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Metabolismo de especies de hongos aisladas de la zona costera frente a Chile central: rol en procesos de respiración y asimilación de nutrientes

(Metabolism of fungal species isolated from the coast off central Chile: the role of respiration and nutrient assimilation processes)

Tesis para optar al grado de Doctor en Oceanografía

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La Tesis de Doctorado en Oceanografía titulada "*Metabolismo de especies de hongos aisladas de la zona costera frente a Chile central: rol en procesos de respiración y asimilación de nutrientes*", del Sr. Marcelo E. Fuentes 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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There is no such thing as a special category of science called applied science; there is science and its applications, which are related to one another as the fruit is related to the tree that has borne it.

Louis Pasteur

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RESUMEN

Los hongos filamentosos marinos han sido recientemente identificados como un componente funcional en sistemas costeros, desarrollando un importante rol en la degradación de materia orgánica e hidrólisis de polímeros de gran tamaño. Sin embargo, el rol ecofisiológico y biogeoquímico de estos hongos ha sido escasamente explorado. Esta tesis doctoral busca determinar el efecto de la temperatura y disponibilidad de nutrientes (como glucosa) sobre la respiración y el crecimiento de hongos filamentosos, además de caracterizar la utilización de substratos de carbono, nitrógeno, fósforo y azufre por estos organismos. Para ello se midieron las tasas de respiración y crecimiento de 5 especies de hongos filamentosos (*Penicillium decumbens*, *Penicillium chrysogenum*, *Acremonium strictum*, *Fusarium fujikuroi* y *Fusarium sporotrichioides*) aisladas desde la zona de surgencia costera frente a las costas de Chile centro sur. El crecimiento fue monitoreado mediante el uso de Microscopía de Epifluorescencia, concentración de ATP y Densidad Óptica, mientras que el consumo de oxígeno fue registrado por medio de un respirómetro con sensores "Optode". Aunque las respuestas encontradas fueron especie específicas, en términos generales tanto la respiración como el crecimiento aumentaron con la temperatura y la concentración de glucosa. El crecimiento de *P. decumbens*, *F. sporotrichioides* y *F. fujikuroi* fue más favorecido (49,8 ± 7,3 %) por la temperatura (entre 9 y 20°C) a altas concentraciones de glucosa (5 g L^{-1} de glucosa). *P. chrysogenum* mostró un patrón particular, el cual estuvo más ligado a la disponibilidad de glucosa que directamente a la temperatura; mientras que el crecimiento de *F. sporotrichioides* y *A. strictum* respondió a una interacción sinérgica entre la temperatura y la concentración de glucosa. Las tasas de respiración fúngicas mostraron valores de Q_{10} en un rango entre 2,2 y 6,7 lo que indica que las tasas respiratorias poseen una fuerte dependencia de la temperatura, especialmente en *A. strictum* y *F. sporotrichioides.* Se obtuvieron los perfiles de utilización de substratos de carbono, nitrógeno, fósforo y azufre para tres especies (*P. decumbens*, *A. strictum* y *F. fujikuroi*). En orden a entender su potencial impacto en la degradación de compuestos de carbono (principalmente carbohidratos y amino ácidos), sus perfiles de utilización de sustratos fue caracterizado usando Biolog Filamentous Fungi (FF) Microplates. Se determinó que estas especies son versátiles, utilizando un amplio rango de fuentes de carbono, mostrando gran capacidad (57,2 % del total) para la utilización de carbohidratos

(monosacáridos, disacáridos, oligosacáridos y polisacáridos), y también utilizaron amino ácidos (0,99 % del total), los cuales produjeron altas tasas de crecimiento, sugiriendo el uso de rutas metabólicas como la glucolisis/gluconeogénesis. La riqueza de substratos reveló una amplia utilización de fuentes de carbono, las cuales produjeron altos "Índices de Shannon" de utilización particular de substratos, con valores de 4,02 para *A. strictum*, 4,01 para *P. decumbens* y de 3,91 para *F. fujikuroi* con diferencias significativas entre 18 de los substratos utilizados por las tres especies. Estas especies mostraron además, altos índices de utilización de substratos de nitrógeno, fósforo y azufre (con glucosa como fuente de carbono), en donde los componentes orgánicos proporcionaron mayor crecimiento de hifas. Los L-amino ácidos (52,6 ± 4,7 %), nucleótidos/nucleósidos (11,6 ± 0,8 %) y aminas (8,7 ± 0,8 %) fueron las fuentes de nitrógeno preferenciales (Cluster I y II), sugiriendo el uso de las vías metabólicas de amino ácidos y/o el metabolismo de las purinas. Otros compuestos como urea, alantoína y ácido úrico produjeron un crecimiento moderado. Los ribonucleótidos de adenosina y guanosina fueron los principales sustratos para la utilización de fósforo, además del uso de cisteína y metionina como principal aporte de azufre al crecimiento de estas especies. Además se observó el crecimiento con variadas fuentes de substratos inorgánicos tales como nitrato, nitrito, tiofosfatos, y tetrationato. Al considerar el rol heterótrofo activo de hongos filamentosos, su importancia en la degradación de materia orgánica y participación en importantes ciclos biogeoquímicos en el océano, sugerimos incluir la comunidad micoplanctónica como un componente integral de la comunidad microbiana en ambientes marinos costeros, para ser incluidos en modelos conceptuales de degradación de materia orgánica y ciclos biogeoquímicos debido al uso de una amplia variedad de compuestos orgánicos e inorgánicos de carbono, nitrógeno, fósforo y azufre.

ABSTRACT

Filamentous marine fungi have recently been identified as a functional component of coastal systems, playing an important role in the degradation of organic matter and hydrolysis of large polymers. However, the ecophysiological and biogeochemical role of these fungi has been scarcely explored. This doctoral thesis seeks to determine the effect of temperature and nutrient availability (such as glucose) on respiration and growth of filamentous fungi, as well as to characterize the use of carbon, nitrogen, phosphorus and sulfur substrates by these organisms. Respiration and growth rates were determined for 5 species of filamentous fungi (*Penicillium decumbens*, *Penicillium chrysogenum*, *Acremonium strictum*, *Fusarium fujikuroi* and *Fusarium sporotrichioides*) isolated from the coastal upwelling zone off central-southern Chile, so as to examine the effects of temperature and nutrient availability (such as glucose). Growth was monitored via epifluorescence microscopy, ATP concentrations and Optical Density; while oxygen consumption was recorded using a respirometer with "Optodes". Although responses were species specific, generally respiration and growth increased with temperature and glucose concentration. Growth of *P. decumbens*, *F. sporotrichioides* and *F. fujikuroi* were most favored (49.8 ± 7.3 %) by temperature (between 9 and 20 °C) when glucose concentrations remained stable (5 g L^{-1} of glucose). *P. chrysogenum* displayed a particular pattern linked to the availability of glucose rather than temperature; whereas growth in *F. sporotrichioides* and *A. strictum* responded to the synergic interaction between temperature and glucose concentration. Fungal respiration rates displayed Q_{10} values between 2.2 and 6.7, indicating that respiration rates were strongly dependent on temperature, especially *A. strictum* and *F. sporotrichioides.* Substrate use profiles for carbon, nitrogen, phosphorus and sulfur were obtained for three species (*P. decumbens*, *A. strictum* and *F. fujikuroi*). In order to understand their potential impact on the degradation of carbon compounds (mainly of carbohydrates and amino acids), their carbon profiles were characterized using Biolog Filamentous Fungi (FF) MicroPlates. These species were found to be versatile, with a large capacity (57.2 % of total) for using a wide range of carbon sources, principally carbohydrates (monosaccharides, disaccharides, oligosaccharides and polysaccharides), but also amino acids (0.99 %), suggesting the use of metabolic pathways, such as glycolysis

/gluconeogenesis. Substrate richness revealed a great capacity for exploiting nutritional sources, reflected by high "Shannon Indices" for particular substrate use, with 4.02 for *A. strictum*, 4.01 for *P. decumbens* and 3.91 for *F. fujikuroi* and significant differences between 18 substrates across all three species. These species also displayed high indices of substrate use complementary to nitrogen, phosphorus and sulfur (with glucose as a carbon source), where organic components accounted for greater hyphal growth. Here, L-amino acids (52.6 ± 4.7 %), nucleotides/nucleosides (11.6 ± 0.8 %) and amines (8.7 ± 0.8 %) were preferential sources of nitrogen (Cluster I and II), suggesting the use of pathways involved in the amino acids and/or purine metabolism. Other compounds, such as urea, allantoin and uric acid produced moderate growth. The ribonucleotides adenosine and guanosine were the main substrate for phosphorus use; and cysteine and methionine were the main sources of sulfur for growth in these species. Moreover, growth was observed with several inorganic substrate sources, such as nitrate, nitrite, thiophosphates, tetrathionate, in the three species. Considering the active heterotrophic role of filamentous fungi, their importance in the degradation of organic matter and their participation in important biogeochemical cycles in the ocean, we suggest the inclusion of the mycoplanktonic community as an integral component of the microbial community in the coastal ocean, to be included in microbial conceptual models of degradation of organic matter and biogeochemical cycles given their use of a wide variety of organic and inorganic carbon, nitrogen, phosphorus and sulfur compounds.

1. INTRODUCCIÓN

El conocimiento científico de la estructura y función de las comunidades de microorganismos en el océano se encuentra en constante evolución desde los años ´60 (Holmes y Anderson, 1963; Pomeroy, 1974; Azam *et al*., 1983; Biddanda, 1986; Herndl y Weinbauer, 2003), donde las bacterias emergen como los principales canalizadores de la materia orgánica disuelta hacia niveles tróficos superiores, y responsables de la mayor parte de la respiración en el océano (Pomeroy, 1974; Azam *et al*., 1983). Sin embargo, el estudio de grupos nuevos altamente abundantes como los virus y arqueas ha abierto nuevas preguntas acerca de la ecología y biogeoquímica del océano (De Long, 1992; Fuhrman *et al*., 1992; Suttle, 2007; Breitbart, 2012; Levipan *et al*., 2012). De esta misma forma, la evidencia reciente que identifica a los hongos marinos como un componente funcional importante de la comunidad microbiana marina (ej. Gutiérrez *et al*., 2011; Jones y Pang, 2012; Wang *et al*., 2014), muestra un eslabón inconcluso en el campo de la microbiología marina, donde su rol ecológico y biogeoquímico en ecosistemas marinos se encuentra en proceso de descubrimiento e integración en los actuales modelos de flujos de energía y nutrientes en el océano.

Las primeras descripciones científicas de hongos marinos realizadas por Durieu y Montagne (1869), correspondieron al descubrimiento del primer hongo marino en rizomas de praderas marinas (praderas de *Posidonia oceanica*). Estos autores destacaron el notable ciclo de vida de *Halotthia posidoniae*, el cual pasa todas sus etapas de vida en el fondo marino. Desde entonces (hace más de un siglo), expertos micólogos han debatido acerca de diferentes temas vinculados a la presencia de hongos en el mar tales como si los hongos pueden, en absoluto, crecer en agua de mar (ej. Murray, 1893). Uno de los primeros informes sobre el parasitismo de un hongo marino (en un alga) se documentó ese mismo año (Church, 1893). Un hito importante en el estudio de los hongos presentes en el medio marino fue el informe de Barghoorn y Linder (1944), que además de las descripciones de hongos de origen marino, representó una veintena de especies, demostrando que los hongos no sólo toleran el agua salada, sino que las condiciones marinas proporcionan un hábitat alternativo, para lo que en ese momento parecía ser un relativamente pequeño número de

hongos. La diversidad de hongos marinos obligados descrita entre 1980 y 1990 incluyó cerca de 530 especies a la taxonomía de ambientes marinos (Jones *et al*., 2009), lo que en la actualidad, con el desarrollo de técnicas moleculares aumentó a un número estimado de 12.060 especies, las que incluyen levaduras, especies no cultivables y derivadas marinas, y un pequeño número de otros hongos superiores (Jones y Pang, 2012). No obstante, aún es poco lo que se sabe acerca de los hongos marinos. Si bien es cierto que los hongos marinos son más discretos y menos numerosos que sus parientes terrestres (Jones y Pang 2012), no podemos dejar pasar el hecho que en la historia evolutiva de hongos, la aparición y diversificación temprana de estos organismos probablemente tuvo lugar en los océanos (Le Calvez *et al*., 2009). Además, estudios recientes han demostrado que los hongos marinos son abundantes actores en los hábitats marinos y participan en diversos procesos biogeoquímicos (Stoeck *et al*., 2007; Alexander *et al*., 2009, Stoeck *et al*., 2009; Edgcomb *et al*., 2011).

