify picophytoeukaryotic taxa. Fluorescent in situ hybridization coupled with tyramide signal amplification (FISH–TSA, Not et al. 2002) was used to quantify three Chloroplastida genera (*Micromonas*, *Bathycoccus*, *Ostreococcus*) using oligonucleotide probes designed in previous work (Not et al. 2004). More general probes were also used in order to target total eukaryotes and total Chloroplastida.

Methods

Study site and hydrographic sampling—Sampling was performed at a fixed time-series station located on the continental shelf ~ 10 km off Dichato, central Chile (Station 18, 36°30.8′S, 73°07.7′W), with a water-column depth of ~ 94 m (Fig. 1). This station is maintained by the Center for Oceanographic Research in the Eastern South Pacific (COPAS), Universidad de Concepcion (UdeC). Water samples were collected (10-liter Niskin bottles) at a nearly monthly frequency from June 2006 to October 2008 (23 sampling dates) on board the R/Vs *Kay-Kay* and *Kay-Kay* II (UdeC). Samples from the surface (0–5 m) were used for the analyses of picoplankton, since most of the time (~ 82%) the chlorophyll *a* maximum was in the upper 10 m. Hydrographic data (temperature and salinity) were obtained using a SeaBird 25 conductivity–temperature–depth–oxygen sensor. Macronutrients and chlorophyll *a*

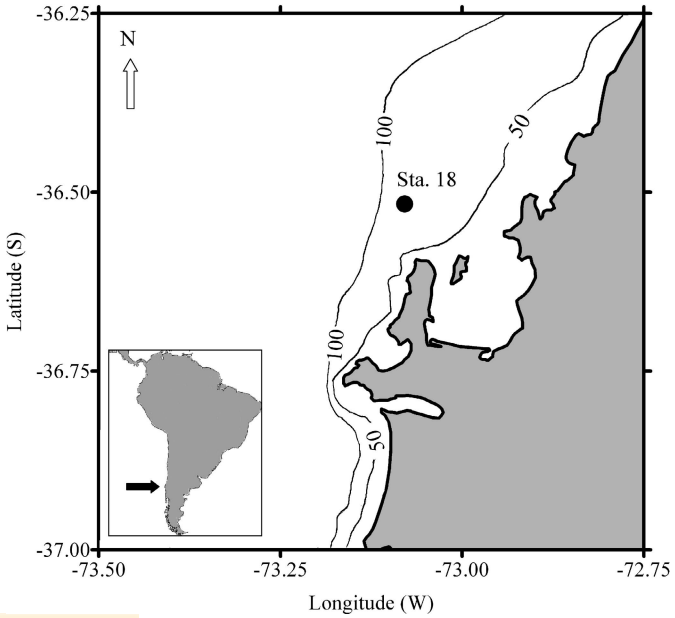

Fig. 1. Location of Station 18 over the shelf in the coastal upwelling area off Concepción (central Chile). Isobaths of 50 m and 100 m are also included.

(total and fractionated through 3-μm filters) were measured using standard methods.

DNA was collected from seawater prefiltered through a 20-μm pore size mesh. With a peristaltic pump, 3 liters of this seawater was filtered through a 3-μm pore size membrane, and microbial biomass was collected on a 47-mm-diameter membrane with a 0.2-μm pore size (Supor 450). The latter was transferred to cryovials and covered with DNA lysis buffer (40 mmol L^{-1} ethylenediaminetetraacetic acid [EDTA], 0.73 mol L^{-1} sucrose, and 50 mmol L^{-1} Tris-HCl pH 8.3), immediately frozen in liquid nitrogen, and stored at −80°C until DNA extraction.

Each sample for FISH–TSA analysis was collected from 45 mL of seawater prefiltered through 3-μm pore size filters and fixed on board with 5 mL of fresh 10% paraformaldehyde for at least 1 h at 4°C. Fixed samples were then filtered onto 25-mm diameter 0.2-μm pore size polycarbonate filters (Millipore) and dehydrated three times with increasing concentrations of ethanol (50%, 80%, and 100%; 2 mL for 3 min each). Filters were stored at −80°C until hybridization.

Samples for flow cytometric analysis were collected from 2 mL of seawater fixed on board with 1% glutaraldehyde and frozen in liquid nitrogen after 10 min at room temperature. Fixed samples were stored at −80°C until analysis.

DNA extraction, polymerase chain reaction (PCR), and cloning—Genetic diversity was assessed on one surface sample per date, on three dates (May, October, and December 2007). May was representative of the autumn–winter condition, when relatively high PPE abundances were found. October and December represented the austral spring and summer conditions, respectively, with relatively low PPE abundances. To obtain the genomic DNA of the

picoplanktonic community, cell lysis was performed by adding lysozyme (1 mg mL^{-1}), proteinase K (0.5 mg mL^{-1}), and sodium dodecyl sulfate (SDS) (1%) to the filters. DNA from the lysate was purified with phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and chloroform–isoamyl alcohol (24 : 1) extractions. It was then precipitated by adding sodium acetate (7.5 M) and isopropanol (0.4 and 1 volume, respectively) and then resuspended in 50 μL of ultrapure DNA- and ribonuclease-free water. Integrity was checked on an agarose gel. DNA was stored at $-20^{\circ}C$ until amplification.

The partial 18S rRNA gene was amplified using the primers EukA (Medlin et al. 1988) and CHLO02r (Zhu et al. 2005), targeting Chloroplastida. This target group was chosen based on preliminary FISH–TSA results demonstrating its importance at the study site (*see* below). The PCR mixture (25 μL final volume) contained $\sim$ 20 ng of environmental DNA template, 200 μmol L^{-1} of each deoxynucleoside triphosphate, 1.5 mmol L^{-1} $MgCl_2$, 0.3 mmol L^{-1} of each primer, 2.5 U of *Taq* DNA polymerase (GoTaq, Promega), and the PCR buffer (1×) supplied with the enzyme. PCR conditions were as follows: initial denaturation at 94°C for 130 s, 30 cycles of denaturation (at 94°C for 30 s), annealing (at 56°C for 45 s), extension (at 72°C for 130 s), and a final extension step (at 72°C for 7 min). PCR products of about 950 base pairs (bp) were purified and visualized after electrophoresis on an agarose gel (1%). Amplified products of three reactions were pooled and concentrated in a volume of 30-μL sterile water. An aliquot of each pooled PCR product was cloned using the kit pGem easy vector (Promega), following the manufacturer-recommended protocol. Positive colonies were selected and grown in multiwell plates with Luria–Bertani medium and glycerol (7%). PCR reamplification was performed with the primers M13F and M13R, using 1 μL of culture as template, to check for the presence of the 18S rRNA gene in the plasmids. Amplified products were visualized using agarose gel electrophoresis.

Sequence analysis—Clones with inserts were sequenced by the Macrogen Inc. sequencing service (Korea). Removal of vector and primer sequences, as well as quality and orientation analyses, were performed using Seqman software (Lasergene 7). Sequences were aligned using the Bosque software (Ramírez-Flandes and Ulloa 2008). The percentage of similarity between sequences was calculated, and sequences with similarities higher than 99.5% were considered to belong to the same operational taxonomic unit (OTU), which corresponds to a species or infraspecies cutoff level (Viprey et al. 2008). One sequence per OTU was sent to the online web tools Bellerephon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and KeyDNAtools (http://keydnatools.com/) in order to identify potential chimeric sequences. The KeyDNAtools website was also used to help assign sequences to a specific clade. With this tool, sequences are screened using 15-bp length oligonucleotide probes (''keys'') generated in silico. The taxonomic specificity, at different hierarchical ranges (supergroups, division, class, order, family, genus, species, and clade), of each key is provided based on a carefully annotated reference 18S rRNA gene eukaryotic database. For each environmental sequence, KeyDNAtools provides the number of ''keys'' found and their taxonomic assignation (Guillou et al. 2008). Sequences were also submitted (May 2009) to Basic Local Alignment Search Tool (BLAST) analysis against the GenBank database (http://www.ncbi.nlm.nih.gov/). An alignment was constructed including the sequences obtained in this study, those retrieved from the BLAST analysis, and sequences from cultured Chloroplastida species. Maximum-likelihood (ML) and neighbor-joining (NJ) algorithms were used to construct phylogenetic trees. Bootstrap values were calculated from 100 iterations. Alignments and phylogenetic trees were performed with Bosque (Ramírez-Flandes and Ulloa 2008). Sequences have been deposited in the Genbank database under accession numbers HM997190–HM997343.

FISH associated with TSA—Filters were covered with 9 μL of hybridization buffer (40% deionized formamide, 0.9 mol L^{-1} NaCl, 20 mmol L^{-1} Tris-HCl pH 7.5, 0.01% SDS, 2% blocking agent [Roche Diagnostic Boehringer]), and 1 μL of horseradish peroxidase–labeled probes (stock at 50 ng μL^{-1}). Specific probes were used to identify and count Chloroplastida and the target genera (*Micromonas*, *Ostreococcus*, and *Bathycoccus*) within this group. For quantifying the whole picoeukaryotic community (photosynthetic and nonphotosynthetic) we used a combination of a general probe that targets total eukaryotes and two probes that target different photosynthetic groups: Chloroplastida (probe CHLO01) and some Chromalveolata algal lineages (probe NCHLO01) (Simon et al. 1995). The details of the probes used are given in Table 1. Hybridization was achieved by incubating the filters at 35°C for 2 h 30 min. Before the signal amplification step, the filters were washed twice with washing buffer (56 mmol L^{-1} NaCl, 5 mmol L^{-1} EDTA, 0.01% SDS, 20 mmol L^{-1} Tris-HCl pH 7.5) at 37°C for 20 min and then put in an equilibration buffer (100 mmol L^{-1} Tris-HCl, pH 7.5, 150 mmol L^{-1} NaCl, 0.074% Tween 20) at room temperature for 15 min. The TSA reaction using the kit New England Nuclear Life Science Products was performed by adding 10 μL of the TSA mix (1 : 1 40% dextran sulfate and amplification diluent, 1 : 50 fluorescein isothiocyanate tyramide with the mixture of dextran sulfate and amplification diluent) and incubating the filters at room temperature in the dark for 30 min. The enzymatic reaction was stopped by washing the filters twice with the equilibration buffer at 55°C. The cells were counterstained with 4′,6-diamidino-2-phenylindole (final concentration 0.5 μg mL^{-1}) mixed with the antifading reagent (Citifluor AF1). A coverslip was fixed over the slides, which were then stored at 4°C until microscopic observation.

Flow cytometry—The abundances of PPEs and *Synechococcus* (no *Prochlorococcus* was detected in the study area) were determined using a FACSCalibur flow cytometer equipped with an ion–argon laser delivering 15 mW at 488 nm (Becton Dickinson). PPEs were discriminated from

Table 1. Probes used in this work. A combination of three probes (EUK1209, CHLO01, NCHLO01) was used to identify and count all (photosynthetic and nonphotosynthetic) picoeukaryotes (Not et al. 2004).

Probe	Sequence (5′–3′)	Target group
EUK1209	GGG CAT CAC AGA CCT G	Eukarya
CHLO01	GCT CCA CGC CTG GTG GTC	Chloroplastida
NCHLO01	GCT CCA CTC CTG GTG GTC	Non-Chloroplastida
CHLO02	CTT CGA GCC CCC AAC TTT	Chloroplastida
MICRO01	AAT GGA ACA CCG CCG GCG	*Micromonas*
OSTREO01	CCT CCT CAC CAG GAA GCT	*Ostreococcus*
BATHY01	ACT CCA TGT CTC AGC GTT	*Bathyococcus*

cyanobacteria by their optical properties: picoeukaryotes emit only red autofluorescence, while *Synechococcus* cells also emit orange fluorescence, as a result of their phycoerythrin content. Light scatter and fluorescence were normalized by adding 1-μm fluorescent beads to the samples. The data generated were processed using the CytoWin software.

Carbon conversion—To estimate the importance of PPEs in terms of carbon biomass, as compared with their counterpart *Synechococcus* in the picophytoplanktonic fraction, we estimated the mean intracellular carbon contents of these two groups for each sampling date from the flow cytometry data. For this, we used the log–log relationship established by Grob et al. (2007) between the mean forward scatter cytometric signal (FSC, normalized to reference beads) and the intracellular carbon content (C) ($C = 2.65 \times (FSC)^2 + 3.27 \times FSC + 2.58$). The intracellular carbon content of each picophytoplanktonic group was multiplied by the number of cells counted by flow cytometry. To estimate the relative contribution of each genus detected by FISH–TSA (*Ostreococcus*, *Bathycoccus*, *Micromonas*) to the picophytoeukaryotic carbon biomass, a conversion factor of 237 fg C μm^{-3} was used. It was proposed by Worden and colleagues (2004) based on carbon–hydrogen–nitrogen measurements of phytoplankton cells from cultures, including *Micromonas pusilla* and *Ostreococcus lucimarinus*. This conversion factor was multiplied by the abundance obtained by FISH–TSA and the cell volume of cultured species (or strains) of each genus obtained from cell lengths in the literature: 0.95 μm for *O. lucimarinus* (strain CCE 9901, Worden et al. 2004), 2 μm for *Bathycoccus prasinos*, and 2 μm for *M. pusilla* (Vaulot et al. 2004).

Nonparametric statistics were used to determine the correlation of picophytoplankton abundances and the PPE's carbon content with biological (chlorophyll $a >$ 3 μm and < 3 μm), chemical (salinity, macronutrients, and nitrogen-to-phosphorus N : P ratio), and physical (temperature) variables. The dependence between the variables was assessed using the Spearman correlation coefficient.

Results

Hydrographic conditions—Surface temperature during the study period ranged from 10.5°C (July 2007) to 16.5°C (December 2006), following the typical cycle of a temperate zone. Despite the influence of colder subsurface water during the austral spring–summer upwelling period (September to March), solar radiation produced higher temperatures in austral spring–summer than in autumn–winter (Fig. 2A). Salinity ranged from 25.9 (July 2006) to 34.4 (February 2008) (Fig. 2A); lower values were related to freshwater inputs by rainfall and/or river discharges. Nitrate and phosphate were well correlated ($p < 0.001$), and values were, on average, slightly lower in spring–summer, mostly because of the high nutrient uptake during phytoplankton blooms (Fig. 2B). Total chlorophyll *a* averaged 5.0 ± 6.8 mg m^{-3}, with a maximum in summer (> 20 mg m^{-3}). The fraction below 3 μm represented, on average, 10% ± 15% of the total chlorophyll *a*, peaking in autumn–winter, with a maximum of ~ 66% in June 2008 (Fig. 3).

Phylogenetic diversity of picoeukaryotes—In total 154 clones were analyzed: 76 from May, 23 from October, and 55 from December. Clones retrieved from May all corresponded to Chloroplastida sequences, whereas 61% and 20% of the clones from October and December, respectively, were affiliated with Chloroplastida. Most of the non-Chloroplastida sequences clustered within Cercozoa: 33% and 76% of the October and December clone libraries, respectively (Table 2). Within Cercozoa, chlorarachniophytes were important in summer, accounting for 66% of the clone library.