Hasta el día de hoy no hay consenso sobre la definición de hongos marinos, aunque es evidente que la agrupación de estos organismos esta basada principalmente como grupo ecológico más que taxonómico (Kohlmeyer y Kohlmeyer, 1979; Hyde *et al*., 2000). Comúnmente se han utilizado descripciones que incluyen hongos "marinos asociados" "derivados marinos", también "marinos facultativos" y "marinos obligados", aunque muy probablemente, lo mejor es distinguir entre hongos aislados de nichos marinos, frente a aquellos que requieren el medio marino (Yarden, 2014). Las descripciones taxonómicas nos muestran que en su mayoría se componen de ascomicetes filamentosos, pero también de basidiomicetes y levaduras (Pang y Mitchell, 2005). Estos hongos pueden ser saprofitos, patógenos o mutualistas como consecuencia de la evolución de la célula fúngica y sus estrategias de alimentación (Kohlmeyer y Kohlmeyer, 1979; Richards *et al*., 2012). Además, poseen reconocidas capacidades para adherirse a sustratos, secretar enzimas, descomponer polímeros biológicos complejos y absorber nutrientes, lo que les permite desarrollarse en una amplia variedad de nichos los cuales incluyen plantas, algas, animales, suelos, sedimentos y ambientes detríticos (Richards *et al*., 2012).

El estudio de comunidades de micro-eucariontes ha mostrado que los hongos ocupan un lugar central en un gran número de hábitats marinos, como aguas pelágicas, aguas profundas, regiones costeras, chimeneas hidrotermales, sistemas anóxicos, regiones frías de hielo, etc. (Gunde-Cimerman *et al*., 2003; Magan, 2007; Damare y Raghukumar, 2008; Manohar y Raghukumar, 2013). En donde la temperatura es uno de los principales factores que afectan la diversidad de hongos marinos, junto con la salinidad (Jones, 2000; Babu *et al*., 2010).

Es ampliamente conocido que la trama trófica microbiana juega un rol crucial en la biogeoquímica del océano (e.g. Bowler *et al*., 2009; Giovannoni y Vergin, 2012). La zona de estudio de la presente Tesis de Grado se encuentra en el sistema de surgencia costera frente a Chile centro sur, el cual presenta altas tasas de producción primaria (e.g. Daneri *et al.,* 2000; Montero *et al.,* 2007) y además se encuentra influenciado por los ríos Biobío e Itata, los cuales aportan nutrientes a la zona costera (Ahumada, 2002; Pantoja *et al*., 2011). Las altas tasas de producción primaria registradas en esta zona, generan alta disponibilidad de moléculas orgánicas para la comunidad microbiana, en donde los estudios de ensamblajes procariontes (bacterias y arqueas) muestran altas biomasas y tasas de producción secundaria (McManus y Peterson, 1988; Troncoso *et al.,* 2003; Cuevas *et al.,* 2004; Levipan *et al.,* 2007; Quiñones *et al.,* 2009), indicando que una fracción significativa de la producción primaria es canalizada a través de la red microbiana (Quiñones *et al.,* 2010). En esta zona además se presenta una alta actividad enzimática extracelular sobre sustratos proteínicos y glucosídicos, producidos en la fracción de tamaño >25 μm, en donde se encuentra una alta biomasa de hongos filamentosos (Gutiérrez *et al.,* 2011). Esta biomasa fúngica presenta una asociación positiva con los incrementos de biomasa fotosintética en aguas superficiales del ecosistema de surgencia costera frente a Chile central, las cuales son comparables a las producidas por bacterias y arqueas durante períodos de surgencia (Gutiérrez *et al*., 2011). No podemos dejar de lado el hecho de que los hongos juegan un papel fundamental en la degradación inicial de polímeros (ej. celulosa, lignina) en residuos de plantas (Kjoller y Struwe, 2002; Baldrian, 2008), lo que los transforma en componentes primordiales en zonas costeras.

Al considerar las microalgas como una de las principales fuentes de sustratos alimenticios en esta zona de surgencia costera y al analizar la composición bioquímica de los principales taxa de microalgas (diatomeas, primnesiofitas, prasinofitas, clorofitas, eustigmatofitas, criptomonas y rodofitas), estos indican que el contenido de azucares es considerablemente variable a través de los grupos, mostrando que la glucosa es el azúcar predominante seguido de la galactosa, manosa y arabinosa, fructosa, ribosa y xilosa (Hellebust, 1974; Brown *et al*., 1997). En consecuencia, estas microalgas generan condiciones nutricionales preferenciales para el crecimiento de hongos filamentosos. Otras investigaciones han mostrado que organismos del fitoplancton poseen altas cantidades de glucosa almacenada en polisacáridos, además de producir exopolímeros (Biersmith y Benner, 1998; Aluwihare y Repeta, 1999; Janse *et al.*, 1999). De esta forma, la respiración de glucosa se presenta como un excelente indicador de la actividad heterotrófica, dado que es el principal y preferido combustible para la producción de ATP en los microorganismos eucariotas (Chambergo *et al*., 2002).

Por otro lado, la lluvia de materia particulada que desciende desde la zona fótica a través de la columna de agua en el océano y que finalmente llega a los sedimentos, puede representar un nicho favorable para el desarrollo de saprótrofos, lo que transforma a hongos filamentosos en un componente fundamental en la cadena alimenticia detrítica (Raghukumar, 2004; 2008). El procesamiento de detritus sigue siendo un tema poco estudiado en la ecología marina, así como el papel de los hongos como saprótrofos en el océano, y por lo tanto es un desafío central para entender el ciclo de nutrientes y el papel de estos organismos en el ciclo del carbono (Richards *et al*., 2012).

Los modelos de degradación de materia orgánica, muestran que la materia orgánica particulada (MOP) se transforma en materia orgánica disuelta (MOD) por la acción de enzimas hidrolíticas generadas por microorganismos, produciendo compuestos de bajo peso molecular. Estos compuestos son absorbidos a través de la membrana celular y posteriormente respirados o usados para la construcción de biomasa celular (Hoppe *et al.*, 1988; Arnosti *et al.*, 1994; Azam, 1998). La MOD es uno de los mayores reservorios de carbono activo en el océano, compuestos de moléculas de diferente labilidad a la

degradación bacteriana (Benner, 2002). La mayoría de las moléculas que componen la MOD presentan tamaños menores a 1000 D, sólo el 1% de los monómeros conocidos puede ser utilizado rápidamente por microbios en el océano (Benner, 2002). En general, estos sustratos deben ser degradados a un tamaño de aproximadamente 600 Da antes de que puedan ser transportados activamente a través de los poros de la pared celular (Weiss *et al*., 1991). En hongos filamentosos, el transporte activo de nutrientes, tales como azúcar, aminoácidos, nitrato, amonio, sulfato y fosfato, implica la separación especial de las bombas de iones principalmente detrás del ápice de las hifas, mientras que las proteínas co-transportadoras están activas cerca de la punta, estas últimas usan el gradiente de concentración de un soluto para obligar a otra molécula o ion en contra de su gradiente de concentración (Kavanagh, 2005). Por lo tanto, la absorción de nutrientes se produce en la punta de las hifas, ya que impulsa continuamente el "recurso fresco", y las mitocondrias situadas detrás del ápice suministran ATP para apoyar la bomba de iones y generar fuerza motriz de protones (Kavanagh, 2005).

En este contexto, esta Tesis Doctoral estudia las potencialidades metabólicas de hongos filamentosos extraídos de la zona costera de surgencia de Chile centro sur, en la cual se presentan cambios drásticos entre regímenes hidrográficos sobre escalas temporales diarias, intraestacional y estacional (Sobarzo *et al*., 2007; Hernández *et al.*, 2012); además de presentar altas tasas de producción primaria (Daneri *et al*., 2000; Montero *et al*., 2007, Quiñones *et al*., 2010). Por esta razón, es importante analizar el efecto de la temperatura y disponibilidad de nutrientes sobre dos procesos fisiológico/ecológicos cruciales como son la respiración y crecimiento de hongos filamentosos marinos. Además se caracterizan los perfiles de utilización de carbono usando un índice metabólico (asimilación de sustratos-crecimiento de micelios) para dilucidar el rol en procesos de consumo de sustratos, con especial atención sobre el efecto de carbohidratos y amino ácidos en el metabolismo de hongos filamentosos. Por último, se analiza el crecimiento de micelios estimulado por sustratos de nitrógeno, fosforo y azufre, los cuales han recibido una mínima atención en relación a la comunidad micoplanctónica y a su rol potencial en los flujos de energía y nutrientes en un ecosistema de surgencia costera altamente productivo e hidrográficamente variable.

2. HIPÓTESIS Y OBJETIVOS

2.1.Objetivo General

Determinar la contribución energética y biogeoquímica que realizan los hongos que habitan el sistema pelágico y bentónico sobre la plataforma continental del Sistema de Corrientes de Humboldt frente a la zona centro sur de Chile.

2.2.Hipótesis de Trabajo y Objetivos Específicos

2.2.1 Objetivo específico 1.- Determinar el efecto de temperatura y concentración de sustratos (glucosa) sobre las tasas de respiración aeróbica de cepas de hongos marinos.

Fundamento de las hipótesis: La comprensión de la dinámica de la respiración a nivel comunitario es limitada debido a la falta de conocimiento acerca de la fisiología de muchas especies y cómo estas se ven afectadas por las condiciones ambientales (Jin y Bethke, 2003; Suberkropp *et al*., 2010). En el caso de las especies de hongos estas se ven afectadas principalmente por la temperatura y disponibilidad de nutrientes (Madan and Thind, 1998). La zona de surgencia de Chile centro sur, presenta cambios en la temperatura del agua en una escala temporal anual, estacional, quincenal, etc. (Grob *et al.*, 2003; Sobarzo *et al*., 2007), lo cual afectaría el crecimiento y respiración de las especies de hongos que habitan en esta zona. Es por este motivo que se plantean las siguientes hipótesis:

Hipótesis 1: Mayores temperaturas (en el rango ambiental) incrementan las tasas respiratorias de cepas de hongos marinos aislados de la zona costera frente a Chile centro sur.

Hipótesis 2: Altas concentraciones de sustrato (glucosa) en el medio incrementan las tasas respiratorias de cepas de hongos marinos aislados de la zona costera frente a Chile centro sur.

2.2.2. Objetivo Específico 2.- Determinar si existen preferencias en la asimilación de carbohidratos (por diferencias en el tamaño) y aminoácidos (debido a los grupos radicales que los componen) y su influencia en las tasas de crecimiento de cepas de hongos filamentosos.