Considering only the Chloroplastida sequences, all but one corresponded to prasinophytes, the latter clustering with chlorophytes (Trebouxiophyceae). Prasinophyte sequences clustered into three of the seven clades proposed by Guillou et al. (2004): Pyramimonadales (clade I), Mamiellophyceae (clade II, now established as a class, Mamiellophyceae; Marin and Melkonian 2010), and Prasinococcales (clade VI). Mamiellophyceae were the most abundant in terms of sequences (~ 97%) and were represented by four genera (Table 2): *Micromonas* (32 clones), *Bathycoccus* (10 clones), *Ostreococcus* (52 clones), and *Crustomastix* (8 clones). In the present study, one clade was present for *Ostreococcus* (clade A) and *Crustomastix* (clade A), and three of the five recognized clades for *Micromonas* (A.BC.1, B.E.3, C.D.5), the first one being more frequent (Fig. 4; Table 3). Interestingly, four OTUs clustered outside of the previously established clades for *Micromonas* with good bootstrap support (Fig. 4). *Micromonas* and *Ostreococcus* were detected during the three periods surveyed, whereas

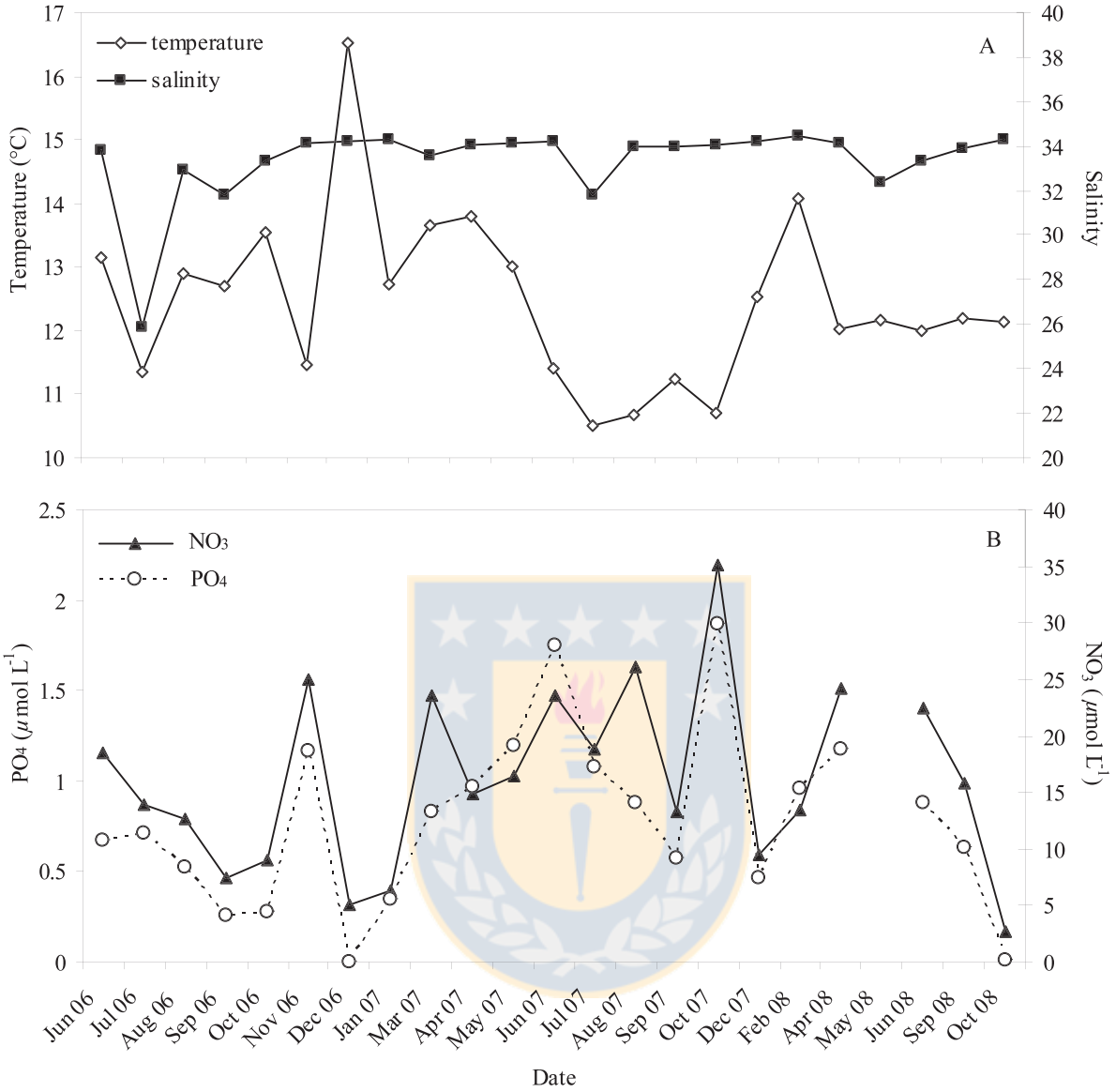

Fig. 2. Variations in (A) physical (temperature, salinity) and (B) chemical (phosphate and nitrate) parameters in the surface waters (0–5 m) of the study area.

Bathycoccus and *Crustomastix* were not observed in summer and autumn, respectively (Table 2). The only spring and summer OTUs (ST100705.F10 and ST120705.G1, respectively) that belonged to *Micromonas* clustered into a single clade (A.BC.1), which also accounted for several autumn clones. *Ostreococcus* was dominant in May (58% of the clones), and its contribution decreased from October (33%) through December (9%). *Micromonas* was only dominant during the spring transition (39%), and *Crustomastix* during summer (64%). Pyraminonadales were detected exclusively in summer, and Prasinococcales and Trebouxiophyceae (Chlorophyta) only appeared in spring (Table 2).

Picoplankton community composition—Photosynthetic picoeukaryote abundance measured by flow cytometry ranged from 10^2 to 10^4 cells mL^{-1}. Seasonal variability was high, with concentrations higher in autumn–winter and lower in spring–summer (Fig. 5A). *Synechococcus* abundance varied between 10^2 and 10^5 cell mL^{-1} (data not shown), and for almost half of the sampling dates, it was one order of magnitude higher than that of their eukaryotic counterpart. No *Prochlorococcus* cells were detected during the entire study period. Abundance of photosynthetic and nonphotosynthetic picoeukaryotes measured by FISH–TSA (Fig. 5A) showed the same seasonal pattern as above, but the range of values was higher (10^3–10^4 cells mL^{-1}). Chloroplastida (probe CHLO02) accounted for up to 100% of the total picoeukaryotic community (74% ± 29% on average). At eight sampling dates, Chloroplastida represented < 70% of the total picoeukaryotes, six of these dates corresponding to spring–summer months. Moreover, for the two December sampling dates, Chloroplastida represented a minor fraction of the total picoeukaryotes (4% and

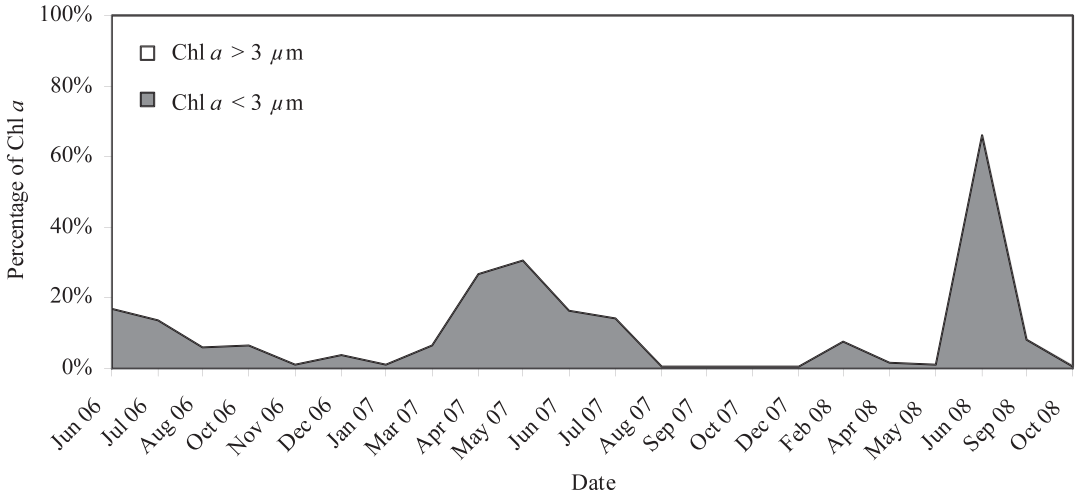

Fig. 3. Relative contribution of two different size fractions (> 3 μm, < 3 μm) to total chlorophyll *a* (Chl *a*), measured in mg m^{-3}, in the surface waters off Concepción.

13%, respectively), consistent with the low number of PPEs detected by flow cytometry.

On average, the abundances of *Ostreococcus* (probe OSTREO01), *Micromonas* (probe MICRO01), and *Bathycoccus* (probe BATHY01) added up to 102% ± 8% of the total Chloroplastida cells (Fig. 5A). Numerically (Fig. 5B), *Ostreococcus* dominated the Chloroplastida community (52.6% ± 19.3%; range, 290–18,742 cell mL^{-1}), followed by *Bathycoccus* (26.9% ± 15.3%; range, 258–7282 cell mL^{-1}) and *Micromonas* (22.9% ± 12.3%, range: 65–9632 cell mL^{-1}).

Carbon biomass—On most sampling dates, PPEs dominated over *Synechococcus* in terms of carbon (Fig. 6). PPEs represent, on average, 57% of the picophytoplanktonic carbon biomass during the study period, the highest percentages being during spring months. The contribution of each genus to PPE carbon varied depending on the PPE cell size (Fig. 7). *Ostreococcus*, although the most abundant genus, contributed the least to carbon biomass, with an average of 13% of the PPE biomass and a maximum of 40% in April 2007 (Fig. 7). *Bathycoccus* made the largest carbon contribution during the study period, with an average of 47% and a maximum of 78% in November 2006. *Micromonas* contributed on average 40% to the PPE's biomass, with a maximum of 70% in August 2006. In general, the highest values of abundance and biomass were observed in autumn and winter, except for *Bathycoccus*, which showed relatively high values during spring months. During winter, PPE's biomass showed the highest values, and *Micromonas* was the largest contributor to this biomass. Considering only spring and summer, PPE abundance was higher during summer, *Ostreococcus* being responsible for this increase, since *Micromonas* as well as *Bathycoccus* showed lower abundances during this season. However, carbon biomass was higher during spring because *Ostreococcus* has a smaller cell size than the other species. Carbon biomass of PPEs showed a significant negative correlation with temperature and salinity (Table 4), these two variables being lower during winter (Fig. 2). The abundances of some picophytoplankton groups, in particular *Synechococcus* and *Ostreococcus*, were negatively correlated with larger phytoplankton chlorophyll *a*. *Ostreococcus* was positively correlated with

Table 2. Number of clones per taxonomic group identified for each sampling date after a phylogenetic analysis of the clone libraries.

		Number of clones		
Division	Group	May 07	Oct 07	Dec 07
Chloroplastida	*Bathycoccus*	9	1	—
(Mamiellophyceae)	*Micromonas*	24	6	1
	Ostreococcus	43	5	1
	Crustomastix	—	1	7
	Prasinococcales	—	1	—
	Pyramimonadales	—	—	2
	Trebouxiophyceae	—	1	—
Cercozoa	Chlorarachniophyta	—	2	37
	other Cercozoa	—	5	5
Radiolaria	Taxopodia	—	1	—
Alveolata	Dinophyceae	—	—	2

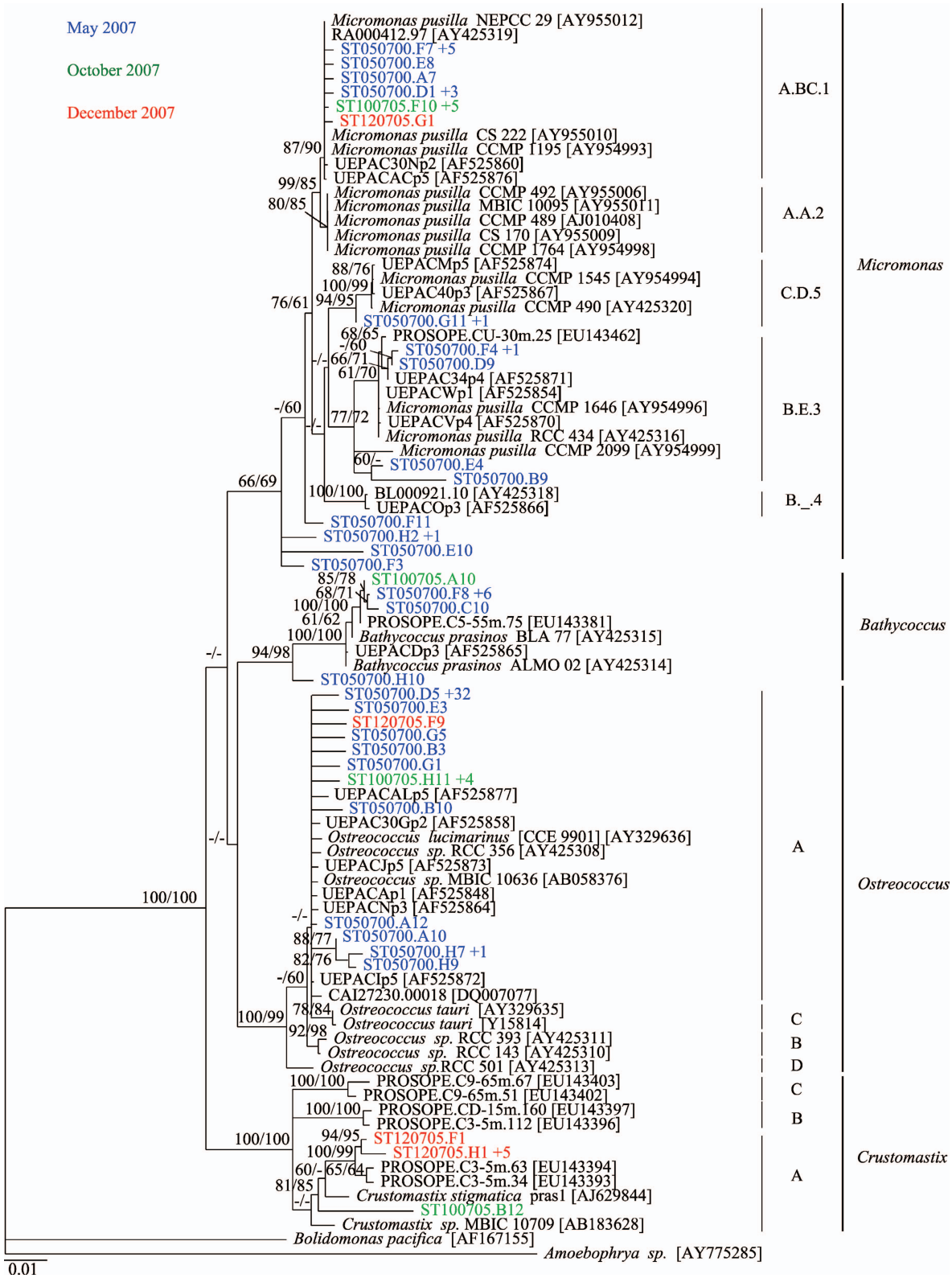

Fig. 4. 18S rRNA gene phylogeny of Mamiellophyceae inhabiting the coastal upwelling ecosystem off Concepción, inferred by ML and considering 996 positions of an alignment of 87 partial and full-length sequences. Significant bootstrap values (≥ 60%) of internal branches, obtained by NJ and ML, are shown in the tree in the order NJ and ML; nonsignificant values are indicated by a dash. Sequences retrieved on the three sampling dates are shown in bold: blue is for May 2007, green for October 2007, and red for December 2007. *Micromonas* clades are according to Worden (2006), *Ostreococcus* are according to Guillou et al. (2004), and *Crustomastix* are according to Viprey et al. (2008).

Table 3. Closest similarity between the clones retrieved in this study and cultured strains of *Micromonas*, *Bathycoccus*, and *Ostreococcus*. Numbers represent percentage of identity (% ID).

Clone	% ID	Closest cultured match
ST050700.F7	99.7	*Micromonas pusilla* CCMP 1195
ST050700.E8	99.7	*Micromonas pusilla* CCMP 1195
ST050700.A7	99.7	*Micromonas pusilla* CCMP 1195
ST050700.D1	99.7	*Micromonas pusilla* CCMP 1195
ST100705.F10	99.9	*Micromonas pusilla* CCMP 1195
ST120705.G1	99.7	*Micromonas pusilla* CCMP 1195
ST050700.G11	99.5	*Micromonas pusilla* CCMP 490
ST050700.F4	99.6	*Micromonas pusilla* RCC 434
ST050700.D9	99.7	*Micromonas pusilla* RCC 434
ST050700.E4	98.4	*Micromonas pusilla* RCC 434
ST050700.B9	97.8	*Micromonas pusilla* RCC 434
ST050700.F11	98.8	*Micromonas pusilla* CCMP 1195 and CCMP 1764
ST050700.H2	97.9	*Micromonas pusilla* CCMP 1195
ST050700.E10	96.7	*Micromonas pusilla* CCMP 1195
ST050700.F3	98.2	*Micromonas pusilla* CCMP 489
ST100705.A10	100	*Bathycoccus prasinos* ALMO 02
ST050700.F8	99.9	*Bathycoccus prasinos* ALMO 02
ST050700.C10	99.7	*Bathycoccus prasinos* ALMO 02
ST050700.H10	97.9	*Bathycoccus prasinos* ALMO 02
ST050700.D5	100	*Ostreococcus* sp. MBIC 10636
ST050700.E3	99.7	*Ostreococcus* sp. MBIC 10636
ST120705.F9	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.G5	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.B3	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.G1	99.7	*Ostreococcus* sp. MBIC 10636
ST100705.H11	99.7	*Ostreococcus* sp. MBIC 10636
ST050700.B10	99.4	*Ostreococcus* sp. MBIC 10636
ST050700.A12	99.9	*Ostreococcus* sp. RCC 356
ST050700.A10	99.3	*Ostreococcus* sp. RCC 356 and MBIC 10636
ST050700.H7	98.6	*Ostreococcus* sp. MBIC 10636
ST050700.H9	98.2	*Ostreococcus* sp. MBIC 10636

nitrogen (nitrate and nitrite) but also with the N : P ratio. The other picoeukaryotic genera did not show any significant correlation with nutrients.

Discussion

The temporal change in the community structure of photosynthetic picoeukaryotes in a temperate coastal upwelling ecosystem of the eastern South Pacific was studied for the first time. We found that Chloroplastida represented the bulk of the picoeukaryotes counted by FISH–TSA. The sum of the three genera *Ostreococcus*, *Bathycoccus*, and *Micromonas* accounted for the Chloroplastida cell counts at all sampling dates. This confirms that these three PPE genera are important genera in upwelling ecosytems (Countway et al. 2010). According to our results, the picoeukaryotic community changed during the annual cycle, both in abundance and diversity. During autumn–winter, PPEs were more abundant, and their abundance decreased as summer approached. Moreover, all clones retrieved from May were Chloroplastida, whereas other lineages appeared in the spring–summer libraries. Thus, along with a decrease in abundance, there was a change in community structure and probably a proliferation of nonphotosynthetic organisms, since total picoeukaryote abundances tended to be higher than PPEs in spring–summer and greatly exceeded them in December 2006. The appearance of nontarget sequences in the clone libraries could be explained by the fact that the primers used are biased toward Chloroplastida but not totally specific. Most of the non-Chloroplastida sequences, which appeared in the warmer months, clustered within Cercozoa, in particular to Chlorarachniophytes. This photosynthetic group, whose sequences contain a single mismatch to the CHLO02 primer, was reported previously in Chloroplastida-biased clone libraries (Viprey et al. 2008). Moreover, the few Dinophyceae and Radiolaria sequences found had two to three mismatches to the Chloroplastida-biased primer. The variability in the number of clones obtained, despite the use of the same protocol and reagents, could be due to differences in clone transformation efficiency.

Chloroplastida, and particularly Mamiellophyceae, were expected to dominate in the study area, since these organisms have been commonly observed in coastal environments (Not et al. 2004; Zhu et al. 2005; Worden 2006) and specifically in upwelling regions (Rodríguez et al. 2006; Countway et al. 2010). They are normally observed in high abundance at the deep-chlorophyll maximum (Not et al. 2005; Countway and Caron 2006). In our case, the maxima in chlorophyll concentration occurred at the

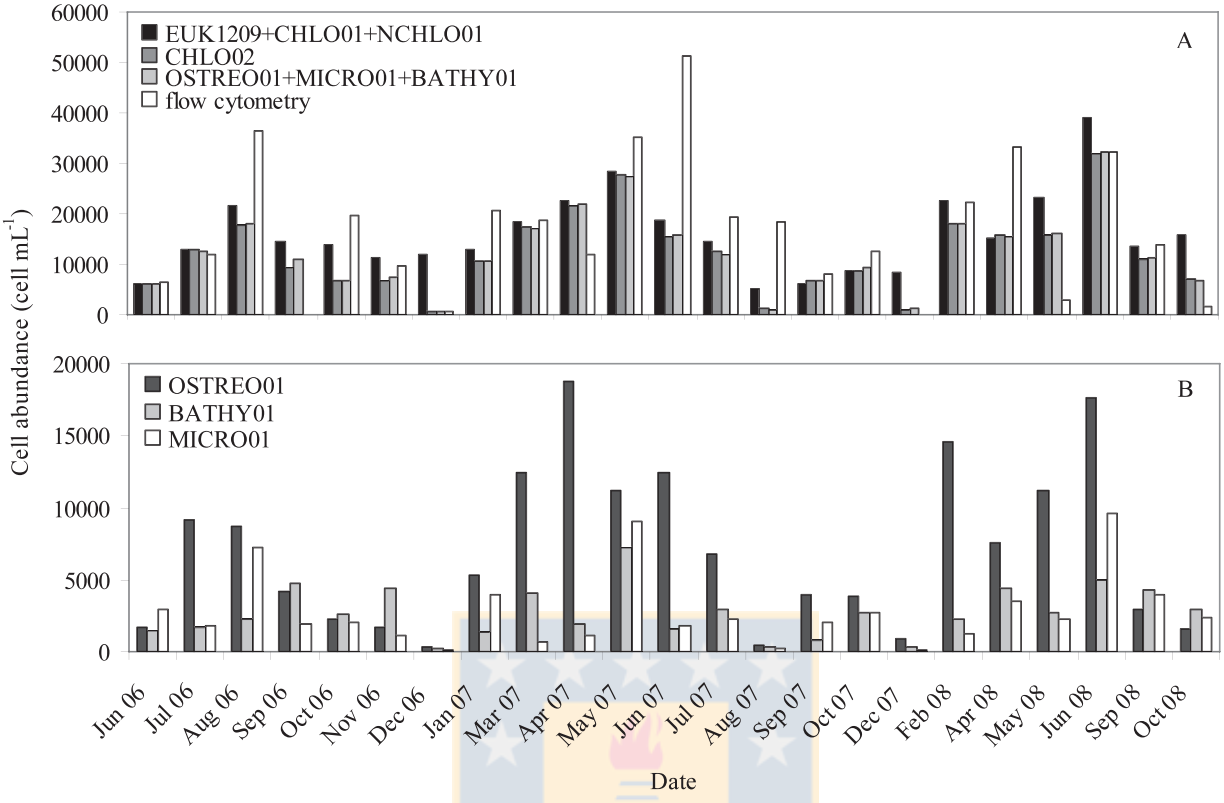

Fig. 5. Abundance of picoeukaryotic cells (expressed as cell mL^{-1}) using FISH–TSA and flow cytometry in the surface waters off Concepción during the study period. (A) Total picoeukaryotes (autotrophs and heterotrophs) were targeted with a mix of three general probes (EUK1209 + CHLO01 + NCHLO01), Chloroplastida with a specific probe (CHLO02), the sum of *Ostreococcus*, *Micromonas*, and *Bathycoccus* with specific probes (OSTREO01 + MICRO01 + BATHY01), and PPE detected by flow cytometry. (B) *Ostreococcus* (OSTREO01), *Bathycoccus* (BATHY01), and *Micromonas* (MICRO01) cells targeted with specific probes. There are no data for flow cytometry on September 2006 and December 2007.

surface most of the time and, therefore, were covered by our sampling scheme. Within the Mamiellophyceae, *Micromonas* has been found to be dominant in nutrient-rich coastal waters (Not et al. 2004, 2005, 2007). Our observation on the importance of *Micromonas* clade A in terms of number of OTUs and clones (Fig. 4) concurred with previous studies (Foulon et al. 2008). Observed *Micromonas* abundances were in the range of those reported before for coastal regions such as the English Channel (Not et al. 2004) but were higher than in the northwest (NW) Iberian upwelling system (Not et al. 2007). Despite its high abundance, *Micromonas* did not numeri-

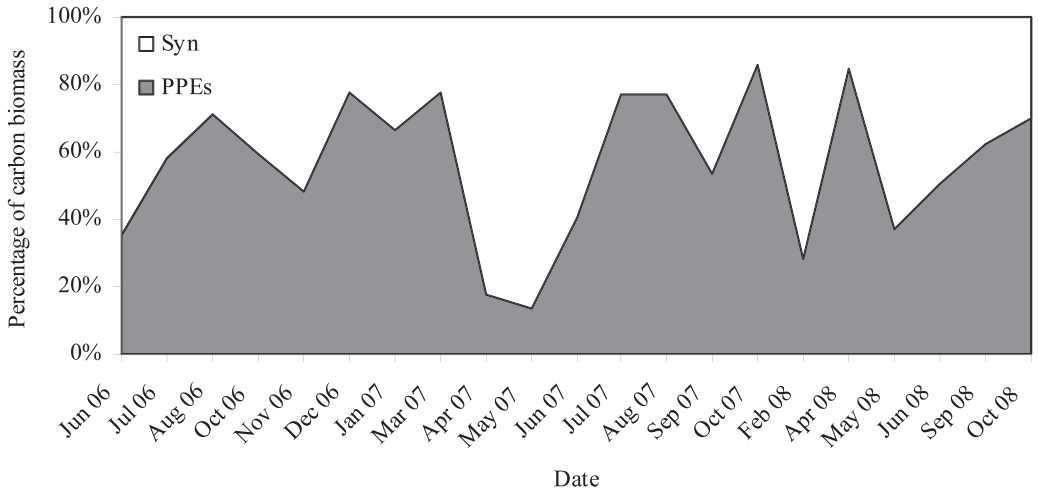

Fig. 6. Relative contribution of *Synechococcus* (Syn) and PPEs to the carbon biomass (μg C L^{-1}) of the picophytoplanktonic fraction during the study period off Concepción. *See* Methods section for conversion factors used.

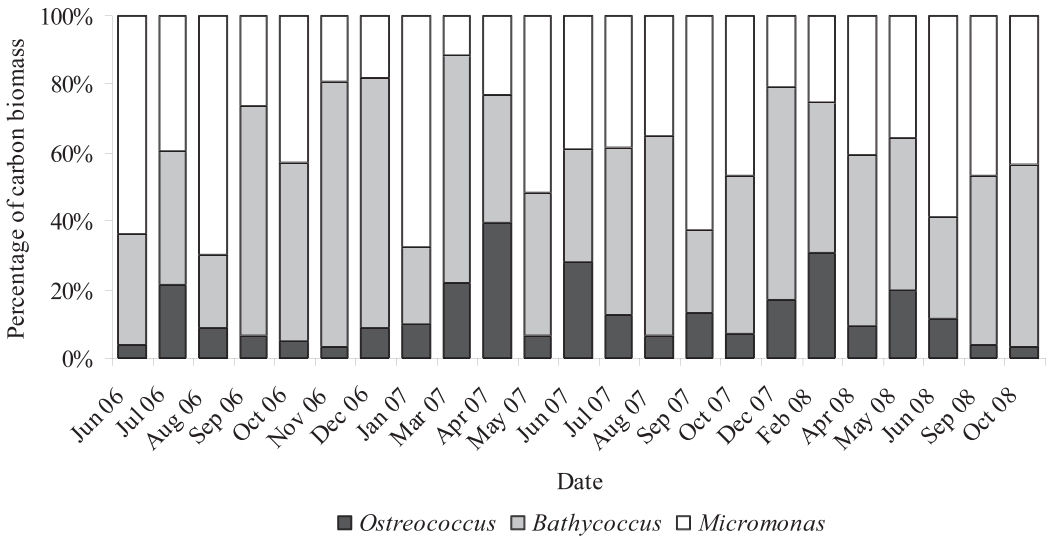

Fig. 7. Relative contribution of *Ostreococcus*, *Bathycoccus*, and *Micromonas* to the picophytoeukaryotic carbon biomass. *See* Methods section for conversion factors used.

cally dominate the PPE community, which contrasts with what has been reported for nearly all other studies in coastal waters of the Northern Hemisphere (Not et al. 2004, 2005, 2008), including those in upwelling regimes (Not et al. 2007). *B. prasinos,* on the other hand, also has been commonly reported in coastal environments, with maximum abundances reaching ~ 2000 cells mL^{-1} in surface waters (Not et al. 2004, 2005). Our observations are comparable, although somewhat higher, with more than 4000 cells mL^{-1}. However, the relative abundance of *B. prasinos* has previously been found to be at most 15% of the Mamiellophyceae community (Not et al. 2004, 2007; Zhu et al. 2005) and always lower than that of *Micromonas*, whereas off Concepcion it was on average ~ 26% of the Mamiellophyceae and surpassed that of *Micromonas* on several sampling dates. Very little is known about the ecology of this species, although comparison of data from two studies that included stations with and without coastal influence (Not et al. 2005, 2008) suggested that *Bathycoccus* reaches maximum abundances in coastal waters. The present study did not uncover any significant relationship between its abundance and environmental variables (Table 4). Finally, *Ostreococcus* inhabiting our study area are members of clade A, a high-light ecotype (Rodríguez et al. 2005) that includes *O. lucimarinus* (strain CCE 9901), which has been isolated from coastal Pacific waters (Worden et al. 2004). *Ostreococcus* occurs generally at very low abundances in coastal environments, with concentrations of no more than 10^2 cells mL^{-1} (Not et al. 2004; Zhu et al. 2005) and at times is absent, as was the case in the NW Iberian upwelling system (Not et al. 2007). However, high concentrations of *Ostreococcus* (e.g., 10^4–10^5 cells mL^{-1}) have been detected in coastal waters during short blooms around Long Island (New York, western North Atlantic; O'Kelly et al. 2003), in the coastal Mediterranean Thau lagoon (Courties et al. 1994), and in the San Pedro Channel (California, eastern North Pacific). The latter is a coastal upwelling area where *Ostreococcus* is present almost year-round but generally in low abundances (Countway and Caron 2006; Countway et al. 2010). In the case of the Chile upwelling ecosystem, we found that *Ostreococcus* was present throughout the study period, with relatively high concentrations (average 6.9×10^3 cells mL^{-1}), although not substantially different from those observed in previous studies during short blooms. A possible explanation for the permanent high abundances we found might be related to the permanent availability of nutrients. Our results suggest that *Ostreococcus* may respond better to new nitrogen (e.g.,

Table 4. Correlation matrix for picophytoplankton groups vs. biological, physical, and chemical variables. All picophytoplankton data concern abundance except for PPE carbon. T, temperature; S, salinity; O_2, oxygen concentration; Chl, chlorophyll *a*.

Picophytoplankton group	T	S	O_2	Chl >3 μm	Chl <3 μm	NO_3	NO_2	NH_4	PO_4	N : P
PPE	0	0	−0.3	−0.2	0.7**	0.4	0.3	−0.2	0.2	0.5*
Ostreococcus	0.1	−0.2	−0.2	−0.5*	0.6**	0.5*	0.5*	−0.1	0.2	0.6**
Bathycoccus	−0.1	−0.3	−0.2	−0.4	0.2	0.3	0.1	−0.2	0.3	0.1
Micromonas	−0.1	−0.2	−0.2	−0.1	0.5*	0.1	0.2	−0.4	0	0.1
Chloroplastida	0.1	−0.2	−0.2	−0.5*	0.6**	0.5*	0.4	−0.1	0.2	0.5**
Total picoeukaryotes	0.3	−0.1	−0.1	−0.4	0.5*	0.2	0.1	−0.1	−0.1	0.3
Synechococcus	0.3	0	−0.1	−0.5*	0.8***	0.3	0.2	−0.4	0	0.5**
PPE carbon	−0.5*	−0.6**	−0.1	0.3	−0.2	0	0.5*	0	0.1	−0.2

* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

NO_3) than *Bathycoccus* and *Micromonas*, which is reflected here in the positive correlations found between *Ostreococcus* and nitrate, nitrite, and the N : P ratio (Table 4); statistically significant relationships with nutrients were not observed for the other two eukaryotic genera, but there was a positive correlation between *Synechococcus* and the N : P ratio as well. Our results are consistent with those of Worden et al. (2004), which have shown slightly higher growth rates of *O. lucimarinus* when nitrate is used as the nitrogen source instead of ammonium. In the Chilean upwelling ecosystem, nitrate is found at relatively high concentrations in the euphotic zone during the entire year (Fig. 2), which may explain why *Ostreococcus* is abundant in this region but absent in the NW Iberian coast (characterized by periodic upwelling events), where nitrate concentrations are lower (Not et al. 2007).