Fundamento de la hipótesis: Los carbohidratos son los principales componentes de la materia orgánica identificada en el océano, dando cuenta de entre 3% a 30% del carbono orgánico disuelto (Gueuen *et al*., 2006; Hung *et al*., 2003), y los amino ácidos son las principales formas de nitrógeno e importantes componentes de carbono orgánico en la mayoría de los organismos marinos (Cowie *et al*., 1992). También representan fracciones significativas de materia orgánica en partículas de sedimentos marinos costeros y en la columna de agua (Cowie *et al*., 1992). Teniendo en consideración que la difusión de sustratos a través de la membrana plasmática depende del tamaño y la estructura química de cada molécula, donde moléculas más pequeñas difunden más rápidamente a través de la bicapa lipídica (Brown, 1996). Con base en estos supuestos se plantean las siguientes hipótesis:

Hipótesis 3: La utilización de azucares más pequeños (<600Da) producirán tasas metabolicas mayores, al ser más fácilmente asimilados por hongos filamentosos de la zona costera frente a Chile centro sur.

Los amino ácidos se clasifican en base a la reactividad de su cadena lateral (grupo R) en: No polares o hidrofóbicos, alifáticos (Gly, Ala, Val, Leu, Iso, Met), Aromáticos relativamente no polares (Phe, Tyr, Try), Polares sin carga los cuales son más hidrofílicos (Ser, Thre, Cys, Pro, Asp, Gln), Positivamente cargados (básicos) (Lys, Arg y His) y Negativamente cargados (Asp y Glu), los cuales son más hidrofílicos. Estas diferencias en reactividad de amino ácidos producirán diferencias en la asimilación de sustratos, donde moléculas más pequeñas y más hidrofílicas difunden más rápidamente a través de proteínas transportadoras (Mishra *et al*., 2014). Basados en esta información se formula la siguiente hipótesis:

Hipótesis 4: Amino ácidos con grupos R polares y/o con carga (hidrofílicos) difunden más fácilmente a través de proteínas de membrana produciendo mayores tasas de crecimiento de hongos filamentosos aislados de la zona costera frente a Chile centro sur.

2.2.3. Objetivo Específico 3.- Caracterizar la asimilación de compuestos orgánicos e inorgánicos de sustratos de nitrógeno, fósforo y azufre de hongos filamentosos marinos provenientes de cultivos puros.

Fundamentación de las hipótesis: Los requerimientos de nitrógeno y fósforo de hongos en general, se suplen de fuentes orgánicas o amonio y no todos los hongos son capaces de consumir nitrato (Deacon, 2005), de esta misma forma amino ácidos como la cisteína y otros compuestos orgánicos suplen los requerimientos de azufre (Kulkarni y Nielsen, 1986). Por lo cual los aportes de compuestos orgánicos deberían favorecer el crecimiento de hongos filamentosos más que los inorgánicos.

Hipótesis 5: Las tasas de crecimiento de cepas de hongos filamentos que habitan la zona costera frente a Chile centro-sur son beneficiadas por la utilización de sustratos orgánicos más que inorgánicos.

Hipótesis 6: Hongos filamentosos aislados de la zona de surgencia frente a Chile centro-sur prefieren fuentes de nitrógeno como el amonio sobre otras como el nitrato y nitrito.

3. MATERIALES Y MÉTODOS

Trabajo de campo y muestreo: Muestras de agua de mar y sedimentos fueron colectadas desde la zona costera frente a Chile centro sur a bordo de la embarcación R/V Kay Kay II, en una estación oceanográfica sobre la plataforma continental localizada a 18 millas náuticas frente a Concepción (36º30.8’ S, 73º07.7’ W; profundidad = 90 m; Diciembre 2008) y dos estaciones cerca de la desembocadura del río Itata: P3-2 (36º 15.1’ S, 72º 52.5’ W; profundidad= 75 m; Enero 2007) y P3-5 (36º 23.6’ S, 72º 54.0’ W; profundidad=10 m; Enero 2007) (Figura 3.1). Las muestras de agua (100-150 mL) fueron filtradas a través de filtros estériles de ester de celulosa de 0,22 µm de poro, que luego se extendieron sobre placas de agar de aislamiento, mientras que las muestras de sedimentos (~0.5 g) fueron vertidos directamente en placas de agar.

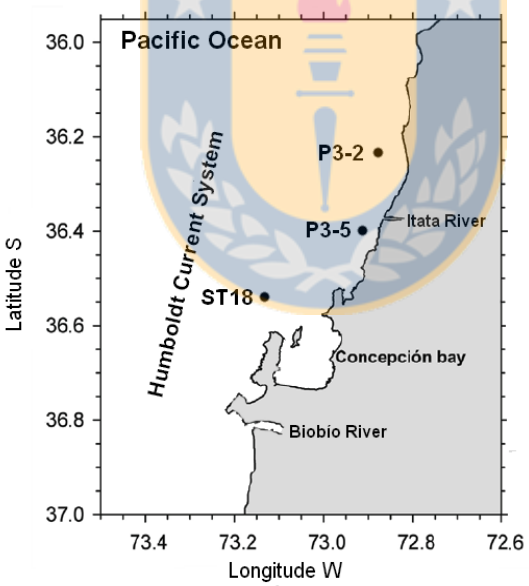

Figura 3.1. Área de estudio.

Aislación e Identificación taxonómica: Todas las muestras fueron cultivadas con agar glucosa y extracto de levadura para la aislación de especies de hongos marinos (Johnson y Sparrow, 1961). Las colonias más abundantes fueron seleccionadas y puestas en agar Emerson’s YpSs agar (Kohlmeyer y Kohlmeyer, 1979) y son parte del banco de

especies de hongos filamentosos marinos del Laboratorio de Geoquímica Orgánica de la Universidad de Concepción. Las especies de hongos marinos usadas en este estudio fueron identificadas a través de análisis de una secuencia parcial del ADN ribosómico de la subunidad nuclear pequeña (SSU rDNA; 18S rDNA) (Chow *et al.*, 1993; Yang *et al.*, 2005). El ADN fue extraído desde los micelios usando kits Power Soil DNA (MO BIO Laboratories Inc.) y 1 µL de la planilla de ADN (template) fue sometido a PCR estándar (Gutiérrez *et al.*, 2010). Los partidores empleados para la amplificación de ADNr fueron NS1 y GC-Fung (May *et al.*, 2001). Los análisis de secuencias BLAST (Basic Local Alignment Search Tool) confirmaron con alta cobertura e identidad (%) la taxonomía de las cinco especies de ascomicetes seleccionadas para este estudio. Estas especies fueron *Penicillium decumbens* y *Penicillium chrysogenum*, ambos aislados de la columna de agua; y *Acremonium strictum*, *Fusarium fujikuroi* y *Fusarium sporotrichioides* aislados desde sedimentos superficiales del área adyacente a la boca del río Itata. Una única cepa de cada especie fue utilizada para cada experimento.

Metodología del Capítulo 1: Efectos de la temperatura y concentración de glucosa sobre el crecimiento y respiración de especies de hongos aisladas desde un ecosistema de surgencia costera altamente productivo.

Condiciones de cultivo: Cada especie fue cultivada en placas petri con agar Emerson's YpSs (Kohlmeyer y Kohlmeyer, 1979) con sulfato de estreptomicina y penicilina (0,15-0,5 g L^{-1}) (Fell *et al*., 2001) para prevenir crecimiento bacteriano, bajo una cabina purificadora de seguridad biológica (LabConco, Logic class II). Estas placas fueron incubadas a 20 °C por cerca de 3 días hasta la producción de esporas. Un barrido de esporas fue tomado y puesto en tubos con agua de mar filtrada para aclimatación previa a la experimentación.

Determinación de biomasa: Para los análisis de biomasa realizados por microscopía de epifluorescencia se utilizo una adaptación del método de tinción con CalcoFluor White (Damare y Raghukumar, 2008; Catrhine y Raghukumar, 2009; Rasconi *et al*., 2009; Gutiérrez *et al*., 2010). Cada especie fue fotografiada (100 imágenes mínimo) usando un microscopio Axioscope 2 plus, Zeiss (aumento 1000x). El tamaño corporal fue

operacionalmente definido como el largo de la hifa (μm) y registrados hasta el día 4-5 para minimizar subestimaciones. Para el cálculo de biomasa se utilizó el programa ImageJ 2x, donde un promedio de 80 imágenes por especie fueron examinados a partir del cual se obtuvo el radio de las hifas para calcular biovolumen, suponiendo que los filamentos son de forma cilíndrica (Cathrine y Raghukumar, 2009). Para la estimación de carbono asumimos una tasa de conversión de 1 μm3 = 1 pg C (Van Veen y Paul, 1979). Para la extracción de ATP se utilizo buffer Tris hervido (hidroximetil aminometano), el cual fue congelado a –20 °C hasta el análisis. El análisis fue realizado usando el ensayo de luciferin-luciferasa de acuerdo a Holm-Hansen (1973), con un luminómetro (GloMax, Promega). La Densidad Óptica (D.O.) fue leída en microplacas de 96 pocillos usando un espectrofotómetro (Elx800, Bio-Tek Instruments) a 450 nm. Por último la D.O. fue registrada y convertida en carbono usando la siguiente regresión lineal (método de mínimos cuadrados; ec. 3.1):

$$Y = 2{,}4549\ x - 0{,}1069 \quad (R^2: 0{,}756;\ F: 27{,}217;\ p<0{,}001;\ n:85) \qquad \text{(ec. 3.1)}$$

Diseño experimental: Se seleccionaron tres temperaturas dentro de rango de la variabilidad ambiental; 9 °C es la temperatura típica cerca del fondo en el área de estudio, 13 °C en la superficie bajo condiciones de surgencia y 20 °C como máximo observado durante primavera-verano. Cuatro concentraciones de glucosa fueron seleccionadas para evidenciar el efecto de este sustrato sobre el crecimiento y la respiración. Dos experimentos de respiración y crecimiento fungal fueron realizados a tres temperaturas con 5 g L^{-1} de glucosa en agua de mar (experimento 1). Experimentos de concentración de glucosa fueron realizados usando las siguientes concentraciones: 0,001 g L^{-1} (5.555 nM glucosa L^{-1}), 0,01 g L^{-1} (55.555 nM glucosa L^{-1}), 0,1 g L^{-1} (555.555 nM glucosa L^{-1}) y 1 g L^{-1} (5.000.000 nM glucosa L^{-1}). Pantoja *et al*., (2011), trabajando en la misma área oceanográfica, estimó una actividad enzimática extracelular máxima de sustratos glucosídicos por la comunidad microbiana de entre 6,9 a 61 nmol $L^{-1}h^{-1}$ (invierno y primavera-verano respectivamente). Una concentración experimental de glucosa de 0,001 a 0,01 g L^{-1} es suficiente para sostener estas tasas enzimáticas durante 7 días de incubación cerrada. Por otra parte, las concentraciones de 0,1 y 1 g L^{-1} fueron usadas para determinar el crecimiento potencial, debido a que los hongos en sistemas acuáticos, crecen asociados a a sustratos nutricionales (Shearer *et al*., 2007). Estas cuatro concentraciones fueron elegidas con el fin de representar

la alta variabilidad en la disponibilidad de nutrientes en la zona costera y en consecuencia, para evaluar los cambios en el crecimiento y la respiración de hongos (Experimento 2) cuando se enfrentan a estas condiciones.

Respiración fúngica: El consumo de oxígeno fue registrado a través de un respirómetro (FIBOX 3, PreSens) con sensores Octode, basado en el principio de extinción de la luminiscencia causada por la colisión entre el oxígeno molecular y las moléculas de colorante luminiscente en el estado excitado (www.loligosystems.com). Los experimentos se llevaron a cabo por triplicado en 5 botellas de 5mL con sensores de punto integrados dentro de cada botella, lo que permitió registral el consumo de oxígeno cada hora y sin tener que abrir las botellas. Para el cálculo de las tasas de consumo de oxígeno se eligió la primera parte lineal de la curva. Para la determinación de la tasa de respiración específica, la tasa de consumo de oxígeno fue dividida por la biomasa fúngica total de cada botella.