The abundance of the photosynthetic picoeukaryote community in the central Chile upwelling area showed a clear seasonal pattern. PPE abundance was higher in autumn–winter and lower in spring–summer. Similar trends have been established for other coastal upwelling ecosystems, such as the Iberian coast (Rodríguez et al. 2006) and the Oregon coast (Sherr et al. 2005). It has also been previously observed that when the larger phytoplankton dominates, picophytoplankton abundances are low (Larsen et al. 2004; Sherr et al. 2005). Here, this also holds as reflected by the negative correlation between the $>$ 3-μm chlorophyll *a* and the abundance of *Ostreococcus* and *Synechococcus*, which are the main contributors to the picophytoplankton abundance in the study area. The reason for this marked seasonal pattern is still poorly understood. One possibility is that picophytoplankters are weak competitors compared with microphytoplankton such as diatoms in high-nutrient environments (Litchman et al. 2007), like those found in upwelling ecosystems. Another possibility is a top-down control by grazing of the heterotrophic microplankton and nanoplankton on the picoplankton fraction (Calbet and Landry 2004; Christaki et al. 2005). While there is no evidence for strong seasonality in the abundance of heterotrophic microplankton and nanoplankton (Böttjer and Morales 2007), grazing rates of heterotrophic nanoplankton on bacteria and cyanobacteria have been shown to be in fact higher during spring–summer in the study area (Böttjer and Morales 2007; Vargas et al. 2007). However, no data are available on specific grazing of PPEs.

The importance of the picophytoplankton community for primary productivity has been assessed and demonstrated in the open ocean (Li 1994; Jardillier et al. 2010), where small cells dominate. But the relevance of this smallest fraction should not be underestimated in coastal environments, especially in highly productive coastal environments, such as upwelling regimes. Cermeño et al. (2006) determined at the Ría de Vigo coastal upwelling system that picophytoplankton accounted for up to 30% of the chlorophyll *a*. We found that the $<$ 3-μm fraction usually contributed less than 20% (on average $\sim$ 11%) of the total chlorophyll *a*, but it did reach up to 66% (June 2008). According to our carbon biomass estimations, more than half of the picophytoplanktonic primary production could be due to eukaryotic picoplankton during most of the sampling dates. This agrees with the observation of Worden et al. (2004), who determined that, on average, PPEs accounted for 76% of the carbon production of the picophytoplankton fraction at a Northern Pacific upwelling coastal system. Both here and in the Northern Pacific (Worden et al. 2004), PPEs contributed a higher carbon biomass than cyanobacteria in this size fraction because of their higher cell size and higher chlorophyll *a* content (Li 1994; Worden et al. 2004). The same is true within the PPE community: *Micromonas* and *Bathycoccus* contributed most to carbon biomass despite being less abundant than *Ostreococcus*. This was recently pointed out by Jardillier et al. (2010), showing that larger photosynthetic picoeukaryotes ($\sim$ 2.8 μm) can account for up to 38% of the total CO_2 fixation in the Atlantic Ocean, despite being almost half as numerically abundant as smaller PPEs ($\sim$ 1.8 μm). One should note, however, that the biovolumes used for the three genera in our study were from cells in culture and are therefore probably overestimated. However, since we are evaluating the relative biomass contribution of each genus, not their absolute contribution to the system, the approach may be valid.

PPE abundances observed off central Chile are similar to those observed previously in coastal upwelling ecosystems (Sherr et al. 2005; Fuller et al. 2006). The PPE contribution to carbon biomass is in the range of what has been reported before for this type of environment, specifically for the NW Iberian upwelling system (Rodríguez et al. 2006). However, the structure of the PPE community from the ecosystem in the eastern South Pacific is somewhat different from those previously observed, as a result of the dominant and persistent abundance of *Ostreococcus*. Although our phylogenetic analysis of the 18S rRNA gene (Fig. 4) revealed that natural populations inhabiting the study area are generally closely related to cultured strains, with similarities above 97% (Table 3), some sequences did not tightly cluster with those previously known for *Micromonas*, *Bathycoccus*, and *Crustomastix*. For example, *Bathycoccus* was not genetically as homogeneous as expected (Guillou et al. 2004, Viprey et al. 2008); one OTU (ST050700.H10) clearly belonged to *Bathycoccus* but clustered out of previous reported sequences with high bootstrap values (Fig. 4).

Further studies are needed to understand why the eukaryotic picophytoplankton decreases in the spring–summer upwelling season but are important during the nonupwelling months. Physiological (e.g., nutrient uptake), ecological (e.g., grazing, virus infection, competition), and even physical (e.g., advection) aspects should be evaluated, as well as the niche partitioning within each genera. The importance of PPEs to the carbon economy of the upwelling ecosystem also needs to be assessed, especially when the larger phytoplankton are less abundant. Although *Ostreococcus*, *Micromonas*, and *Bathycoccus* were the main components of the PPE fraction, attention should be paid to the other photosynthetic groups present in the study area, such as Chlorarachniophytes. Their significant presence in the clone libraries occurred when the abundances of *Ostreococcus*, *Micromonas*, and *Bathycoccus* were

low. Finally, *Ostreococcus* appeared as the dominant PPE in the Chile upwelling ecosystem. Elucidating whether this is based on particular genetic characteristics, e.g., when compared with lineages found in the eastern North Pacific (Countway and Caron 2006; Countway et al. 2010), or on ecological aspects will help in understanding the basis for its global distribution.

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4.2. Diversidad y variabilidad temporal de la comunidad de pequeños protistas en una zona costera de mínimo oxígeno estacional

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Resumen

Las Zonas de Mínimo Oxígeno (ZMO) marinas albergan una gran diversidad de comunidades microbianas que son cruciales en los ciclos biogeoquñimicos a nivel global. Mientras que los procariontes que habitan las ZMO han sido bien estudiados, los eucariontes unicelulares (protistas) han recibido mucha menos atención, a pesar de que controlan a las comunidades microbianas y en gran medida el flujo de carbono a través de diversas y complejas interacciones tróficas (ej. predación, parasitismo, saprotrofía). En este trabajo se analizó la diversidad genética de los protistas más pequeños (< 3 μm, los picoeucariontes) que habitan la zona afótica de la columna de agua en una ZMO costera estacional frente a Chile Central. Utilizamos multiples partidores para secuenciar el gen 18S ARNr en cuatro fechas de muestreo a lo largo de un año (enero, abril, julio y octubre). Un total de 1.772 secuencias parciales y 349 OTUs fueron analizados. Syndiniales fue el grupo más representado en todas las librerías de clones, sin embargo en enero (verano austral) grupos prominentes también fueron Dinophyceae y Fungi. En abril (otoño autral) la muestra estuvo enriquecida en Labyrinthulomycetes, en su mayoría representado por secuencias sin afiliación con grupos previamente descritos. En julio (invierno austral), cuando la columna de agua está más oxigenada, la muestra fue la más diversa en términos de grupos taxonómicos. En octubre (primavera austral) Ciliophora parece ser un grupo importante. Dentro de Syndiniales, el Grupo II predominó sobre el Grupo I en deficiencia de oxígeno, pero el ultimo sobrepasó al primero cuando la columna de agua estaba oxigenada. La presencia fluctuante de descomponedores (Fungi y Labyrinthulomycetes), parásitos (Syndiniales) y fagótrofos (ej. ciliados), de acuerdo a las condiciones de la columna de agua en la ZMO estacional frente a Chile Central sugiere roles ecológicos de los picoeucariontes heterótrofos que deben ser considerados.

Running head: Diversity of protists at a seasonal OMZ

Diversity and Temporal Variability of the Small Protist Community at a Seasonal Coastal Oxygen Minimum Zone

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ABSTRACT

Protists control microbial communities and the carbon flux through different and complex ecological interactions. The genetic diversity of small protists (i.e. picoeukaryotes) inhabiting the aphotic water column at a coastal seasonal Oxygen Minimum Zone (OMZ) site off Central Chile was surveyed. We used a multiple-primer approach to sequence the 18S ribosomal ribonucleic acid (rRNA) gene in four sampling periods throughout a year (January, April, July and October). A total of 1,772 partial length rRNA sequences and 349 OTUs were analyzed. Syndiniales was the most represented group in all samples, although in January (austral summer) prominent groups were also Dinophyceae and Fungi. April (austral autumn) sample was enriched in Labyrinthulomycetes, and the majority had no association with any cultivated organisms. The July (austral winter) sample, when the water column is oxygenated, was the most diverse. In October (austral spring) Ciliophora seems to be important. Within the Syndiniales, Group II was predominant over Group I when oxygen was low, but the reverse was observed in July. The inferred ecological function of the different groups found suggest that parasitism, phagotrophy, and saprotrophy play an important role in this ecosystem, and their relative importance may vary through the year with changes in hydrographic conditions.

Keywords
Protists; coastal upwelling; seasonal Oxygen Minimum Zone; phylogenetic diversity; heterotrophs

INTRODUCTION

Although the ancestral environment in which the first eukaryotic cell originated would have held roughly 1% of the oxygen of the present atmosphere (Fenchel & Finlay 1995), aerobic respiration has been crucial in eukaryotic evolution. The use of oxygen as a terminal acceptor is the most thermodynamically efficient energy metabolism (Fenchel & Finlay 2008), this is why the vast majority of eukaryotic organisms (particularly multicellular ones) depend on this element, and those who are able to live in oxygen-deficient or anoxic environments are mainly unicellular (protists). Oxygen thus plays an essential role in the distribution of unicellular eukaryotes in the environment, the structure of the microbial community, and on their functional interactions such as trophic relations and carbon fluxes (Fenchel & Finlay 2008). Diversity studies on protist communities in oxygen-deficient conditions are a major aspect of interest, since the knowledge of the communities inhabiting oxygen deficient areas of the oceans may be an approach to the eukaryotic cell origin. On the other hand, if the capability of living under hypoxic or anoxic conditions has been acquired during the evolution, new eukaryotic lineages might be found that are exclusive to these kind of environments (Bérney et al. 2004). Moreover, low-oxygen environments could harbour rare species with unusual metabolisms for eukaryotic cells, like denitrification (Glock et al. 2013; Risgaard-Petersen et al. 2006).

Despite the vast knowledge of the eukaryotic cell, the extent of protistan diversity and ecological function is still largely unexplored. Several anoxic environments have been surveyed for protist diversity and surprisingly a high richness and diversity of organisms have been found, with several of them representing putative new phylogenetic groups (Jeon et al. 2006; Stoeck et al. 2003; Stoeck & Epstein 2003). These environments include sediments around fumaroles (Takashita et al. 2005), fjords (Behnke et al. 2006 and 2010; Zuendorf et al. 2006), and other confined or stratified basins such as the Black Sea (Wylezich & Jürgens 2011), the Cariaco Basin (Stoeck et al. 2003), and the L´Atalante hypersaline anoxic basin of the Mediterranean Sea (Alexander et al. 2009). However, areas of intermediate depth and hypoxic levels of dissolved oxygen (below ~5 μM) (Kamykowski & Zentara 1990) that arise when O_2 consumption is high and ventilation is low, have received less attention. Globally, these "Oxygen Minimum Zones" (OMZ) are wider on eastern margins of the oceans due to the upwelling of nutrient-rich waters that supports high primary production and consequently rapid remineralization of this organic material, leaving intermediate depth waters with a deficiency of oxygen (Helly & Levin 2004). These areas are of particular interest because of the influence they have on the global climate, since high respiration and denitrification rates make it a source of CO_2 and N_2O which are both important greenhouse gases (Zher & Ward 2002). Most of the key biogeochemical reactions occurring within OMZs are driven by bacteria and archaea. However, eukaryotic microbes control bacterial and archaeal abundance through predation, and therefore the carbon flux through the food web. On the other hand, heterotrophic protists have been underestimated in ecosystem and carbon cycle models and they are involved in many trophic interactions such as predation, parasitism, and saprotrophy (Worden et al. 2015) that are now regarded as critical for oceanic foodwebs (De Vargas et al. 2015).

Recently, Parris et al. (2014) analyzed the microbial eukaryote diversity of the vast and permanent OMZ off Northern Chile, describing vertical variation in protist composition that support the idea of adaptation to permanent oxygen depletion. In contrast, the OMZ associated with the Central Chile coastal environment is influenced by a seasonal upwelling regime; therefore, oxygen-depleted conditions are generally restricted in time to the upwelling events

occurrence (austral spring and summer months) and in space to the subsurface layer (~under 20 m) (Sobarzo et al. 2007). The effect of changing levels of dissolved oxygen in the water column on the protist community is poorly known in these kind of environments and the fluctuations of the community structure in response to oxygen could reaffirm the specialization of certain protistan taxa to oxygen-deficient conditions or could indicate the presence of taxa capable of living in variable oxygen conditions. Niche specialization of protist communities in space and time according with the biogeochemical conditions, including oxygen gradients, has been an issue of discussion and it seems that some protistan organisms are restricted to specific habitats (Edgcomb et al. 2011; Orsi et al. 2011).

Picoeukaryotes represent the smallest protists, comprising cells which diameter is less than 3 µm. These cells are crucial in the primary productivity of the open ocean (Li 1994, Grob et al. 2007), and several picophytoplanktonic species of the class Mamiellophyceae are known to be widely distributed (Collado-Fabbri et al. 2011, Not et al., 2004, Zhu et al. 2005), but knowledge about the heterotrophic counterpart of this size fraction is still limited. In recent years, the perception of eukaryotic diversity has changed as a result of research focused on these small organisms, unveiling a vast representation of almost all the major taxonomic lineages (López-García et al. 2001, Moon-van der Staay et al. 2001). Since this fraction is less well-known and has the highest potential for the presence of new groups, we focused our investigation on picoplanktonic eukaryotic cells and assessed their genetic diversity in the aphotic layer of the OMZ off Central Chile, analyzing the variability of the picoeukaryote community, taxa richness and putative effect of dissolved oxygen changes during an annual cycle in the water column.

MATERIALS AND METHODS

Sample collection

Sampling was performed at a fixed time-series station (Station 18: 36º30.8'S 73º07.7'W) maintained by the Center for Oceanographic Research in the Eastern South Pacific (COPAS), Universidad de Concepcion (UdeC). The sampling site is located on the continental shelf ~10 km off Dichato, Central Chile, with a water-column depth of ~93 m (Fig. 1). Water samples were collected monthly during the year 2010 on board the R/V *Kay-Kay* II (UdeC), using 10-liter Niskin bottles and at discrete depths from the surface (0 m) to 80 m. Samples from 50 m were used for the analyses of picoeukaryotes, this depth is considered deep enough to be affected by the presence of an OMZ and not so deep that it is affected by the sediment. Hydrographic data (temperature and salinity) were obtained using a SeaBird 25 conductivity-temperature-depth-oxygen (CTDO) sensor. Macronutrients and chlorophyll *a* concentrations were measured using standard methods (Morales et al. 2007).

For desoxyribonucleic acid (DNA) extraction purposes, water samples were collected from seawater prefiltered through a 20-µm pore size mesh. Then, using a peristaltic pump, three liters of this seawater were filtered through a 3-μm pore size membrane and microbial biomass was collected on a 47-mm diameter membrane with a 0.2-µm pore size (Supor® 450). The latter was transferred to cryovials and covered with lysis buffer (40 mM ethylenediaminetetraacetic acid [EDTA], 0.73 M sucrose, and 50 mM Tris-HCl pH 8.3), immediately frozen in liquid nitrogen, and stored at -80 ºC until DNA extraction.

DNA extraction, amplification and cloning

Four samples from 50 m were selected to perform the genetic diversity analysis of the picoeukaryotic fraction: January, April, July and October; the selection was made based on the water column conditions (physical, chemical and biological profiles) shown in each month (see Results section). In order to obtain the picoplanktonic community genomic DNA, cell lysis was performed by adding lysozyme (1 mg mL^{-1}), proteinase K (0.5 mg mL^{-1}), and sodium dodecyl sulfate (SDS) (1%) to the filters. DNA from the lysate was purified with phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1) extractions. It was then precipitated by adding sodium acetate (7.5 M) and isopropanol (0.4 and 1 volume, respectively) and resuspended in 50 µl of ultrapure DNA and RNAse free water. Integrity was checked on an agarose gel. DNA was stored at -20 ºC until amplification.

The 18S ribosomal ribonucleic acid (rRNA) gene was amplified using four different primer sets: A) Euk528F (5´-CGG TAA TTC CAG CTC C-3´) and U1391R (5´-GGG CGG TGT GTA CAA RGR-3´), B) Euk82F (5´-GAA DCT GYG AAY GGC TC-3´) and U1391R, C) 6CilF (5´-AAY CTG GTT GAT CCT GCC AG-3´) and 1511CilR (5´-GAT CCW TCT GCA GGT TCA CCT AC-3´), and D) Euk328 (5´-ACC TGG TTG ATC CTG CCA G-3´) and Euk329 (5´-TGA TCC TTC YGC AGG TTC AC-3´) followed by a nested reaction with Euk360F (5´- CGG AGA RGG MGC MTG AGA-3´) and U1517R (5´-ACG GCT ACC TTG TTA CGA CTT-3´). The primer sets A and B constitute a combination of a eukaryotic-biased primer and a universal one, primer set C is biased toward ciliates, and primer set D is biased toward eukaryotes and the nested primer set is a combination of eukaryotic biased and universal primers. The Polymerase Chain Reaction (PCR) mixture (25 µl final volume) contained ~20 ng of environmental DNA as template, 200 µM of each deoxynucleoside triphosphate (dNTP), 1.5 mM $MgCl_2$, 0.3 mM of each primer, 2.5 U of *Taq* DNA polymerase (GoTaq®, Promega), and the PCR buffer (1x) supplied with the enzyme. PCR conditions were as follows: initial denaturation at 95 ºC for 180 s, 30 cycles of denaturation (at 95 ºC for 45 s), annealing (at 56 ºC for 60 s), and extension (at 72 ºC for 120 s), and a final extension step (at 72 ºC for 7 min). PCR products were purified and visualized after electrophoresis on an agarose gel (1%). Amplified products of two reactions were pooled and concentrated in a volume of 30 µL sterile water. An aliquot of each pooled PCR product was cloned using the kit pGem easy vector (Promega), following the manufacturer-recommended protocol. Positive colonies were selected and a PCR re-amplification was performed with the primers M13F and M13R, using a pinch of each positive colony as template, to check for the presence of the 18S rRNA gene in the plasmids. Amplified products were visualized using agarose gel electrophoresis.

Sequence analysis

Clones with inserts were sequenced by the Macrogen Inc. sequencing service (Korea). Removal of vector and primer sequences, as well as quality and orientation analyses, were performed using Seqman software (Lasergene 7). Sequences were aligned using MUSCLE (Edgar 2004) in the Bosque software (Ramírez-Flandes and Ulloa 2008). The percentage of similarity between sequences was calculated and sequences with similarities higher than 99% were considered to belong to the same Operational Taxonomic Unit (OTU). One sequence per OTU was sent to the on line web tools Bellerephon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) in order to identify potential chimeric sequences. Taxonomic affiliations of the sequences were obtained

using BLASTN against the Silva rRNA database (July 2011). Sequences were also submitted (December 2013) to Basic Local Alignment Search Tool (BLAST) analysis against the GenBank database (http://www.ncbi.nlm.nih.gov/). An alignment was constructed including the sequences obtained in this study, those retrieved from the BLAST analysis, and sequences from cultured species from the main groups present in the clone libraries. FastTree algorithm was used to construct phylogenetic trees (Price et al. 2010). Phylogenetic trees were performed with Bosque (Ramírez-Flandes and Ulloa 2008).

RESULTS

Water column conditions

The general physical-chemical-biological conditions of the water column at the sampling site during the times selected to make eukaryotic clone libraries are shown if Figure 2. During January (Fig. 2A) a typical austral summer condition in temperate waters is observed, with relatively high surface temperature and a shallow and pronounced thermocline, high phytoplankton production (~10 mg Chl-*a*/m^3), and the formation of an OMZ at approximately 20 m. Bacterioplankton abundance curve decreases at the OMZ and recovers at 50 m. During April (Fig. 2B) a typical autumn condition is observed. Surface temperature decreases, the absence of upwelling events, and the weak wind mixing maintains a stratified water column where the phytoplankton production is lower (< 1 mg Chlo-*a*/m^3), the thermocline and the oxycline are deeper (approximately 30 m), and oxygen levels from 40 m reach even lower values than in the summer condition (< 2 μM). In fact, the lowest oxygen values are detected during this period. Bacterioplankton abundance is high at depth, reaching the highest value of the whole study period at 50 m. During July (Fig. 2C) a typical austral winter condition is observed, winds cause mixing and a weak and deep oxycline, so that the entire water column becomes oxic. Primary production is the lowest of the period and the bacterioplankton abundance profile is less variable than on the other sampling dates, although it decreases in depth. During October (Fig. 2D) a further increase in the surface temperature and the formation of a thermocline is observed, primary production increases and there is a new development of an OMZ. However, unlike January and April, the OMZ is deeper and at 50 m the oxygen concentration is ~5 μM, almost twice the concentration in January. Bacterioplankton numbers decrease drastically with depth and at 50 m is the lowest abundance of the four sampling dates (< 300,000 cells/ml).

Clone libraries

A total of 1,772 partial-length rRNA gene sequences from 16 clone libraries, corresponding to four sampling dates and 4 different primer sets, were analyzed. We retrieved 488 sequences amplified with primer set A (99 from January, 171 from April, 120 from July and 98 from October), 551 with primer set B (94 from January, 152 from April, 148 from July and 157 from October), 496 with primer set C (142 from January, 154 from April, 91 from July and 109 from October) and 237 with primer set D (78 from January, 103 from April, 38 from July and 18 from October). No chimeras were found, and sequences affiliated to archaea, bacteria and metazoa were excluded for further analysis. We obtained from the primer set A 34 metazoa and 3 archaea sequences; from the primer set B 33 metazoa, 1 bacteria and 19 archaea; from the primer set C 4

metazoa and 387 archaea; and from the primer set D 9 metazoa. Considering the remaining sequences, from the clone libraries constructed with the primer set A we obtained 451 clones, with the primer set B we obtained 498 clones, from clone library C 105 clones, and clone libraries D produced 228 clones. We pooled all the data together and obtained 349 OTUs (99% similarity) and we concentrated on the presence/absence of groups. Most of the OTUs (~88%) were obtained exclusively by a single pair of primers, the rest was obtained by two or more pairs of primers coincidentally (Figure 3A). On the other hand, considering the OTUs per sampling period, most of them were present exclusively in one month and less than 2% were shared between months (Figure 3B).

The abundance of OTUs at each sampling period and its identity is shown in Figure 4. A total of 89 OTUs were present in the January sample, the most prominent groups were Syndiniales, Dinophyceae and Fungi. The April clone libraries had 95 OTUs and were enriched in a particular Stramenopile group: Labyrinthulomycetes. The July sample, when the water column is more oxygenated, was the richest in terms of OTUs and the community seems to be more diverse, with the co-ocurrence of several groups, although Syndiniales is more frequent. Of the 90 OTUs retrieved in October, Syndiniales is predominant, but is enriched in Ciliophora. Based on the classification proposed by Adl et al. (2012), four of the 5 super-groups described for eukaryotes are present on our clone libraries (Opisthokonta, Excavata, Sar, and Archaeplastida) and some groups with uncertain phylogenetic classification (Haptophyta, Telonemia, Cryptophyceae, Ancyromonadida, Biliphytes, and Centrohelida).

Sar, Stramenopiles. Labyrinthulomycetes were by far the most abundant stramenopiles present in the clone libraries (272/total clones), in particular during April (263 clones), and most of these sequences retrieved clustered together within Thraustochytriaceae, apparently forming a group relatively far from cultivated species, representing putative new organisms (Fig. 5). Diatomea was relatively important during July and January, and most of the sequences fell into class Mediophyceae, close to typical large chain-forming phytoplankton (e.g. *Thalassiosira* and *Chaetoceros*). Other algal classes were detected, in particular Chrysophyceae and Pelagophyceae, the first occurred in all the samples and was more abundant in April, and the latter was present only during July. Sequences related with the group of heterotrophic uncultured flagellates designated MArine STramenopiles (MAST, Massana et al. 2004) appeared in all the samples. Different clades have been defined for this group and several of these were present in our clone libraries: during January MAST-3 and MAST-20 were detected, during April MAST-4 and -8, during July MAST-1B and -1C, -3, -4, and -8, during October MAST-1B and -1C, and -4. In general, MAST was more represented during July and October. Few clones of known heterotrophic groups occurred in the libraries: Bicosoecida (in January, April and July) and Peronosporomycetes (in January). Some sequences that appeared during January and October showed the closest identity to a group within Ochrophyta recently named MOCH (Marine OCHrophyta), in particular the clade MOCH-4, that probably represent a new heterotrophic lineage, as they seem to be aplastidic (Massana et al. 2014).

SAR, Alveolates. Considering all the samples together, Syndiniales was the group with the highest representation in the libraries and was relatively abundant during all the sampling dates. Groups-I, -II, -III y -V of syndiniales were found, and group-II was the most abundant during summer, autumn and spring, and group-I was most abundant in winter. Group-V was not observed in summer. Group-II was more abundant in April, when it accounted for more than

82% of the Syndiniales clones, but had more OTU representation in January (Fig. 6). Several clades within the group-II were present, but some were exclusive of certain periods (e.g. clades 12, 16 and 26 in July or 21 and 44 in April), some sequences clustered within group-II but did not show a clear affiliation with any clade previously described. In Group-I there were no exclusive clades, but in January and April there were fewer clades present. On the other hand, a relatively large proportion of the alveolates were part of Dinophyceae, especially from the January libraries, most of them were Gymnodinophycidae, but related mostly to environmental clones, several retrieved from other oxygen deficient sites. There were some sequences obtained in our study that clustered within unknown Dinophyceae clusters. Ciliophora was detected in all the samples, but they were especially abundant in October, when they almost doubled the number of Syndiniales in terms of clone number. Most of the ciliates in January, April and July were Spirotrichia, although some Litostomastea appeared in July, but the bulk of ciliate clones in October are related to a different group: Phyllopharyngea, particularly Suctoria, and the sequences we retrieved cluster all together in a group with non-cultivated representatives. An Ellobiopsidae (incertae sedis in Alveolata) clone also appeared in the summer sample.

SAR, Rhizaria. Only few cercozoan and radiolarian clones appeared in our clone libraries, but they were both present at the four samples used in this study. Radiolaria was represented by Polycystinea (January and October), Acantharia (April), RAD-A (July), and RAD-B (April, July and October). Cercozoa was represented mainly by Thecofilosea and Silicofilosea, but none of them was detected in April. Thecofilosea was relatively abundant in October, with all clones clustering close to *Cryothecomonas*.

Opisthokonta. Most of the Opisthokonta clones were Fungi, although there were very few Holozoa in January and July that clustered within Choanomonada (choanoflagellates). Considering just the Fungi sequences, 93.1% were Ascomycota, the rest was Basidiomycota and Chytridiomycota (basal fungi). Basidiomycota was represented by Ustilaginomycetes in April and July, and our clones were related to environmental sequences retrieved from the deep ocean and the Arabian Sea OMZ (Fig. 7). Chytridiomycota was detected in October and related to environmental sequences retrieved from marine sediments. Ascomycota was absent in July and October, but very abundant in January and April, especially in January. Within Ascomycota, Sordariomycetes was predominant; we also found in summer Eurotiomycetes, Dothiomycetes, and Saccharomycetes, while in autumn we found Eurotiomycetes and Saccharomycetes (Fig. 7).

Other eukaryotes. The super-group Excavata was also retrieved from our clone libraries, represented by the groups Jakobida and Diplonemea. Only during autumn were there sequences with 99% of identity with *Jakoba libera*, while only in winter and spring we found Diplonemea sequences with high percentages of identity with environmental clones retrieved from deep Pacific Ocean samplings. The super-group Archaeplastida was present during all the sampling months, but was very abundant during winter and most of the clones were Chlorophyta, in particular Mamiellophyceae, and closely related to the genera *Micromonas*, *Bathycoccus* and *Ostreococcus*. The recently proposed super-group CCTH (Cryptophyta, Centroheliozoa, Telonemia, Haptophyta, and perhaps including Biliphytes) (Burki et al. 2009) was represented mainly by Haptophyta, that was more abundant in winter with clones with 99% of identity with *Phaeocystis globosa*; and Cryptophyta, that was present exclusively in the winter sample, some clones very close to *Teleaulax* species (100% of identity) and others clustered within

Katablepharidales. Ocasionally, some very few clones of Telonemia in January, Centroheliozoa in April and Biliphytes in July were detected.

DISCUSSION

The goal of this research was to survey the protistan diversity in a seasonal OMZ coastal area during four sampling dates in a year, and consider the contrasting physical-chemical-biological conditions in the water column. Since one of our objectives was to cover the largest diversity from our clone libraries and retrieve as many phylotypes as possible, we chose the use of a multiple-primer approach (Stoeck et al. 2006). It is well known that the PCR steps introduce a bias when studying a microbial community based on sequences recovered from the environment. Primers, even the so-called "universal", do not amplify equally the different groups (Potvin & Lovejoy 2009). This is particularly true in eukaryotes, as it is common that different taxa vary in their number of copies of the 18S rRNA gene (Zhu et al. 2005). Our results confirm the effectiveness of the multi-primer approach in achieving richer clone libraries (Jeon et al. 2006), since only one OTU was shared simultaneously by all the pair of primers, and ~88% of the 349 OTUs were retrieved only with one primer pair, thus by using several primer sets instead of one we encompass a greater diversity of picoeukaryotes. However, the clone libraries originated from different pairs of primers that were enriched roughly evenly at higher taxonomic ranks, even from the primer set biased toward ciliates, so first, it is possible that the use of several primers enhances the diversity coverage at lower taxonomic levels. In fact, Edgcomb et al. (2011) found that four different primer sets had differential recovery of ciliate taxa, but the number of clones for total ciliates was almost even; and second, the ciliate primers are not specific and exclusive for that group. We also have to mention that the pair of primers biased for ciliates (primer set C) amplified mostly archaeal sequences, as far as we know this has not been highlighted by previous studies that have used these primers (Behnke et al. 2006; Stoeck et al. 2007; Zuendorf et al. 2006).

The samples that we used for the present study were from 50 m depth at a highly productive coastal site, so the photic layer is shallow and never reaches the sampled depth. Considering this, it is expected that the active picoeukaryotic community is constituted basically by heterotrophic organisms and that sequences related with eminently phototrophic groups present in our clone libraries are exogenous cells derived from the photic zone that are there through mixing of the water column and/or in transit to the sediments (Stoeck et al. 2007). Most of the clones related to typical algal lineages (e. g. Bacillariophytina, Pelagophyceae, Chlorophyta) were present in the austral wintertime (July), when northerly winds are predominant, favoring mixing of the water column through downwelling events (Sobarzo et al. 2007). The phytoplankton community during winter off the Concepción Bay is dominated by smaller cells, including picophytoeukaryotes, that reach their highest abundances in this period, Mamiellophyceae (Chlorophyta) is the main component of these community (Collado-Fabbri et al. 2011). This is consistent with what was shown by our clone libraries and supports the idea of photic zone derived cells.