Análisis estadístico: El supuesto de normalidad se puso a prueba para cada conjunto de datos utilizando pruebas de Shapiro-Wilk y de normalidad de Kolmogorov-Smirnov. Las relaciones entre el contenido de carbono, D.O. y ATP; entre la respiración y la biomasa; y entre la respiración y el tamaño corporal específico, fueron evaluados por regresión lineal (mínimos cuadrados, valor alfa: 0,05). En el experimento 1 un análisis de varianza (ANOVA) de tres vías fue aplicada al crecimiento y las tasas de respiración a diferentes temperaturas (factores: temperatura, día, especies) y una prueba post-hoc de Tukey con una prueba de diferencia honestamente significativa (HSD). En el experimento 2, un ANOVA de tres vías se aplicó al crecimiento en las diferentes (4) concentraciones de glucosa y 2 temperaturas, durante 7 días (factores: temperatura, concentración de glucosa, días) y se llevó a cabo una prueba post-hoc Tukey HSD. Se aplicó la prueba de Bartlett para homocedasticidad. Todos los análisis estadísticos se realizaron con el software SYSTAT 12©.

Metodología del capítulo 2: Perfil de utilización de carbono de las especies de hongos filamentosos *Fusarium fujikuroi*, *Penicillium decumbens* y *Acremonium strictum* aislados desde ambientes costeros marinos.

Preparación de la suspensión: Para el perfil de utilización de carbono se llevaron a cabo los siguientes pasos. Un cultivo puro de una especie fúngica se cultivó en una placa de agar de extracto de malta al 2%, hasta la formación de los conidios. Los conidios se tomaron a través de un barrido suave de la superficie de la placa de agar y se suspendieron en fluido de inoculación (libre de carbono) a una densidad específica. La densidad inicial fue la misma para todas las especies, medida como densidad óptica mínima registrada a 450 nm. Se pipetearon 100 µL de esta suspensión en cada pocillo de las microplacas Filamentous Fungi (FF) de Biolog (Bochner, 2001; Tanzer *et al*., 2003). Detalles de los substratos en el Anexo 1.

Sustratos de carbono: La microplaca FF se incubó a 20 ° C durante 192 horas, midiendo cada 24 h a través del uso de un espectrofotómetro (ELx800, Bio-Tek Instruments) a 490 nm y 750 nm en triplicado. La microplaca Biolog FF posee 96 pocillos con diferentes compuestos de carbono incluyendo un control (agua). Esta placa utiliza Iodonitrotetrazolio violeta (INT) como un colorante redox para medir colorimétricamente la actividad mitocondrial (valor redox) resultante de la oxidación de fuentes de carbono metabolizable (Tanzer *et al*., 2003; Bochner, 2009). La reducción de INT y la producción de formazán de color purpura es irreversible y la acumulación de formazán medida espectrofotométricamente refleja cuantitativamente la oxidación del sustrato. La lectura de 750 nm mide la turbidez, lo que refleja la producción de micelio de cada sustrato ensayado. Se determina el valor redox corregido (CRV) nm (490 nm-750 nm) (Kubicek *et al*., 2009).

Análisis de datos: Posteriormente los sustratos analizados se agruparon utilizando los criterios aplicados por Tosiah (2013) según los tipos de sustratos. Además como una medida de la cantidad de sustratos utilizados por hongos (riqueza sustratos) y la diversidad de sustratos, que engloba la riqueza de substratos y la intensidad del uso de sustratos, el cual fue calculado utilizando el índice de Shannon (H):

$$H = -\sum_{i=1}^{N} pi(\ln pi) \qquad \text{(ec. 3.2)}$$

pi es el proporción de la actividad microbiana en el substrato *i* en la actividad microbiana total, y N = 95 es el número de sustratos en una placa FF (Zak *et al*., 1994; Tam *et al*., 2001; Stefanowicz, 2006). El valor H describe la capacidad de las especies de hongos para degradar más o menos tipos de sustratos, actuando así como un índice de la diversidad fisiológica. En este caso, los hongos filamentosos que son capaces de degradar más sustratos y / o para degradar estos con eficiencia similar tendrían mayores valores de H.

Un análisis de conglomerados de unión se aplica a los valores redox (como un porcentaje relativo al total) entre las especies a las 96 h, que corresponde a la fase de crecimiento lineal en la mayoría de las fuentes de carbono. Esto se llevó a cabo utilizando Statistica 6.0© (Stat Soft, Inc.). Un ANOVA de una vía se utilizó para detectar diferencias en la densidad óptica (490-750nm) entre las especies.

Metodología del capítulo 3: Utilización de sustratos de Nitrógeno, Fósforo y Azufre por hongos filamentosos aislados de la zona costera de surgencia frente a Chile centro sur.

Preparación de la suspensión: Los perfiles de utilización de Nitrógeno (PM3), Fósforo y Azufre (PM4) se realiza de la misma forma que en las microplacas Biolog FF de carbono (preparación de la suspensión capitulo 2). Sin embargo, las placas Phenotype Microarray (PM) requieren de glucosa como fuente de carbono base y no incorporan Iodonitrotetrazolio, por lo que miden crecimiento por turbidez registrado a 750 nm, además de necesitar en el caso de las PM3, sulfato de sodio y fosfato de potasio (pH 6,0).

Sustratos de nitrógeno, fosforo y azufre: PM3 y PM4 fueron incubados a 20 °C por 120 horas y leídos cada 24 horas usando un espectrofotómetro (Elx800, Bio-Tek Instruments) a 750 nm por triplicado. El panel de prueba de la microplaca Biolog PM3 se compone de 96 pocillos con diferentes fuentes de nitrógeno, incluyendo un control y las microplacas PM4 se compone de 59 pocillos con sustratos de fósforo (más un control, pocillo A-1) y 35 pocillos con sustratos de azufre (más un control, pocillo F-1) (Bochner, 2001; Tanzer *et al*., 2003). (Detalles de los substratos en el Anexo 1).

Análisis de datos: Un análisis de conglomerados de unión se aplicó a los valores de crecimiento a 750 nm entre las especies a las 96 h, utilizando Statistica 6.0 (Stat Soft, Inc.).

Un ANOVA de una vía se utilizó para detectar diferencias en la densidad óptica (750nm) entre las especies durante los 5 días de cultivo.

El índice de Shannon H (ec. 3.2), fue utilizado, considerando un número de sustratos analizados (N) de: 95 sustratos para nitrógeno, 59 sustratos para fósforo y 35 sustratos para azufre (Zak *et al*., 1994; Tam *et al*., 2001; Wang *et al*., 2011; Muñiz *et al*., 2014). Finalmente las rutas metabólicas de los sustratos que producen mayor crecimiento fueron determinadas con la ayuda de la Kyoto Encyclopedia of Genes y Genomes (KEGG; www.genome.jp/kegg), la cual posee una colección de mapas de vías para el metabolismo de carbohidratos, amino ácidos, nucleótidos, etc.

4. CAPÍTULOS DE RESULTADOS

4.1. Capítulo 1: Efectos de la temperatura y concentración de glucosa sobre el crecimiento y respiración de especies de hongos aisladas desde un ecosistema de surgencia costera altamente productivo.

Manuscrito publicado en la revista Fungal Ecology: Fuentes, M.E., Quiñones, R.A., Gutiérrez, M.H and Pantoja, S. 2014. Effects of temperature and glucose concentration on the growth and respiration of fungal species isolated from a highly productive coastal upwelling ecosystem. Fungal Ecology, 13:135-149.
http://www.sciencedirect.com/science/article/pii/S1754504814001275

Resumen: Las tasas de respiración y crecimiento fueron medidas en cinco especies de hongos (*Penicillium decumbens, P. chrysogenum, Acremonium strictum, Fusarium fujikuroi* y *F. sporotrichioides*) aislados desde el sistema de surgencia costero frente a Chile centro sur, para determinar los efectos de la disponibilidad de glucosa y temperatura. El crecimiento fue monitoreado por microscopía de epifluorescencia, medidas de ATP y densidad óptica. El consumo de oxígeno fue registrado a través de un respirómetro con sensores Optode. Aunque se encontraron respuestas especie especificas, en general la respiración y el crecimiento incrementan con la temperatura y con la concentración de glucosa. EL crecimiento de *P. decumbens, F. sporotrichioides* y *F. fujikuroi* fue más favorecido por la temperatura cuando la glucosa permanece estable. *P. chrysogenum* tuvo un patrón de crecimiento particular, que parece estar más ligado a la disponibilidad de glucosa que directamente a la temperatura. El crecimiento de *F. sporotrichioides* y *A. strictum* responde a la interacción sinérgica entre la temperatura y la glucosa. Valores de Q10 para la respiración fúngica variaron entre 2.2 a 6.7, indicando una fuerte temperatura-dependencia de las tasas de respiración, especialmente en *A. strictum* y *F. sporotrichioides*.

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Effects of temperature and glucose concentration on the growth and respiration of fungal species isolated from a highly productive coastal upwelling ecosystem

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ABSTRACT

Respiration and growth rates were measured in five species of fungi (*Penicillium decumbens*, *P. chrysogenum*, *Acremonium strictum*, *Fusarium fujikuroi* and *F. sporotrichioides*) isolated from the upwelling system off the coast of central-south Chile to determine the effects of glucose availability and temperature. Growth was monitored by epifluorescence microscopy, ATP measurements, and optical density. Oxygen consumption was recorded via a respirometer with Optode sensors. Although species-specific responses were found, overall both respiration and growth increased with temperature and glucose concentration. Growth of *P. decumbens*, *F. sporotrichioides* and *F. fujikuroi* was more favoured by temperature when glucose remained stable. *P. chrysogenum* had a particular growth pattern, which seemed to be more linked to glucose availability than directly to temperature. Growth of *F. sporotrichioides* and *A. strictum* responded to the synergistic interaction between temperature and glucose. Values of Q_{10} for fungal respiration ranged from 2.2 to 6.7, indicating a strong temperature-dependence of respiration rates, especially in *A. strictum* and *F. sporotrichioides*.

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Introduction

Marine fungi constitute an ecological group mostly made up of filamentous ascomycetes, but also some basidiomycetes and yeasts (Pang and Mitchell, 2005). Fungi may be saprotrophic, pathogenic or mutualistic as an evolutionary consequence of the fungal cell and its feeding strategies (Adomas et al., 2008; Martin et al., 2011). Several ascomycete species in the marine environment are pathogens of algae (Kohlmeyer and Kohlmeyer, 1979). For example, certain fungi can cause abnormal growth in macroalgae (Richards et al., 2012).

The ecological success of fungi is linked to the fungal cell wall, composed of chitin/cellulose, which reinforces the fungal cell and allows it to resist the osmotic pressure produced during feeding, and to survive in diverse and heterogeneous environments (Richards et al., 2012). These adaptations have led to high metabolic rates and rapid growth (Bartnicki-García, 1979). Fungi are ubiquitous and capable of occupying every ecological niche (Richards et al., 2012). They are able to develop in nutrient-rich environments, such as those provided by plants and animals, soils, sediments and detrital environments, where they can adhere to substrata, secrete enzymes, decompose complex biological polymers and absorb nutrients (Richards et al., 2012). Temperature is one of the major factors affecting the diversity of marine fungi, along with salinity (Jones, 2000; Babu et al., 2010).

In the ocean, the rain of particulate matter from the photic zone down through the water column and ultimately reaching bottom sediments can represent a favorable niche for saprotrophs, making them an important component at the bottom of the detrital food chain and critical for the survival of detrivorous animals (Raghukumar, 2004; 2008). Indeed, fungi can be the dominant microeukaryotes in particular environments, such as methane seeps (Takishita et al., 2006) and deep water sediments (Damare and Raghukumar, 2008). This opportunistic strategy of fungal nutrition leads to a rapid response to changes in substrate conditions (Lindahl et al., 2010) and the production of structures for resistance and dispersal that allow fungi to persist despite limited growth under adverse environmental conditions (e.g. extreme coastal continental Antarctic, sea ice) (Gunde-Cimerman et al., 2003; Magan, 2007). The processing of detritus remains a poorly-studied subject in marine ecology, as well as the role of fungi as saprotrophs in the ocean, and thus is a central challenge in understanding nutrient cycling and the role of these organisms in the carbon cycle (Richards et al., 2012).