Another set of sequences detected was related to groups known to have phototrophic, heterotrophic and/or mixotrophic representatives. Dinoflagellates, within Alveolata, have very few species that are exclusively phototrophic, and in marine environments heterotrophic (non plastid containing) species could be dominant (Gomez 2012). Moreover it has been suggested

that all dinoflagellates are capable of phagotrophy (Sherr et al. 2007). Dinoflagellate sequences are frequent in deep-sea and oxygen-deficient waters surveys (Sauvadet et al. 2010), we have to consider though that they could be overestimated due to the large number of 18S rRNA gene copies or that they could be nonindigenous phototrophs (Stoeck et al. 2007). But beyond the presumable overestimation of this group, it is highly possible that the dinoflagellates found are playing an active role, and in this sense special consideration should be given to several groups with no cultivated organisms and close affiliation with clones retrieved from oxygen deficient areas. Chrysophyceae, within Stramenopiles, are organisms known as "golden algae", but have several mixotrophic and heterotrophic genera. Some of the sequences that we obtained showed close affiliation (99% identity) with *Paraphysomonas* sp., colourless heterotrophic cells that feed via phagocytosis on bacteria and other particles, but the rest are only related to environmental clones within new clades and their ecological role is still unknown. Cryptophyta and Haptophyta, two closely related groups (Burki et al. 2009), are nearly all photosyntethic, but mixotrophy is common and some species are heterotrophic, in fact, several of the Cryptophyte sequences retrieved were Katablepharid, a group of heterotrophic flagellates. Haptophyte sequences, on the other hand, were probably photosynthetic, since they were affiliated with the algal genera *Phaeocystis* and *Chrysochromulina*, *Phaeocystis* was especially abundant during July, which is consistent with the possible presence of exogenous cells coming from the photic zone in wintertime.

Most of the sequences found, as was expected, were related with heterotrophic groups. In this sense, we can classify them, according to their ecological role (trophic strategy), as: parasites, phagotrophs and decomposers (saprotrophs degrading organic matter). Considering all our clone libraries, the most frequent organisms were alveolates, specifically Syndiniales. This group is composed exclusively of marine parasites and very few species have been described, but sequences within this group are extremely common and abundant in molecular surveys made on diverse marine environments, specially phylotypes related to groups I and II (Guillou et al. 2008), and the same trend is shown in this work. We can make a relation between the oxygen levels and the relative abundance of group I and II: during January and April, when the oxygen reached the lowest values, group II is by far predominant over group I, and only when the water column is well oxygenated (July) does group I surpass group II (Fig. 6). Our results are a bit different from what has been previously described, because group I has been indicated as predominant in anoxic and suboxic ecosystems (Guillou et al. 2008), moreover, group II has been absent in some surveys on oxygen deficient environments (Behnke et al. 2006, Zuendorf et al. 2006). Parris et al. (2014) also found group II to be predominant at the permanent OMZ off Northern Chile, so this discrepancy could be based on the host organisms and their specificity to some habitats, in this case, the Eastern South Pacific OMZ, although the clade composition is different. It seems that group II organisms parasitize only dinoflagellates, while group I organisms seem to be more generalists, infecting a wider diversity of organisms including radiolarians, ciliates, and fish eggs (Dolven et al. 2007; Massana 2011). But considering the enormous genetic diversity of Syndiniales estimated from environmental sequences, we still ignore the extant of organisms parasitized. Moreover, it has been suggested that these parasites do not co-evolve with their host, making them very opportunistic and capable of infecting a diverse spectrum of organisms (Guillou et al. 2008). So it may be that the apparent specialization of group I in low-oxygen environments is an artifact of the limited number of environments studied and the lack of knowledge about parasite life cycles and their hosts. Ellobiopsidae are another parasite group that is found in January and known to be ectoparasites of pelagic

crustaceans, especially copepods, that reduce fecundity; as they produce spores, it is possible that these small cells have been captured in our clone libraries (Albaina & Irigoien 2006). Fungi, on the other hand, can also live associated with other living organisms parasitizing them. Chytridiomycota account for most of the pelagic fungal parasites known and they can infect phyto- and zooplankton (Jobard et al. 2010, Sime-Ngando 2012). Chytrids, nonetheless, seems to be particularly sensitive to low oxygen levels, since chytrid-like sequences are not common in suboxic and anoxic environments (Jebaraj et al. 2010; Jobard et al. 2010; Richards et al. 2012); in our study we detected a couple of sequences in January and one sequence in October related with Chytridiomycota, both conditions were suboxic.

Sequences related with phagotrophic organism were very common. Within Alveolata, Cilliophora are known consumers of bacteria and so they represent a major trophic link in the microbial food web, and they seem to be important consumers in oxygen-deficient environments, where they are often the most abundant group found in terms of clones retrieved (Alexander et al. 2009; Behnke et al. 2006 and 2010; Zuendorf et al. 2006), this is also supported by the fact that several ciliate species are anaerobic. In our study, ciliates only dominated the clone libraries during October (56% of the clones retrieved) and had the highest representation in terms of OTUs. The ciliate community during this sampling date is phylogenetically homogeneous, considering that the bulk of the clones retrieved are Phyllopharyngea and fell particularly within the Suctoria group. Marine Suctoria species are commonly epibionts forming symbiotic relationships with crustaceans (Fernandez-Leborans & Tato-Porto 2000) and are represented by sessile microbes that produce planktonic cells by budding to reproduce themselves (Zhao et al. 2013), so the clones retrieved might correspond to swarmer forms of Suctoria. The super-group Rhizaria, mainly ameboid protists that feed via phagocytosis, was poorly represented in our clone libraries, and few Radiolaria sequences appeared. Most of the rhizarian sequences retrieved from marine environments are radiolarian (Massana 2011), in spite of the fact that radiolarian species are part of the microplankton, so little is known about the organisms behind the environmental clones, in our case some uncultured radiolarian groups were retrieved, especially the RAD-B clade. Cercozoa, less common rhizarian clones on environmental picoplanktonic libraries, was mostly represented by the genera *Cryothecomonas* and could be important during October. Although there are parasitoid *Cryothecomonas* species, it seems that most of them are predatory, and it has been observed in marine samples from in situ hybridization analyses that most of the *Cryothecomonas* cells are smaller than previously thought, suggesting that there are new species in the < 5 μm size fraction or unknown life stages in this genus (Thaler & Lovejoy 2012). Within Stramenopila the bacterivorous group MAST (MArine STramenopiles) was also found, although not as abundant as one would expect, considering that they are usually well represented in marine clone libraries (Massana et al. 2006). Different clades have been described for this group and it seems that oxygen is a crucial parameter that restricts their ecological distribution (Massana et al. 2013). In our survey we found three clades (and subclades) known to have anoxic sequences: (sub) clade 1C, 3 and 20; the latter being typical and perhaps exclusive of anoxic habitats (Massana et al. 2013).

Decomposer groups detected on our clone libraries are mainly two: Fungi and Labyrinthulida (Stramenopiles). Fungi usually have the saprotroph way of feeding, which implies the production of exoenzymes to degrade organic matter and take up the nutrients osmotrophically (Richards et al. 2012), so the presence and diversification of Fungi in the water column requires nutrient-rich substrate, such as the decaying particulate organic matter in subsurface waters below a highly productive photic zone, like the one present during January in

our study site, when we found higher representation of Fungi-like sequences; in fact, Gutierrez et al. (2011) found at the same site (station 18) the highest abundance of hyphae and concentration of a fungal biomarker coinciding with the most productive periods. Is interesting though, that the closest affiliation of many of our sequences are cultivated non-marine species (Fig. 7), this might indicate enormous underestimation of Fungi in the ocean and that the databases do not capture the full environmental diversity. On the other hand, and very distant in the Eukaryotic tree, is the stramenopile group Labyrinthulida, known as fungoids, because of the same trophic strategy. Evidence of the importance of this group in marine ecosystems has increased in the last decades, together with the extension of their phylogenetic diversity from molecular surveys; new sub-groups have been observed, especially in oxygen deficient environments (Raghukumar & Damare 2011). Some studies have revealed also that Labyrinthulomycetes are present in high numbers in the water column and that they can occasionally even exceed the bacterial biomass (Raghukumar & Damare 2011), although they are seasonally variable and related with high photosynthetic production (Li et al. 2013, Raghukumar & Damare 2011). This is consistent in some ways with our findings; Labyrinthulomycetes appeared to be very abundant in only one sampling date (April). Our results suggest that when the system is more productive and there is more organic material in deeper layers, the decomposer picoeukaryotic community is favored, but Fungi and Labyrinthulomycetes populations, competing for the same ecological niche, might vary according to some environmental parameters (such as oxygen) or according to the quality and composition of the organic matter they can degrade. Labyrinthulida sequences retrieved in this study seems to represent a new clade within Thraustochytrids (Fig. 5), a group of biotechnological interest because it includes important producers of polyunsaturated fatty acids (Miller et al. 2007).

Our results revealed that the picoeukaryotic community inhabiting the deep layer of a coastal water column associated with a seasonal upwelling regime and, therefore, seasonal OMZ, varied over time during the period of a year. This variability is evident not only in terms of higher taxonomic levels (i.e super-group, class, order), but also in terms of lower taxonomic levels (i.e. genera or species), since most of the OTUs we obtained were not shared between sampling periods (Fig. 3B). The shifts in community structure between the samplings can be a response of the community to several parameters, but are likely to be associated with O_2 concentration. Previous studies have shown that protists exhibit habitat specialization following biogeochemical gradients, even in sites near to each other, and in relation to oxygen availability (Orsi et al. 2011; Orsi et al. 2012; Parris et al. 2014). We found that not only is the taxon distribution variable, but so are the trophic strategies, which opens a wide variety of questions, considering the scarce knowledge of the organisms behind most of the sequences found and the novelty of some groups. With respect to phagotrophs, represented here mainly by ciliates, some rhizaria and MAST, we still do not know exactly what organisms they prey on, if they have any specificity, and what their consumption rates are. Massana et al. (2009) found using a combination of fluorescent *in situ* hybridization and fluorescently labeled bacteria that different MAST clades had different prey preference and ingestion rates. Since bacteria and archaea are the most likely prey of these pico-sized consumers, picoeukaryotes should be of major interest for study, considering the important biogeochemical processes carried out by non-eukaryotic microbes inside the OMZ. Parasites and decomposers, on the other hand, play a crucial role in the carbon cycle; they can increase the pool of dissolved organic matter by releasing cellular contents of their hosts and, therefore, modify the microbial loop and the carbon flux to the sediments, and even increase the CO_2 release in the case of decomposers degrading refractory

organic matter (Jobard et al. 2010; Sime-Ngando 2012). Heterotrophic protists must be included in the marine carbon flux models (beyond being “bacterivorous flagellates”), so it is crucial to increase the volume of sequence and genomic data, and develop efforts in cultivate them to define their physiological ranges and ecological niches, and even their metabolic pathways; this combined with efforts to know their actual abundances, should be the approach taken to understand their actual role in the environment, especially in OMZs.

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FIGURE LEGEND

Fig. 1. Location of Station 18, time-series sampling station over the coastal shelf off Concepción (Central Chile).

Fig. 2. Profiles showing physical-chemical-biological characteristics of the water column at each sampling date. Clone libraries were sampled at 50 m depth. **A.** January. **B.** April. **C.** July. **D.** October.

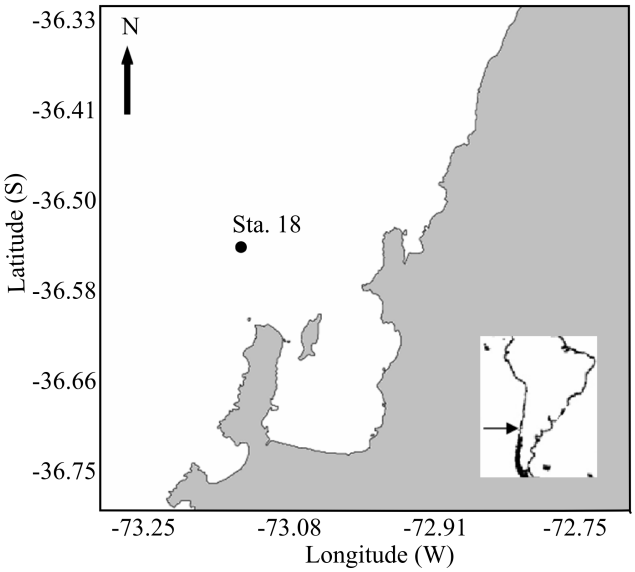
Fig. 3. Diagrams showing the distribution of the 349 OTUs obtained in the four clone libraries. **A.** Distribution of OTUs per pair of primers. **B.** Distribution of OTUs per sampling period.

Fig. 4. Abundance of OTUs of the most representative taxonomic groups identified on each sampling date.

Fig. 5. 18S rRNA gene phylogeny of Stramenopile group Labyrinthulida inhabiting the study site, inferred by FastTree algorithm. One sequence per OTU is represented on the tree, and colored icons correspond to each sampling date (red: January; yellow: April; blue: July; orange: October). Within Labyrinthulida, support values over 0.75 are shown.

Fig. 6. Abundance of Syndiniales Group I and II in terms of OTUs retrieved at the four dates sampled in this study.

Fig. 7. 18S rRNA gene phylogeny of Fungi inhabiting the study site, inferred by FastTree algorithm. One sequence per OTU is represented on the tree, and colored icons correspond to each sampling date (red: January; yellow: April; blue: July; orange: October). Support values over 0.75 are shown.



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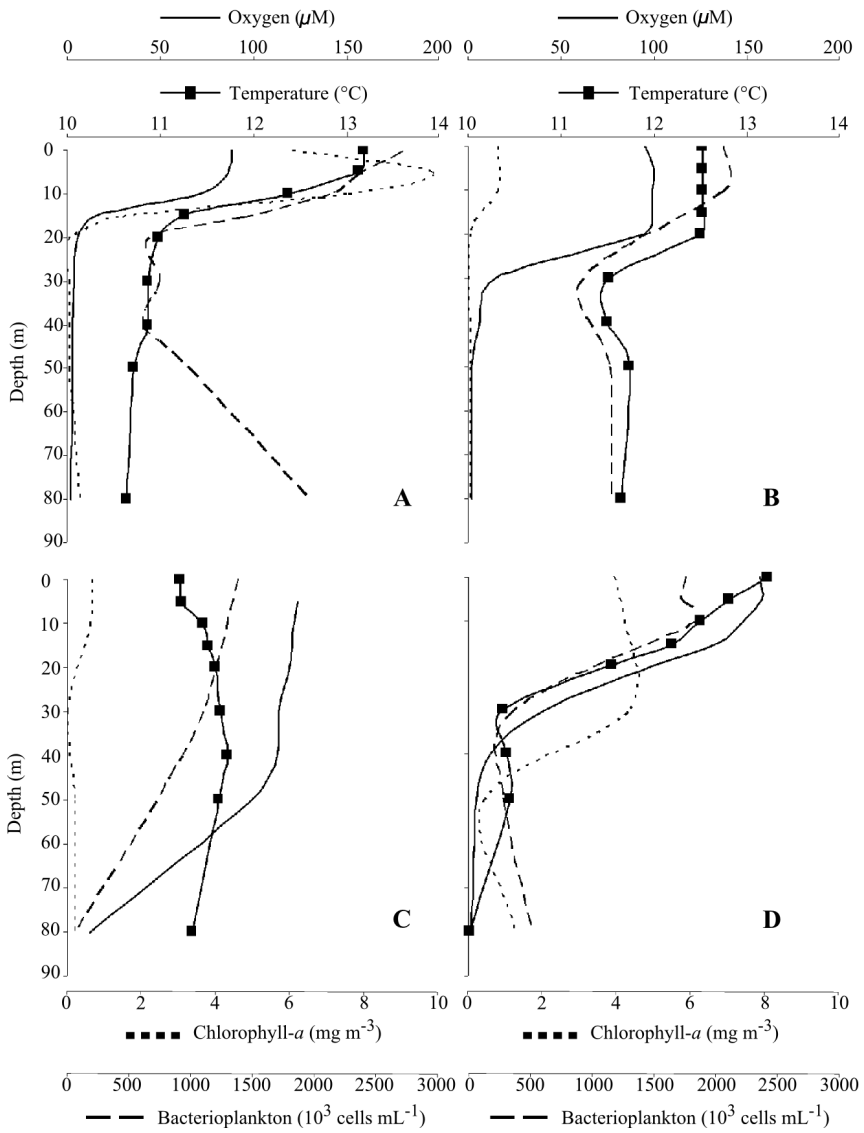