More than 500 species of marine fungi many of which have the capacity to decompose lignocellulose (Hyde et al., 1998) have been isolated from different marine environments (Jones, 2000). Lignocellulose is composed of structural blocks of glucose in combination with xylose and other minors (Mabee et al., 2006). Evidence of cellulolytic activity in seawater suggests the presence of a sufficient quantity of glucose for the growth and metabolism of microorganisms that take part in cellulolytic activity (Greene and Barnett, 1951; Sguros et al., 1962). The concentrations of neutral free sugars in the ocean are generally very low (<50 nM; Rich et al., 1996; Skoog et al., 1999); in the surface ocean (<100 m) total hydrolyzable neutral sugars were found to range between 200 and 800 nM (Benner, 2002). Neutral monosaccharides such as glucose have been examined more widely since the introduction of techniques capable of measuring their concentrations in seawater (Mopper et al., 1992).

It is widely known that the microbial realm plays a crucial role in the biogeochemistry of the ocean (e.g. Bowler et al., 2009; Giovannoni and Vergin, 2012). In the upwelling coastal ecosystem off central-south Chile, some of the highest primary productivity rates ever measured have been reported (e.g. Daneri et al., 2000; Montero et al., 2007). Moreover, studies on prokaryote assemblages in this zone have shown substantial biomass (bacteria and archaea) and very high secondary production (McManus and Peterson, 1988; Troncoso et al., 2003; Cuevas et al., 2004; Levipan et al.,. 2007; Quiñones et al., 2009), indicating that a significant fraction of the primary productivity is channeled through the microbial food web (Quiñones et al., 2010). The recent discovery, by Gutierrez et al. (2011), of very high biomass and high extracellular enzyme activity on proteinaceous and glucosidic substrates by microscopic filamentous fungi in this ecosystem raises the question of the ecological and biogeochemical roles played by fungi within the microbial realm.

There is a positive association between the increase in fungal biomass and periods of high autotrophic biomass in surface waters of upwelling ecosystems in central-south Chile (Gutiérrez et al., 2011). Often phytoplankton groups have high quantities of glucose stored in polysaccharides and produce exopolymers (Biersmith and Benner, 1998; Aluwihare and Repeta, 1999; Janse et al., 1999), making them a potential source of substrate (i.e. mono(poly)saccharides) for fungal growth. During active upwelling periods, fungal biomass is comparable to that of bacteria and archaea in central-south Chile and increases after the maximum of phytoplankton biomass (Gutiérrez et al., 2011). Since glucose is the principal and preferred fuel for ATP production in eukaryotic microorganisms (Chambergo et al., 2002), glucose respiration can be considered as an indicator of heterotrophic activity.

The highly productive coastal upwelling area off Concepción (central-south Chile) presents drastic changes between hydrographic regimes over temporal scales of days (tides, summer sea breeze), to intraseasonal (upwelling events, river discharge pulses) and seasonal (coastal wind patterns, radiation) (Sobarzo et al., 2007; Hernández et al., 2012). All variations in physical and hydrographic forcing can lead to changes in the distribution and composition of phytoplankton (Iriarte et al., 2012), which is closely linked to the distribution of filamentous fungi (Gutiérrez et al., 2011). The effects of variations in temperature and nutrient availability on marine filamentous fungi have received minimal attention, despite their potential role in nutrient and energy fluxes of the coastal ecosystem.

Herein we tested the following hypotheses: (i) an increment in water temperature produces higher growth and respiration rates of filamentous fungi inhabiting the upwelling coastal ecosystem off south-central Chile, and (ii) an increment of glucose concentration in the culture medium produces higher growth and respiration rates of filamentous fungi from the upwelling coastal ecosystem off south-central Chile.

Our research contributes to the exploration of the biogeochemical role of marine fungi in the microbial realm by understanding the basic interplay between some abiotic (temperature, glucose concentration) factors and two crucial physiological/ecological processes, growth and respiration, in five species of fungi from this ecosystem.

Methodology

Field work and sample collection

Seawater and sediment samples were collected from the coast off central-south Chile on board R/V Kay Kay II, at an oceanographic station on the continental shelf located 18 nautical miles from the shore (36°30.8′ S, 73°07.7′ W; depth = 90 m) and at two stations near the Itata River mouth: P3-2 (36° 15.1′ S, 72° 52.5′ W; depth = 75 m) and P3-5 (36° 23.6′ S, 72° 54.0′ W; depth = 10 m). Seawater samples (100–150 ml) were filtered through sterile 0.22 μm pore cellulose ester filters (Advantec MFS), which were then spread on isolation agar plates, while sediment samples (~0.5 g) were directly poured onto agar plates.

Isolation and taxonomic identification

All samples were cultured with glucose agar and yeast extract for the isolation of marine fungal species (Johnson and Sparrow, 1961). The most abundant colonies were selected and cultured in Emerson's YpSs agar (Kohlmeyer and Kohlmeyer, 1979) and were added to the fungal species bank of the Laboratory of Marine Organic Geochemistry of the University of Concepción. The species of marine fungi used in this study were identified by analysis of a partial sequence of the nuclear small subunit ribosomal DNA (SSU rDNA; 18S rDNA) (Chow et al., 1993; Yang et al., 2005). DNA was extracted from mycelia using the Power Soil DNA Kit (MO BIO Laboratories Inc.), and 1 μl of the template DNA was subjected to standard PCR (Gutiérrez et al., 2010). PCR products were then subjected to Denaturing Gradient Gel Electrophoresis (Fischer and Lerman, 1983) to verify the purity of cultures. The primers employed for rDNA amplification were NS1 and GC-Fung (May et al., 2001). Sequences were compared with fungal rDNA sequences included in the SILVA database (http://www.arb-silva.de) via BLASTALL sequence match routines and using Megablast on the non-redundant Genbank database (http://blast.ncbi.nlm.nih.gov). The BLAST (Basic Local Alignment Search Tool) sequence analysis confirmed with high coverage and identity (%) the taxonomy of the five ascomycete species selected for this study. Additional details of the fragments sequenced are given in Table 1.

Five fungal species were identified: *Penicillium decumbens* (Thom, 1910) and *P. chrysogenum* (Thom, 1910), both isolated from the water column; and *Acremonium strictum* (Gams, 1971), *Fusarium fujikuroi* (Nirenberg, 1976) and *F. sporotrichioides* (Sherbakoff, 1915), isolated from surface sediments. A unique strain from each species was used in the experiments (Table 1). The species selected for analysis are ubiquitous ascomycetes recorded in numerous systems, and have been classified into two groups: *F. fujikuroi*, *F. sporotrichioides* and *P. decumbens* have been identified as plant, soil and grain pathogens (Yoshida et al., 1992; Brown and Gordon, 2001; Mateo et al., 2002; Wiśniewska et al., 2011; Swer et al., 2011); while *A. strictum* and *P. chrysogenum* are described as marine species in the WoRMS database (http://www.marinespecies.org) and have been isolated from the marine environment in previous studies (Costello et al., 2001; Julianti et al., 2011; An et al., 2013).

Culture conditions

Each species was cultured in a Petri dish with Emerson's YpSs agar with streptomycin sulfate and penicillin (0.15–0.5 g l^{-1}) (Fell et al., 2001) to avoid bacterial growth, within a Purifier biological safety cabinet (LabConco, Logic class II). These plates were incubated at 20 °C for almost 3 d until spore production. Smears of spores were taken and placed into tubes with filtered seawater for acclimatization prior to experimentation.

Experimental design

Three temperatures within the environmental range were selected; 9 °C is the typical temperature in the study area near the bottom, 13 °C at the surface under upwelling conditions, and 20 °C of the maximum observed temperature during spring-summer. Four glucose concentrations were selected to determine the effect of this substrate on fungal growth. Two growth and respiration experiments were carried out at three temperatures with 5 g l^{-1} of glucose in seawater (Experiment 1). Experiments were carried out using the following glucose concentrations: 0.001 g l^{-1} glucose

Table 1 – Identified fungal isolates and stations of sample collection. WC: water column, SF: sizes of the fragments sequenced in base pairs (bp)

Matrix and depth	Station/fungal isolate	BLAST match sequence			
		Closest Genbank neighbour (GenBank accession no.)	SF	Coverage (%)	Identity (%)
WC, 8 m	ST 18/2	*Penicillium chrysogenum* (GU295948)	333	87	100
WC, 30 m	P3-2/148	*Penicillium decumbens* (GU573852)	270	87	100
Surface sediment	P3-5/246	*Fusarium sporotrichioides* (DQ680059)	242	83	100
Surface sediment	P3-5/234	*Fusarium fujikuroi* (HM165488)	305	94	100
Surface sediment	P3-5/241	*Acremonium strictum* (U43968)	326	98	99

(5 555 nM glucose), 0.01 g l^{-1} glucose (55 555 nM glucose), 0.1 g l^{-1} glucose (555 555 nM glucose) and 1 g l^{-1} glucose (5 000 000 nM glucose). Pantoja et al. (2011), working in the same oceanographic area where we collected our fungal strains, estimated an enzymatic extracellular hydrolysis of glucosidic substrates by the microbial community of 20–100 nM $h r^{-1}$ (winter and spring-summer respectively). An experimental concentration of glucose (0.001–0.01 g l^{-1}) was chosen because it is sufficient to sustain these hydrolysis rates after 7 d of closed incubation. On the other hand, we used experimental glucose concentrations of 0.1 and 1 g l^{-1} to assess the growth potential of the study species. It is important to note that fungi in aquatic environments grow associated with nutritional substrates (Shearer et al., 2007). The four described glucose concentrations were chosen to represent the high seasonal variability of nutritional substrates in the coastal zone and, accordingly, to assess the changes in fungal growth and respiration (Experiment 2) when confronting these conditions.

The spores were diluted with an optical density (O.D.) concentration close to the minimum (0.085), and then placed in 5 ml tubes with liquid medium (Meyers, 1966). In experiment 1, a fixed glucose concentration of 5 g glucose l^{-1} was used. Three tubes of each experimental temperature were destructively sampled daily for 7 d at 9, 13 and 20 °C. Respiration was measured for each tube and then a sample was extracted for biomass analysis via epifluorescence (1 ml), O.D. (150 µl) and ATP (500 µl). A control was used containing culture medium plus antibiotics. In experiment 2, four glucose concentrations were used. A simple culture medium containing 1 g glucose and 0.22 g $(NH_4)_2SO_4$ in 1 l aged filtered seawater was diluted to obtain glucose concentrations of 1 g l^{-1}, 0.1 g l^{-1}, 0.01 g l^{-1} and 0.001 g l^{-1}. These were incubated at 9 °C and 20 °C in microplates in triplicate. Respiration was measured for each tube and then a sample was extracted for biomass analysis via OD450 nm measurements every 24 hr for 6 d.

Biomass determination

The most appropriate method for determining fungal biomass is debatable. In this study, we used microscopy, ATP content and optical density. To analyze an assemblage of fungal species from natural field samples, the use of microscopy is adequate, although it can underestimate the total carbon content and is a time-intensive approach. Optical density is recommended for the routine counting of pure cultures in the laboratory. In the case of biomass greater than 18 µg C, O.D. is recommended, whereas ATP is useful for lower concentrations. With diluted samples like the ones collected from the water column in coastal waters, microscopic analysis was the best option. However, this alternative is very time-consuming and thus not convenient when working with many fungal cultures. On the other hand, marine fungi are scarcely known, and therefore it is still not clear which is the best and/or most convenient method to estimate biomass and ultimately growth. We think that reporting the results of the three methods used for estimating biomass and comparing them provides useful information for other researchers working with marine fungi.