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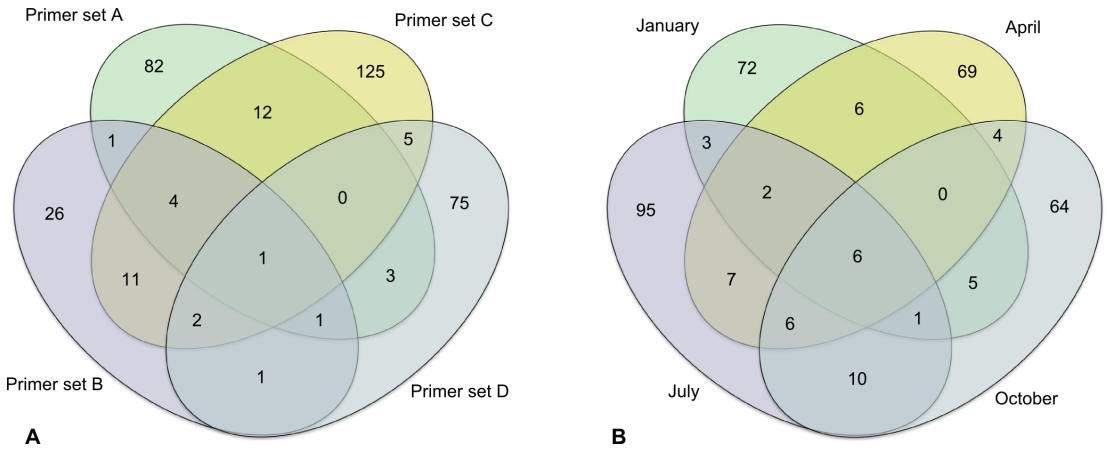

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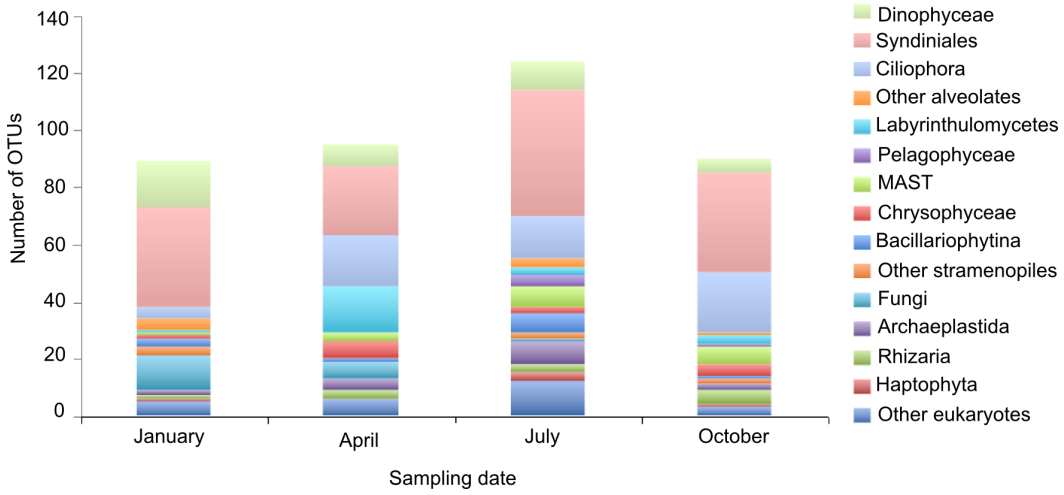

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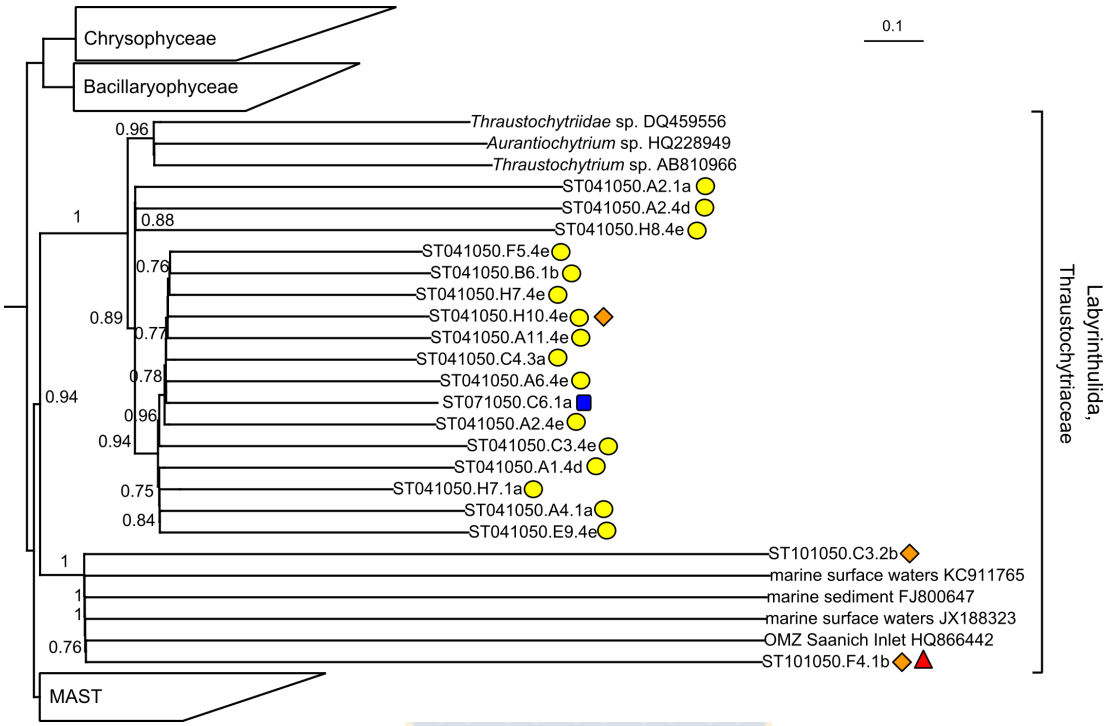

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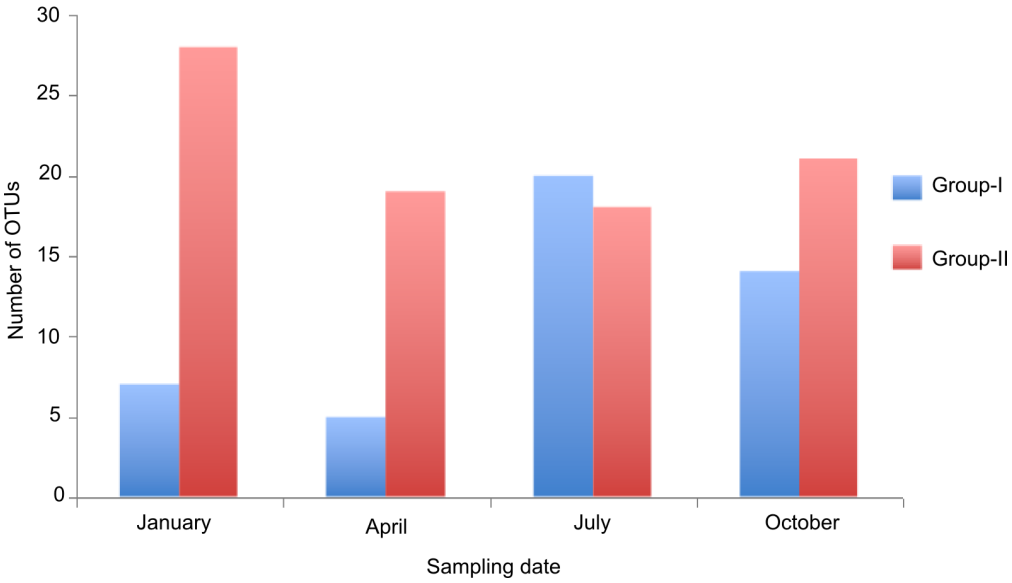

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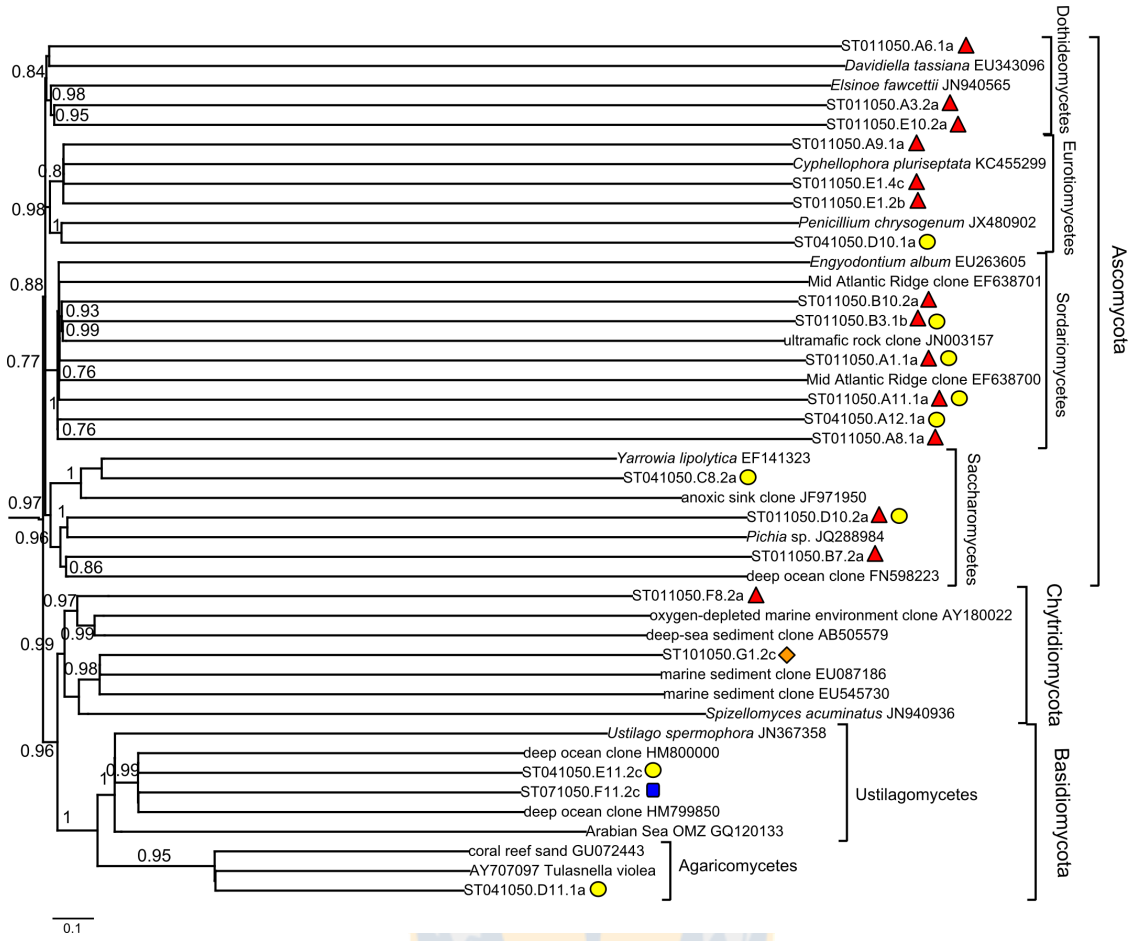

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5. DISCUSION

A partir de fines de los años 90´s hasta hoy se ha generado una inédita expansion del conocimiento acerca de la vida microbiana. Se ha podido acceder, en gran medida gracias al desarrollo de técnicas moleculares, a la inmensa diversidad microbiana, la diversidad de habitat que han colonizado y la importancia que tienen en procesos biogeoquímicos globalmente. Sin embargo, este aumento en el estudio de los organismos microbianos se ha enfocado principalmente a la fracción procarionte (bacterias y archaeas). Curiosamente, los protistas (organismos eucariontes unicelulares), siendo tan microorganismos como los procariontes, han sido objeto de menor interés científico y los avances en su estudio han sido más lentos, a pesar de su rol ecológico crucial (Caron et al. 2009). En el ambiente marino, los protistas fotosintéticos son los principales responsables de la productividad primaria, por ende, de la inyección de carbono orgánico al océano y, en acoplamiento, de la biogeoquímica de otros elementos como el nitrogeno, el fósforo y el sílice (Field et al. 1998). Es relativamente conocido el rol del fitoplancton eucarionte de gran tamaño (ejemplo diatomeas) en las tramas tróficas marinas, pero es escaso el conocimiento acerca del picofitoplancton eucarionte, a pesar de ser los principales contribuyentes en la fijación de CO_2 en los vastos ambientes oligotróficos oceánicos (Grob et al. 2007b, Jardillier et al. 2010). Por otro lado, el funcionamiento del resto del sistema depende en gran medida del proceso opuesto (y complementario) a la fotosíntesis, que es la oxidación del carbono mediante la respiración de materia orgánica. Este proceso es llevado a cabo fundamentalmente por los organismos heterótrofos que, en el caso de los protistas, son muy diversos, no solo filogenéticamente (Fig. 1.2) sino también en términos de estrategias tróficas. La predación es la forma más conocida de alimentación heterótrofa y las otras estrategias nutricionales han sido mucho menos estudiadas, lo que no significa que sean menos importantes para el sistema. La actividad saprófita y parasítica de protistas en general no es considerada en modelos de tramas tróficas marinas (Worden et al. 2015), a pesar de que existe cada vez más evidencia de la presencia y actividad de hongos (principales eucariontes descomponedores) (Gutierrez et al. 2010 y 2011) y de que la mayoría de las secuencias eucariontes extraídas del ambiente marino pertenecen a alveolados marinos parásitos (Guillou et al. 2008). Esto no hace más que confirmar la

necesidad de aumentar nuestro conocimiento acerca de las comunidades microbianas eucariontes, sobre todo de la fracción más pequeña de tamaño, que es la más críptica.

En esta tesis se buscó caracterizar la estructura comunitaria de la fracción eucarionte de la comunidad microbiana picoplanctónica que se encuentra en una area costera de Chile Central. El area de estudio tiene características que la hacen tener gran relevancia en términos biogeoquímicos a nivel global. Se trata de una zona de surgencia costera con regimen estacional, siendo un lugar donde existe una alta tasa de fijación de carbono atmosférico (altamente productiva biológicamente) y, por tanto, altas tasas de degradación de material orgánico. Una de las principales implicancias de esto es el desarrollo de una ZMO subsuperficial siguiendo la estacionalidad de la surgencia, donde se albergan procesos particulares como la desnitrificación. En general, la estructura de los ciclos biogeoquímicos del C y el N en el area de estudio es de alto interés científico, no solo por las rutas que siguen estos elementos en la trama trófica, sino también por la influencia que tienen en el clima global y sus cambios a futuro. Todas las reacciones biogeoquímicas claves en este aspecto son llevadas a cabo por microorganismos, y el rol de la comunidad microbiana eucarionte es fundamental, ya sea directamente a través de la fotosíntesis o la degradación de material orgánico, o indirectamente a través del control de otros organismos (uni o pluricelulares) por predación o parasitismo. Siendo parte fundamental del funcionamiento ecológico del sistema, los protistas picoeucariontes no han sido poco caracterizados, esta tesis representa el primer estudio de la estructura comunitaria de picoeucariontes en la zona y es uno de los pocos que describe la comunidad picoeucarionte en ZMOs, sobre todo con variabilidad estacional.