For the biomass analysis via epifluorescence microscopy, an adaptation of the CalcoFluor White staining method was employed, in which the extracted sample was filtered with a black polycarbonate filter of 0.22 µm and immediately dyed (Damare and Raghukumar, 2008; Catrhine and Raghukumar, 2009; Rasconi et al., 2009; Gutiérrez et al., 2010). Each species was photographed (minimum 100 images) using an Axioscope 2 plus microscope, Zeiss (magnification 1000×). Fungal body size was operationally defined as hypha length (µm). A slight underestimation may have taken place given that the mycelia form a thin layer on the filter and the picture only captures the surface. However, this underestimation is small, given that the pictures were taken after only 4 d of incubation at the beginning of mycelium formation. For biomass calculation the ImageJ 2× program was used; a transformation of the RGB image to 8-bits was applied to each group and a threshold adjustment (B&W, Intermodes) was used to carry out the particle analysis and to obtain the surface area of hyphae of each sample. Moreover, an average of 80 images per species were examined from which the radius of hyphae was obtained to calculate biovolume, assuming that filaments are cylindrical in shape (Cathrine and Raghukumar, 2009). For estimating carbon we assumed a conversion rate of 1 $\mu m^3 = 1$ pg C (Van Veen and Paul, 1979).

The extraction of ATP was carried out using a boiled Tris buffer (hydroxymethyl aminomethane), which was frozen at −20 °C until analysis. Analysis was carried out using a luciferin-luciferase assay according to Holm-Hansen (1973), with a luminometer (GloMax, Promega). O.D. measurements were carried out using turbimetric measurement via chromatography (Banerjee et al., 1993; Meletiadis et al., 2001). For this, 150 µl of each sample was taken in triplicate and stirred to homogenize prior to reading. The sample was read in 96-well microplates using a spectrophotometer (Elx800, Bio-Tek Instruments) at 450 nm. For each well a correction was made by subtracting background O.D. Lastly, O.D. was recorded and converted into carbon using the regression equation described in Fig 1.

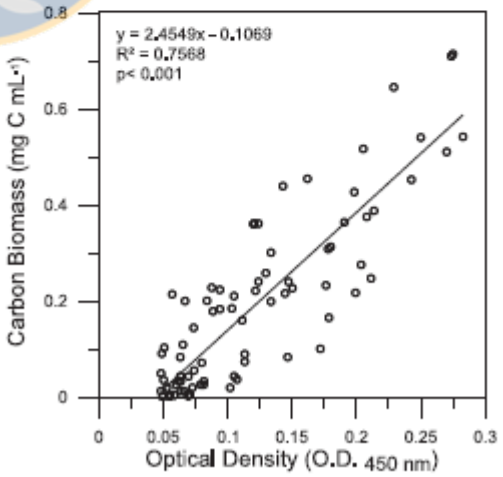

Fig 1 – Relationship between optical density and carbon biomass of fungal hyphae from *P. decumbens*, *P. chrysogenum*, *A. strictum*, *F. fujikuroi* and *F. sporotrichioides*. Least Square Regression, confidence level 95 %, n = 85.

Fungal respiration

Oxygen consumption was recorded using a respirometer (FIBOX 3, PreSens) with Optode sensors, based on the principle that molecular oxygen quenches luminescence. Experiments were carried out in triplicate in 5 ml bottles with integrated spot sensors inside each bottle, which allowed oxygen consumption to be recorded every hour without having to open the bottles. Culture medium and aged filtered seawater were included in the experimental design as a control and blank, respectively. Sample stabilization took 2.5 min and measurements were carried out during 1 hr or up to 3 hr when respiration rate was low.

For the calculation of oxygen consumption rates, the first linear part of the curve was chosen where gases were stabilized within the bottle and the first 5–10 min were omitted awaiting the stabilization of the instrument, where clear variation in oxygen was displayed. For the determination of the specific respiration rate, the oxygen consumption rate was divided by the total biomass of each bottle.

Statistical analyses

The assumption of normality was tested for each data set using Shapiro–Wilks and Kolmogorov–Smirnov normality tests. The relationships between carbon content, O.D., and ATP, between respiration and biomass, and between specific respiration and body size were assessed by linear regression (least squares, confidence 0.95). In experiment 1, a three-way analysis of variance (ANOVA) was applied to growth and respiration rates at different temperatures (Factors: temperature, day, species) with an Honestly Significant Difference (HSD) Tukey Test for post-hoc testing. In experiment 2, a three-way ANOVA was applied to growth at four different glucose concentrations and two temperatures, by 7 d (Factors: temperature, glucose concentration, days) and a post-hoc test was carried out with an HSD Tukey Test. The Bartlett test for homoscedasticity was applied. All statistical analyses were performed using SYSTAT 12.

Results

A significant regression of carbon biomass on optical density was observed (Fig 1). Growth experiments using ATP measurements also produced a significant regression of biomass estimates on optical density (linear regression, slope = 1.78, $R^2 = 0.744$, $p < 0.001$).

Fungal growth rates at 9 °C were low (21–51 μg C hypha day^{-1}), slightly greater at 13 °C (except for *P. chrysogenum*), and were significantly stimulated at 20 °C, with rates between 66 and 153 μg C hypha day^{-1} (Table 2). Among species, *A. strictum* displayed the greatest increase in growth rate at 20 °C, 153 μg C hypha day^{-1}, followed by *F. sporotrichioides* (Fig 2, Table 2). The growth rate of *P. chrysogenum* did not change dramatically with temperature (Fig 2D). Between *P. decumbens* and *F. sporotrichioides* growth rates were not significantly different at 9 and 13 °C (Fig 2C and E), but a significant difference was detected at 20 °C.

Fungal growth under different concentrations of glucose as carbon source and under two experimental temperatures (Experiment 2) displayed significant differences in the five species analyzed during 7 d of incubation (Table 2).

There were no significant differences in growth between the five studied species at 0.001 and 0.01 g l^{-1} glucose, where carbon biomass reached 0.32 mg C and 0.45 mg C at 9 and

Table 2 – ANOVA and post-hoc Tukey Tests of growth rate experiments of fungal species (mg C day^{-1}). Number of biomass samples taken for the estimation of growth rates in experiment 1 = 72; Number of biomass samples taken for the estimation of growth rates in experiment 2 = 168. G.C. = Glucose concentration (g l^{-1}). ** indicates significant differences in growth rates among species within a row; Numbers in superscripts indicates no significant differences in growth rates between experiments (identified with the same number) conducted at different temperatures. Letters in superscripts indicate no significant differences in growth rates between experiments (identified with the same letter) conducted at different glucose concentration. Alpha used in ANOVA and post-hoc Tukey Tests = 0.05. In brackets = standard deviation

Exp. 1		*A. strictum*		*F. fujikuroi*	*P. decumbens*		*P. chrysogenum*	*F. sporotrichioides*		*p*
9 °C		0.051 (0.012)		0.021 (0.004)	0.032 (0.002)		0.041 (0.007)^{m}	0.044 (0.004)^{n}		**
13 °C		0.072 (0.0003)		0.059 (0.0013)	0.036 (0.0003)		0.031 (0.006)^{m}	0.037 (0.004)^{n}		**
20 °C		0.153 (0.005)		0.066 (0.011)	0.094 (0.012)		0.076 (0.009)	0.102 (0.004)		**
2-way ANOVA Temp × Days										
F-ratio (*p*-value)		22.978 (<0.001)		14.492 (<0.001)	13.433 (<0.001)		5.873 (<0.001)	19.623 (<0.001)		
Exp. 2	G.C.									
9 °C	1	0.082 (0.002)	1	0.104 (0.003)	0.132 (0.001)	3	0.100 (0.001)	0.076 (0.004)		**
	0.1	0.016 (0.003)^{a}		0.017 (0.003)^{d}	0.023 (0.002)		0.014 (0.004)^{i}	0.024 (0.003)		
	0.01	0.010 (0.002)^{b}	2	0.012 (0.003)^{d,e}	0.011 (0.006)^{g}		0.011 (0.003)^{i}	0.012 (0.006)^{k}	4	
	0.001	0.013 (0.001)^{a,b}		0.012 (0.001)^{e}	0.010 (0.003)^{g}		0.013 (0.002)^{i}	0.015 (0.003)^{k}		**
20 °C	1	0.084 (0.026)	1	0.113 (0.007)	0.120 (0.017)	3	0.152 (0.026)	0.197 (0.034)		**
	0.1	0.056 (0.001)		0.049 (0.001)	0.038 (0.011)^{h}		0.035 (0.003)^{j}	0.033 (0.005)^{m}		**
	0.01	0.013 (0.003)^{b,c}	2	0.043 (0.001)^{f}	0.039 (0.012)^{h}		0.030 (0.004)^{j}	0.012 (0.005)	4	
	0.001	0.018 (0.003)^{c}		0.043 (0.003)^{f}	0.034 (0.010)^{h}		0.028 (0.007)^{j}	0.039 (0.003)^{m}		
3-way ANOVA Temp × Conc × Days										
F-ratio (*p*-value)		4.902 (<0.001)		11.329 (<0.001)	14.937 (<0.001)		2.694 (<0.001)	3.729 (<0.001)		
Tukey Test Temp × days										
q value (*p*-value)		5.217 (<0.001)		46.173 (<0.001)	30.680 (<0.001)		7.273 (<0.001)	18.290 (<0.001)		

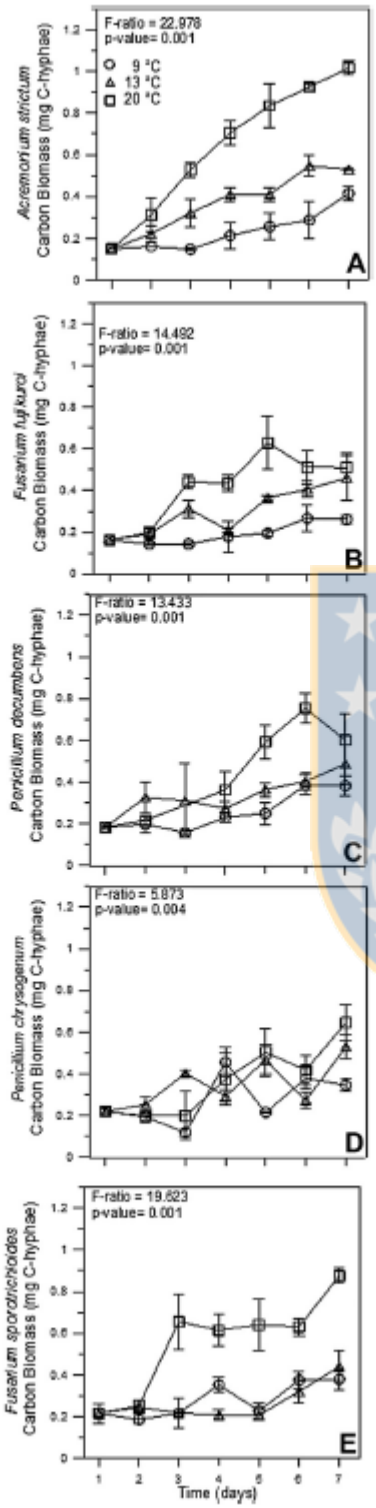

Fig 2 – Growth of marine fungal species at 9 °C, 13 °C and 20 °C: (A) *A. strictum*, (B) *F. fujikuroi*, (C) *P. decumbens*, (D) *P. chrysogenum* and (E) *F. sporotrichioides*. Glucose concentration in the culture medium was 5 g l^{-1}. Bars show standard deviations; Two way ANOVA (factors = temperature and days), n = 72; bars show standard deviations.