Uno de los objetivos de esta tesis fue la descripción de la comunidad picofitoplanctónica en la capa fótica de la columna de agua en la zona de estudio. Tal como se esperaba, y confirmando la primera hipótesis, la comunidad picofitoeucarionte está dominada por miembros de Chloroplastida, específicamente Mamiellophyceae, y más específicamente por los 3 géneros más importantes cuantitativamente en ambientes costeros: *Micromonas*, *Bathycoccus* y *Ostreococcus*. Lo novedoso de nuestros resultados es que *Ostreococcus* se encuentra como dominante en términos de abundancia celular durante todo el año, presentándose como un componente clave en la comunidad picofitoplanctónica en la zona de

estudio. *Ostreococcus* normalmente está presente en zonas costeras, pero previamente ha sido observado en altas abundancias de manera esporádica ("peaks") (Countway & Caron 2006, O´Kelly et al. 2003), no con altas abundancias de manera constante en el tiempo como es este caso. Es interesante que Vaulot et al. 2012 observaron en otro sitio de la costa de Chile Central mediante una combinación de "sorting" por citometría de flujo, WGA y pirosecuenciación 454, que la mayor parte de las lecturas correspondía a *Bathycoccus*. Es posible que las condiciones locales en la Bahía de Concepción sean particularmente favorables a *Ostreococcus*, pero también es necesario considerar que las estaciones costeras que incluyó el crucero BIOSOPE 2004 en que se tomaron las muestras para aquel estudio se encontraban en otro punto de la costa de Chile (33ºS aproximadamente) y que el muestreo se realizó en época estival, cuando esperaríamos que la comunidad picofitoeucarionte no estuviera en su mayor desarrollo. Nos inclinamos a pensar que la explicación a esta dominancia permanente de *Ostreococcus* tiene que ver la fuente de nitrógeno, ya que en la zona de estudio las concentraciones de nitrato son relativamente altas durante todo el año, incluso en períodos de no-surgencia cuando la producción se supone está sustentada por fuentes de nitrógeno regenerado (amonio). Existen antecedentes de que *Ostreococcus* responde bien a fuentes de nitrógeno "nuevo" (Worden et al. 2004), lo que se suma a la correlación positiva que encontramos en esta tesis entre la abundancia de *Ostreococcus* y las concentraciones de nitrato y nitrito. No existen muchos antecedentes acerca de la biogeografía de *Ostreococcus* y de los factores que regulan su distribución, pero actualmente se reconocen claramente 2 clados dentro de este género: clado OI y clado OII (Demir-Hilton et al. 2011). Estos 2 clados tienen probablemente importantes diferencias fisiológicas, ya que muy rara vez coinciden en un mismo ambiente, OI parece ser un clado costero y OII un clado oceánico (Demir-Hilton et al. 2011, Simmons et al. 2016). Simmons et al. (2016) realizaron un estudio biogeográfico de ecotipos de picoprasinofíceos en el Pacífico Norte y establecieron que las mayores abundancias de células del clado OI, que es el que encontramos de manera exclusiva en nuestro ambiente de estudio, se relacionan con el aumento de nitrato y amonio en la columna de agua. Se ha sugerido previamente que la diferencia ecotípica entre estos 2 clados tiene que ver con la adaptación a la luz (Rodriguez et al. 2005, Six et al. 2008), sin embargo, al parecer la concentración de nutrientes sería un factor que controla la distribución de cada uno de estos clados (Simmons et al. 2016).

Existe una clara estacionalidad en la abundancia celular de la comunidad picofitoplanctónica en la zona de estudio, tendencia observada en otras zonas costeras caracterizadas por una surgencia estacional (Rodríguez et al. 2006, Sherr et al. 2005). La abundancia de picofitoeucariontes es mayor en los meses de otoño-invierno y menor en primavera-verano. Es evidente por la correlación negativa entre la clorofila-*a* > 3 µm y la abundancia de *Ostreococcus* y *Synechococcus* (principales contribuyentes a la abundancia picofitoplanctónica en este caso) que cuando el fitoplancton de gran tamaño domina, las abundancias de picofitoplancton son bajas. Podríamos explicar esta alternancia en gran medida por la disponibilidad de nutrientes (Marañon et al. 2015). Si el picofitoplancton crece a una mayor tasa que el fitoplancton de mayor tamaño a bajas concentraciones de nutrientes, pero este ultimo tiene tiene una tasa de crecimiento máximo mayor que el primero, entonces el picofitoplancton dominará sobre el fitoplancton de mayor tamaño en bajas concentraciones de nutrientes y los segundos dominarán sobre los primeros en mayores concentraciones de nutriente. El picofitoplancton habrá llegado a su maxima tasa de crecimiento en una concentración de nutrientes en que el fitoplancton de mayor tamaño aún puede seguir creciendo (Lalli & Parsons 1997). Ahora bien, las concentraciones de nitrato que se detectan en la zona de estudio durante los meses de primavera-verano, cuando domina el fitoplancton de gran tamaño, son muy bajas, porque el input de nitrato dado por la surgencia ha sido rápidamente consumido por estos organismos. ¿Podría entonces, bajo estas condiciones de bajo nutriente, desarrollarse secundariamente la población picofitoplanctónica? Aquí es donde podría ser clave la razón N/P, que tiende a ser más baja durante los meses de primavera-verano, lo que favorece a organismos que crecen en "bloom", ya que tienen mayor requerimiento de fósforo por la mayor producción de ácidos nucleicos que implica el crecimiento exponencial; no así a los que tienen una estrategia de vida más "sobreviviente", como el picofitoplancton, que tendría una mayor producción de pigmentos y proteínas en relación al microfitoplancton y, por tanto, requeriría más nitrógeno (Arrigo 2005).

En relación al segundo objetivo de esta tesis, que fue caracterizar la comunidad picoeucarionte de la capa profunda del sitio de muestreo, podemos decir que, siendo la comunidad picoeucarionte de la capa profunda eminentemente heterótrofa durante todo el año, existe una variación en los grupos taxonómicos dominantes y en la funcionalidad de la

comunidad. Destaca la presencia de grupos saprófitos, como serían los hongos y los fungoides Labyrinthulomycetes, durante los meses de enero y abril, lo que coincide con un mayor flujo de materia orgánica desde la capa fótica, debido a la gran producción primaria predominante en los meses de primavera-verano. Gutierrez et al. (2011) determinan en el mismo sitio de estudio la biomasa fúngica durante un periodo de casi 3 años, y ésta se concentra claramente entre los meses de septiembre y marzo, disminuyendo drásticamente el resto del año. El peak que encontramos de Labyrinthulomycetes es en abril, por alguna razón este grupo, que tendría un nicho ecológico muy similar al de los hongos, a pesar de estar muy distantes en el árbol filogenético eucarionte, se vería favorecido en este momento de muestreo. La razón por la cual hay un cambio en la comunidad saprófita picoeucarionte de un momento de muestreo a otro puede obedecer a multiples factores, entre ellos la cantidad y calidad de la materia orgánica presente, o la concentración de oxígeno, que es la más baja registrada en los 4 muestreos. Es fundamental estudiar y eventualmente aislar los organismos detrás de las secuencias aisladas de Labyrinthulomycetes, no solo porque se trataría de secuencias de organismos previamente no descritos, sino también por la potencial importancia biotecnológica que representan. Los Labyrinthulomycetes, específicamente del grupo de los Thraustochytridos, que es el grupo al que se afilian las secuencias aisladas en el marco de esta tesis, son productores de ácidos grasos poliinsaturados (AGPI) y carotenoides de gran importancia comercial (Miller et al. 2007). De hecho, Pino et al. (2015) aislaron algunas cepas de estos fungoides marinos desde algunas playas en la costa cercana a nuestra zona de estudio, comprobando su capacidad de producir AGPIs en cultivos.

Es difícil poder determinar la acción específica del oxígeno como modelador de la comunidad picoeucarionte en la capa profunda del sitio de estudio. El único momento de muestreo que muestra una clara diferencia en la concentración de oxígeno es el mes de julio, cuando la columna de agua está bien oxigenada. Es en este muestreo que se observa la mayor diversidad de grupos taxonómicos, destacando la presencia de Syndiniales del grupo I, protistas de acción parásita sobre una amplia diversidad de hospederos (Dolven et al. 2007). Sin embargo, es importante considerar que la oxigenación de la columna de agua está dada en gran medida por la mezcla y hasta eventos de hundimiento de agua, por lo tanto, es altamente probable la presencia exógena de organismos desde aguas superficiales, lo que es evidente por

la presencia de secuencias de grupos eminentemente fotosintéticos en el muestreo de julio. Es difícil en este caso poder "limpiar" los resultados obtenidos de la presencia de organismos provenientes de la capa superficial, y tal vez la mayor diversidad esté dada por el ingreso de organismos exógenos a la capa afótica de la columna de agua. De todos modos, la fagotrofía como función se destaca en los meses de julio y octubre, con la mayor presencia de grupos MAST y con una gran dominancia del grupo Ciliophora. Es durante estos muestreos, además, que se encuentra la menor concentración de bacterioplancton, lo que puede relacionarse con mayores tasas de predación sobre esta fracción microbiana (Cuevas et al. 2004).

La presencia de organismos picoeucariontes en las costas de Chile Central había sido estudiada previamente en cruceros por el Pacífico, específicamente el BIOSOPE (mencionado previamente). Los estudios de picoeucariontes en el marco del BIOSOPE 2004 fundamentalmente se enfocaron a la fracción fotosintética (Masquelier & Vaulot 2008, Shi et al. 2009 y 2011, Vaulot et al. 2012) y muestran en general relativamente bajas abundancias de picofitoeucariontes en superficie (los muestreos se hicieron en época estival), cuya identidad sería Mamiellophyceae (Shi et al. 2009), particularmente *Bathycoccus* (Vaulot et al. 2012). Por otro lado, ha existido un monitoreo de picofitoeucariontes por citometría de flujo en el marco de la serie de tiempo COPAS en la Estación 18 desde el año 2002 a la fecha, lo que ha sido fundamental para tener una línea base del estudio de estos organismos. Sin embargo, la resolución taxonómica de la comunidad picofitoeucarionte y su variación en el tiempo nunca había sido develada, y menos aún la presencia de picoeucariontes heterótrofos. Esta tesis doctoral entrega información valiosa acerca de la estructura comunitaria picoeucarionte en un sistema variable estacionalmente. En cuanto a la comunidad picofitoeucarionte, es necesario realizar mayores estudios del que parece ser un componente clave: *Ostreococcus*; especialmente intentos de aislamiento y cultivo, para comprender mejor a esta cepa de la célula eucarionte más pequeña que se conoce. El picofitoplancton puede no ser considerado importante en un sistema como el estudiado, donde la gran mayoría de la producción se concentra en los meses donde el microfitoplancton domina la comunidad fotosintética, sin embargo, el sistema se mantiene el resto del año básicamente por la fijación de CO_2 por parte del nano y picofitoplancton (Vargas et al. 2007). La red trófica en estos periodos es más bien microbiana y la mayor parte del carbono sería reciclado en la zona fótica (Vargas et al. 2007),

pero hay autores que proponen que la transferencia de carbono picofitoplanctónico a la capa profunda podría ser mayor a lo que clásicamente se ha asumido (Richardson & Jackson 2007). Por otro lado, nuestro análisis de la comunidad picoeucarionte heterótrofa confirma que los protistas consumidores son mucho más que flagelados bacterívoros como clásicamente se les considera en los modelos tróficos marinos. Hemos detectado organismos saprófitos y es necesario conocer qué tan importantes son en la degradación de la material orgánica particulada, si compiten por el mismo sustrato con las bacterias o incluso entre ellos (hongos vs Labyrintúlidos). La acción parásita de los protistas también debe ser considerada en los flujos y destinos del carbono orgánico, ya que al matar a sus hospederos, los parásitos liberan sustrato para la acción microbiana y modifican la red trófica (Sime-Ngando 2012).

Sin duda, el real desafío que tenemos como científicos es lograr dar un paso más allá y avanzar desde la biología y el comportamiento de los organismos o las poblaciones a comprender su rol en la dinámica de procesos de escala global y en los potenciales cambios en el ecosistema. Sin embargo, para esto lo mínimo es contar con una línea base de información acerca de la diversidad, dinámica e interacciones de los integrantes del ecosistema marino. En este sentido, los protistas han sido ampliamente desestimados, a pesar de la importancia que tienen en los modelamientos de flujos de carbono a través de sus diversos comportamientos tróficos (Caron et al. 2009, Worden et al. 2015). La pregunta es cómo abordar el estudio de los organismos eucariontes unicelulares, sobre todo los de menor tamaño, si la gran mayoría no ha sido aislada ni menos cultivada. En la era de la genómica y de la proteómica estos estudios parecieran responder directamente muchas preguntas ecológicas; los procesos mediados biológicamente dependen básicamente de la acción de proteínas, que a su vez son codificadas por el ADN genómico, de este modo se pueden estudiar procesos sin necesidad de aislar organismos. En el caso de los protistas es menos directa esta relación, ya que la manifestación de su batería de proteínas no es solo a nivel de metabolismo, sino también, por ejemplo, a nivel de receptores y estructuras celulares específicas necesarias para su alimentación (Worden et al. 2015). A diferencia de su contraparte procarionte, los eucariontes tienen una gran cantidad de genes “accesorios” con función desconocida (Worden & Allen 2010), esto se suma a que no existe un catálogo conocido de genes que estén involucrados en la manifestación de la compleja alimentación heterótrofa protista, es decir, es difícil relacionar

un gen con un comportamiento trófico (ejemplo: parasitismo). En definitiva, es necesario adquirir mayor información acerca de la biología celular de protistas, tanto a nivel estructural como fisiológico, y combinar esta información con muestreos en distintos ambientes para abarcar su diversidad y distribución. Esto permitirá cuantificar el real aporte de estos organismos en el ecosistema marino, podrán ser integrados en los modelos de flujos de carbono. Ya existen estudios que sugieren que en un escenario de mayor temperatura y estratificación como el que se espera con el calentamiento global las ZMOs aumentarían (Keeling et al. 2010) y, por otro lado, el picofitoplancton podría adquirir un mayor rol productivo en el oceano (Li et al. 2009). Se requiere conocimiento urgente de distintos ambientes marinos para tener información base y poder estimar eventualmente el impacto de los cambios globales que están sucediendo en las distintas poblaciones protistas.

6. CONCLUSIONES

1. En la bahía de Concepción existe una variación en la estructura comunitaria picoeucarionte tanto fotosintética como heterótrofa a lo largo del ciclo anual, de acuerdo a las condiciones cambiantes de una columna de agua en una zona marcada por la presencia de surgencia costera estacional.

2. En superficie, la comunidad picoeucarionte está compuesta mayoritariamente por miembros del grupo Chloroplastida, más específicamente mamielofíceos de los géneros *Bathycoccus*, *Micromonas* y *Ostreococcus*. Esta comunidad picofitoeucarionte es abundante en períodos de no-surgencia (otoño-invierno) y disminuye drásticamente en períodos de surgencia, cuando el fitoplancton de mayor tamaño domina la alta producción primaria.

3. El género *Ostreococcus* domina numéricamente la comunidad picofitoeucarionte durante todo el año, a diferencia de otros sitios costeros, donde se han observado altas abundancias solo de manera esporádica. Postulamos que la presencia permanente de *Ostreococcus* tiene que ver con que la concentración de nitrato es relativamente alta durante todo el año, incluso en períodos de no-surgencia, y este género parece responder bien a las fuentes de nitrógeno "nuevo".

4. El aporte productivo del picofitoplancton en el sistema es en promedio 10% de la clorofila total, pudiendo llegar a más del 60% en ciertos periodos; siendo el picofitoplancton eucarionte responsable en promedio del 57% de esta biomasa carbónica. En particular, el género *Bathycoccus*, a pesar de no ser dominante en abundancia, hace la mayor contribución.

5. En la capa profunda la comunidad picoeucarionte es eminentemente heterótrofa y es altamente variable en distintos momentos del año en términos de grupos dominantes. Si consideramos todas las muestras analizadas, el grupo Syndiniales es el más representado en las librerías de clones, sin embargo, destaca la presencia de Dinophycea y Fungi en verano, Labyrinthulomycetes en otoño y Ciliophora en primavera. La muestra de invierno, cuando la columna de agua está bien oxigenada, es la más diversa y presenta grupos fotosintéticos

propios de superficie, lo que puede ser explicado por la mezcla e incluso el hundimiento de agua en período invernal.

6. Funcionalmente también existe una variación en la comunidad heterótrofa que habita la capa profunda del area de muestreo. Se destaca la presencia de grupos saprófitos (Fungi y Labyrinthulomycetes) durante y después del período de mayor productividad. La acción parásita representada por Syndiniales está presente durante todos los muestreos, sin embargo, su composición está claramente determinada por el oxígeno. La predación destaca como función en primavera, con la dominancia de ciliados.

7. Los resultados de esta tesis representan la primera descripción de la comunidad picoeucarionte en el area de estudio y representa una base para abordar próximos estudios ecológicos de protistas.

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