20 °C, respectively (Fig 3). At 0.1 g l^{-1} glucose concentration, biomass values reached 0.42 and 0.65 mg C. Incubations with 1 g l^{-1} glucose were associated with high increments of average biomass, reaching 1.39 mg C (*F. sporotrichioides*) and 1.07 mg C (*P. chrysogenum*) from 4 d onwards. Significant differences were observed between the growth rates of *P. chrysogenum*, *F. fujikuroi* and *F. sporotrichioides* with 1 g l^{-1} glucose, which produced biomass differences relative to the two incubation temperatures (9 °C and 20 °C) of 54.9 %, 18.5 % and 11.2 %, respectively (Fig 3).

Differences in growth rates caused by the interaction between temperature and glucose concentration were significant for the five studied species. However, in the case of *A. strictum* and *P. decumbens*, the observed growth differences were mainly due by the effect of glucose concentration (Table 2). The temperature dependency of the growth of *A. strictum* was lost when the glucose concentration was lower than 1 g l^{-1} (Figs 3 and 4).

Significant differences in oxygen consumption rates at different temperatures (Experiment 1) were found between *A. strictum* and *F. sporotrichioides*, and in both species the respiration rates were significantly greater at 20 °C than at lower temperatures (Fig 4A and E, Table 3). The highest respiration rate of *A. strictum* was 479 ± 55 µmol O_2 l^{-1} at 20 °C, which was considerably greater than those of the other studied species at any experimental temperature (Fig 4A). However, the five studied species had high respiration rates, greater than 200 µmol O_2 l^{-1}.

Respiration rates in the experiments with glucose concentrations (Experiment 2) of 0.01 and 0.1 mg l^{-1} showed maximum oxygen consumption of 107.9 and 150.6 µmol O_2 hr^{-1}, respectively (Table 3, Fig 5). The oxygen consumption rates of *A. strictum*, *F. fujikuroi*, *P. decumbens* and *P. chrysogenum* showed significant differences due to the combined effect of temperature and glucose concentration. In contrast, the differences in the respiration rates of *F. sporotrichioides* was clearly modulated only by glucose concentrations (Table 3).

Q_{10} values ranged from 2.2 to 6.7 (Table 4), indicating a strong temperature-dependence of respiration rates especially in *A. strictum* and *F. sporotrichioides*.

The increase in biomass showed increments in oxygen consumption (Fig 6). Incubations for the determination of specific respiration rates (i.e. oxygen consumption rates per biomass) were carried out with organisms from spores until forming a defined mycelium after 3 d or 4 d depending upon the species. Younger and smaller organisms had high oxygen consumption rate to biomass ratios, and thus had higher specific respiration rates than older hyphae. The relationship between log specific respiration rate and log body size (hypha length) could be represented by a linear equation ($p < 0.001$) with a slope of −0.26, although only 24 % of the variance was accounted for (Fig 7).

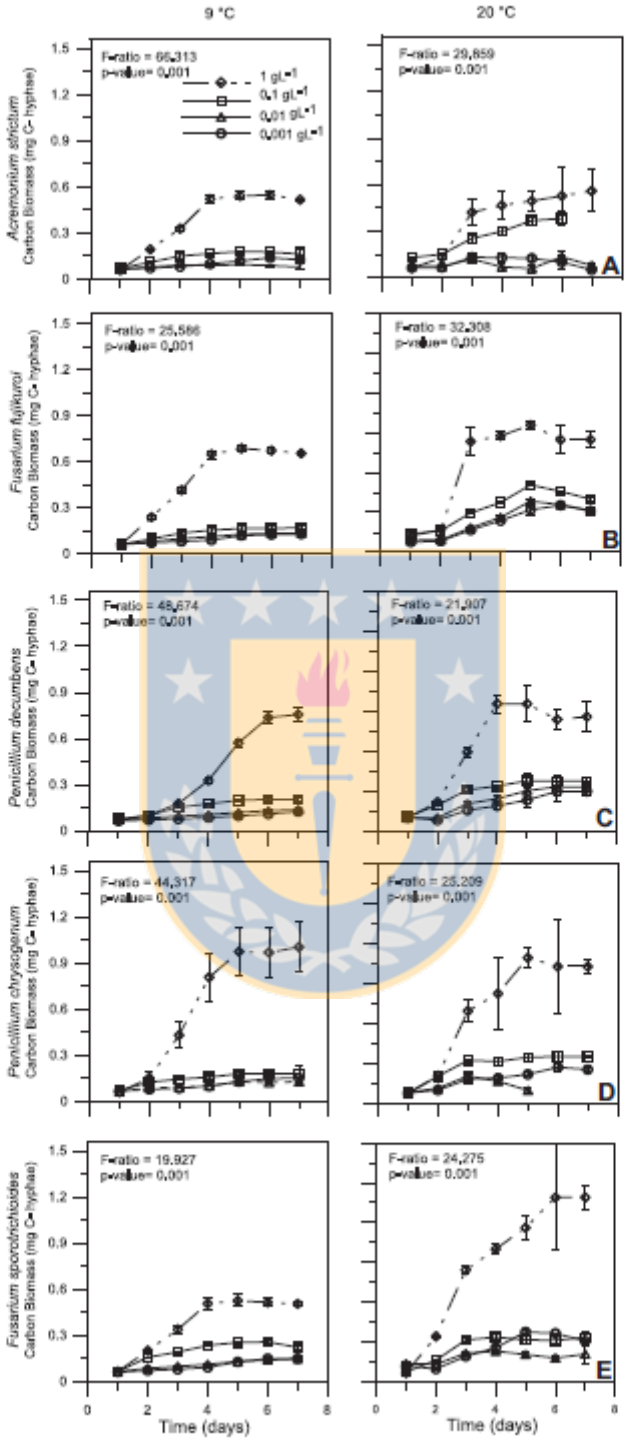

Fig 3 – Growth of marine fungal species at two incubation temperatures (9 and 20 °C) and four glucose concentrations (1 g l^{-1}, 0.1 g l^{-1}, 0.01 g l^{-1}, 0.001 g l^{-1}) in (A) *A. strictum*, (B) *F. fujikuroi*, (C) *P. decumbens*, (D) *P. chrysogenum* and (E) *F. sporotrichioides*. Two way ANOVA (factors: glucose concentration and days), n = 84, bars show standard deviations.

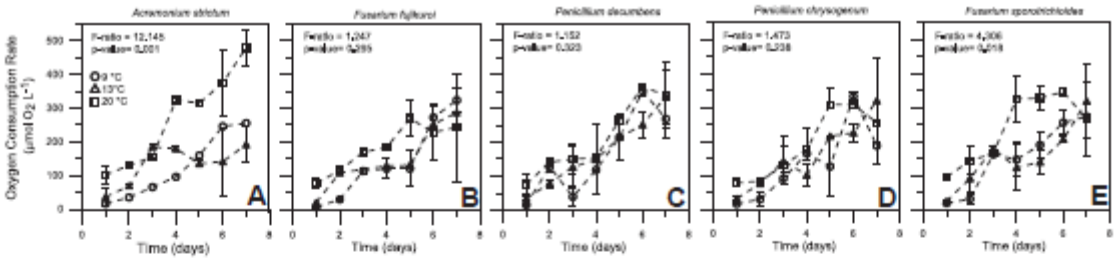

Fig 4 – Oxygen consumption rates of marine fungal species at temperatures of 9 °C, 13 °C and 20 °C, of (A) *A. strictum*, (B) *F. fujikuroi*, (C) *P. decumbens*, (D) *P. chrysogenum* and (E) *F. sporotrichioides*. Two way ANOVA (factors = temperature and days), n = 63, bars show standard deviations.

Discussion

In the coastal zone off central-south Chile, temperature follows an annual cycle associated with surface heating in which temperatures of 11.5–12.5 °C are characteristic of surface waters (0–30 m) during the austral winter, corresponding to Eastern South Pacific Transition Water (ESPTW) (Sobarzo et al., 2007). On the other hand, during March, surface temperatures are around 18 °C, similar to periods of upwelling relaxation. Close to the bottom on the continental shelf, temperatures are near to 9 °C during the spring months (Parada et al., 2001; Grob et al., 2003; Sobarzo et al., 2007). The fundamental factors that regulate fungal growth are temperature and the availability of nutrients (Madan and Thind, 1998), and according to the results of the present study, environmental temperature changes may produce considerable effects on the growth of filamentous fungi. Growth rates of fungal species inhabiting bottom sediments and the water column during upwelling periods were low (21–51 µg C hypha d^{-1}), while increments in temperature led to considerable increases in fungal growth rate, which could potentially reach values up to 153 µg C hypha d^{-1}. In fact, with the exception of *P. decumbens*, Q_{10} ranged between 3.9 and 6.85 in the studied fungal species (Table 4), which is considerably higher than values reported for other micro-organisms in aquatic systems. For instance, a value close to 2 has been reported for the Q_{10} of the metabolic rates of micro-organisms, such as prokaryotes (White et al., 1991), protists (Choi and Peters, 1992), phytoplankton (Eppley, 1972) and zooplankton (Ikeda et al., 2001). However, Q_{10} values estimated in our study are similar to those found for Arctic zooplankton (Q_{10} = 6.51; Alcaraz et al., 2013). The estimated Q_{10} for our species suggest that fungal metabolism in the coastal zone off central-south Chile is highly dependent on temperature.

The fungal biomass reported in this study is at the upper limit of values found in the field for the study zone (Gutierrez et al., 2010, 2011). Ideal conditions for growth of filamentous

Table 3 – ANOVA and post-hoc Tukey Tests of respiration rate (µmol O_2 l^{-1} hr^{-1}) experiments of fungal species. Data correspond to average respiration rates obtained during experimental days 3, 4 and 5. N for experiment 1 = 72; N for experiment 2 = 84. G.C. = Glucose concentration (g l^{-1}). "" indicates significant differences in respiration rates among species within a row; Numbers in superscripts indicates no significant differences in respiration rates between experiments (identified with the same number) conducted at different temperatures. Letters in superscripts indicate no significant differences in respiration rates between experiments (identified with the same letter) conducted at different glucose concentrations. Alpha used in ANOVA and post-hoc Tukey Tests = 0.05. In brackets = standard deviation

Exp. 1		*A. strictum*	*F. fujikuroi*	*P. decumbens*	*P. chrysogenum*	*F. sporotrichioides*	p
9°C		220.4 (35.7)	253.7 (91.5) [1]	285.6 (59.3) [2]	215.9 (51.9) [3]	246.2 (28.2) [4]	
13°C		156.4 (26.8)	235.0 (71.2) [1]	273.1 (43.4) [2]	254.0 (49.9) [a] [3]	222.2 (38.6) [4]	
20°C		399.3 (72.0)	244.2 (17.1) [1]	329.0 (44.1) [2]	291.1 (26.6) [a]	311.5 (36.7)	**
2-way ANOVA Temp x Days							
F-ratio (p-value)		12.145 (0.001)	1.247 (0.295)	1.152 (0.323)	1.473 (0.238)	4.306 (0.018)	
Exp. 2	G.C.						
9°C	0.1	112.6 (18.6)	70.3 (6.9) [c]	122.5 (22.5)	64.4 (7.5)	75.7 (7.2)	g **
	0.01	74.2 (29.6) [b]	65.8 (10.1) [c]	41.1 (4.1)	52.1 (3.6) [5]	61.2 (8.3)	h **
20°C	0.1	108.5 (17.7)	105.9 (19.7) [d]	74.5 (13.9) [e]	80.0 (6.1) [f]	88.2 (14.3)	g **
	0.01	65.5 (9.1) [b]	86.1 (3.2) [d]	70.5 (17.8) [e]	74.8 (25.7) [f] [5]	68.9 (25.9)	h **
3-way ANOVA Temp x Conc x Days							
F-ratio (p-value)		49.57 (<0.001)	15.70(<0.001)	183.46 (<0.001)	9.55 (<0.001)	33.16 (<0.001)	
Tukey Test Temp x days							
q value (p-value)		61.49 (<0.001)	18.95 (<0.001)	233.74 (<0.001)	23.71 (<0.001)	74.38 (<0.001)	

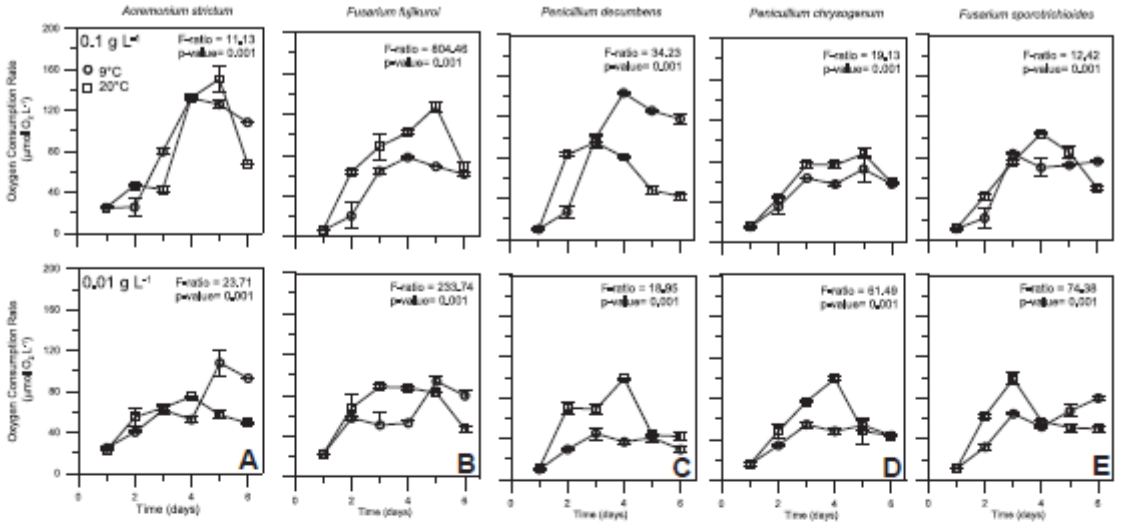

Fig 5 – Oxygen consumption rates of marine fungal species at two incubation temperatures (9 and 20 °C) and 4 glucose concentrations (1 g l^{-1}, 0.1 g l^{-1}, 0.01 g l^{-1}, 0.001 g l^{-1}) in (A) *A. strictum*, (B) *F. fujikuroi*, (C) *P. decumbens*, (D) *P. chrysogenum* and (E) *F. sporotrichioides*. Two way ANOVA (factors: glucose concentration and days), $n = 84$, bars show standard deviations.

marine fungi are potentially produced during periods of high autotrophic biomass in surface waters in the coastal upwelling system of central-south Chile (Gutiérrez et al., 2011). This is likely to be due to phytoplankton excretion of glucose-based exopolymers and stored polysaccharides (Biersmith and Benner, 1998; Aluwihare and Repeta, 1999; Janse et al., 1999). In addition, in this area there are high levels of organic matter input due to fresh water discharges along the coast near Concepción (Sánchez et al., 2008; Iriarte et al., 2012).

Biomass estimations of filamentous aquatic fungi have been determined from ergosterol (Seitz et al., 1979) or chitin (Sharma et al., 1977); however, these techniques are time-consuming and, therefore, less suitable for routine and rapid analyses. Our results show, for the first time for marine fungi, that there are strong associations in biomass estimates between O.D. and microscopy (i.e., biovolume and carbon conversion) and between O.D. and ATP. We highly recommend the use of O.D. to estimate biomass in laboratory analyses with pure strains due to its accuracy and precision, as well as its rapid application.

The five species analyzed showed increases in their growth and respiration rates at greater glucose concentrations (Fig 2) with respiration rates over 200 µmol O_2 l^{-1} hr^{-1} when incubations were conducted at 5 g l^{-1} glucose (Fig 4). The nutrient concentration in water has been shown to affect fungal activity, particularly sporulation (Suberkropp, 1998; Ferreira et al., 2006) and oxygen consumption (Gulis et al., 2004).

The growth of *F. sporotrichioides* is modulated by the synergistic interaction between temperature and glucose (Table 3). Generally, fungal biomass and activity – and consequently decomposition rates – increase with temperature and nutrient concentration (Ferreira and Chauvet, 2011).

The growth rates of *P. decumbens*, *P. chrysogenum* and *F. fujikuroi* are clearly temperature-dependent (Figs 2 and 3); nevertheless, their respiration rates are temperature-dependent only at low glucose concentrations (Fig 5). Lack of temperature-dependence of fungal respiration has been reported in other fungi, such as mesophilic ascomycetes (Maheshwari et al., 2000). The biochemical basis of this is not clear, but can be related to branched respiratory chains as a function of both cytochrome and alternative pathways in fungi (Joseph-Horne and Hollomon, 2000; Joseph-Horne et al., 2001). This physiological characteristic may be significant in terms of their growth in nature because these species of fungi may be able to maintain an optimal metabolic rate over a

Table 4 – Oxygen consumption rate Q_{10}. SD: standard deviation. 1–4: between day 1 to 4 (F-ratio = 0.356 and p-value = 0.83)

Q_{10}	20-9 °C	*A. strictum*	*F. fujikuroi*	*P. decumbens*	*P. chrysogenum*	*F. sporotrichioides*
Average day 1–4 (SD)		0.43 (0.21)	0.32 (0.16)	0.44 (0.22)	0.53 (0.26)	0.44 (0.22)
Maximum Q10 value		6.7	3.9	2.20	4.53	6.85

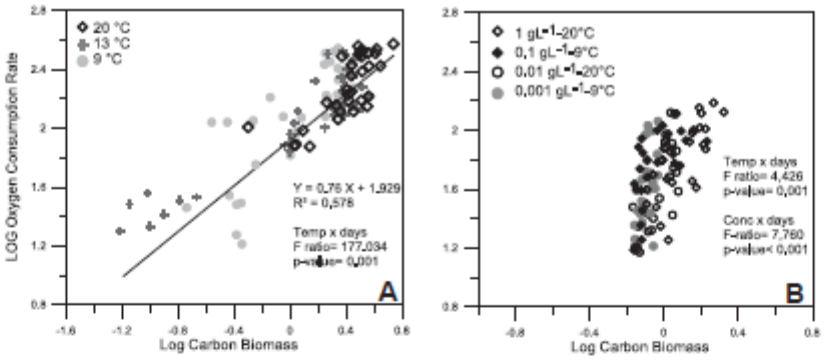

Fig 6 – Relationship between log carbon biomass and log oxygen consumption rate (least square regression, n = 214), (A) Data points correspond to temperatures of 9, 13 and 20 °C and a glucose concentration of 5 g l^{-1} (Exp. 1) and (B) data points correspond to four glucose concentrations. Note that the x-axes of both graphs are scaled differently.

broad range of temperatures, ensuring a competitive advantage over those that lack this ability (Prasad et al., 1979).

The highest fungal biomass reported for the study zone is close to 40 μg C l^{-1}, associated with temperatures of 12–13 °C, and located at the chlorophyll maximum (about 20 m depth) (Gutiérrez et al., 2011). During our experiments, at glucose concentrations under 0.01 g l^{-1}, biomass only reached average values of 290 μg C l^{-1}. Under natural conditions, fungal productivity can also be limited by ecological interactions, such as competition with bacteria (Mille-Lindblom et al., 2006; Frey-Klett et al., 2011) and mycotrophy (McMahon et al., 1974; Kagami et al., 2004; Lepère et al., 2007), and physical processes, such as burial (Janssen and Walker, 1999; Cornut et al., 2010).

There are a few studies that report respiration rates for terrestrial fungi and far fewer for the marine environment.

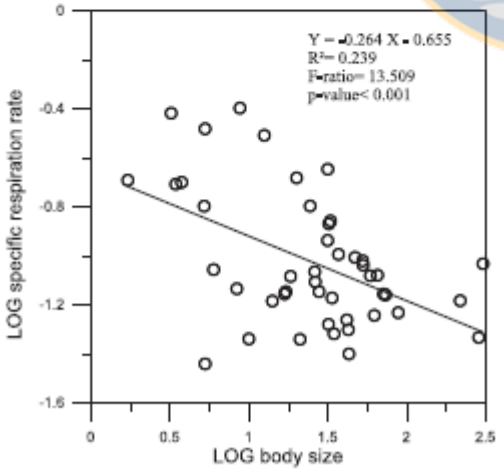

Fig 7 – Relationship between respiration rate as μmol O_2 l^{-1} mg C and mycelium size (μm; length) of fungal hyphae from *P. decumbens*, *P. chrysogenum*, *A. strictum*, *F. fujikuroi* and *F. sporotrichioides*. Least squares regression, confidence 0.95, n = 52.

Seto and Tazaki (1975) reported respiration rates of the saprotrophic fungus *Trichoderma lignorum* as high as 100–110 mg CO_2–C g^{-1} hyphae-C hr^{-1}, which are an order of magnitude lower than the values reported in this study. On the other hand, in incubations of the fresh water fungus *Phlyctochytrium punctatum* under starvation, respiration rates ranged from 22.6 μmol O_2 l^{-1} hr^{-1} (not starved) to 16.5 μmol O_2 l^{-1} hr^{-1} (starved), when glucose significantly stimulated oxygen consumption (Belsky et al., 1984). These rates are in the range of those determined in this study at 48 hr. The lack of standard presentation of respiration rates in the literature further complicates detailed comparisons between studies.

The total respiration rate of the ocean has been a point of controversy for many years due to the ongoing debate over sources and sinks of carbon in the ocean (e.g., Williams, 1998; del Giorgio and Williams, 2005; Laruelle et al., 2010). Knowledge of respiration at a community level is limited due to the lack of knowledge of the physiologies of many species and how they are affected by environmental conditions (Jin and Bethke, 2003; Suberkropp et al., 2010). Respiration is considered to be an important indicator of the physiological activity of a community, given that heterotrophic organisms acquire their energy from the oxidation of carbon compounds (Varó et al., 1993; Ikeda et al., 2001; Irwin et al., 2007). From Rubner (1883), a relationship between metabolic rates and body mass was defined by a power equation, which is valid for the great majority of organisms, where metabolic rate is equal to body mass to the power of 3/4 (Kleiber, 1961; West et al., 2002). In this study, the oxygen consumption rate of fungi had a slope of 0.76 (equivalent to $^3/_4$ power) and is within the typical average of exponents, explaining the enormous variability in metabolism from single-celled organisms to metazoans (Peters, 1983; Prothero, 1986). Moreover, mass-specific metabolic rates and the majority of biological rates, such as respiratory rates and cardiac stride frequencies, are on a scale of $M^{-1/4}$, with M as body mass (Lindstedt and Calder, 1981; McMahon and Bonner, 1983; Peters, 1983; Calder, 1984; Schmidt and Nielsen, 1984), which coincide with values reported in this study (i.e. −0.26; approx. −1/4). This exponent (−1/4) may be related to the characteristics of filamentous

fungal growth. As fungi grow, branching hyphae are formed, which produce associations with the hyphae from itself and from other individuals, creating a mycelium with a specific available geometric area inversely proportional to its size (Znidarsic and Pavko, 2001). This may generate a lower surface area and hence produce a decrease in gas, matter and heat exchange with the environment. Therefore, as biomass (filaments) increases, there should be a decrease in specific respiration rate.

According to our results the growth and respiration of filamentous fungi isolated from the upwelling system off central-south Chile may be strongly influenced by the presence of high concentrations of substrate (glucose) and changes in water temperature. The coastal ecosystem where *P. decumbens*, *P. chrysogenum*, *A. strictum*, *F. fujikuroi* and *F. sporotrichioides* were collected is highly variable both in terms of temperature and availability of organic matter (e.g., González et al., 2000; Montero et al., 2007; Sobarzo et al., 2007). Future research will be necessary to elucidate the *in situ* response of these filamentous fungi to these forcing factors in this highly productive ecosystem.

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4.2. Capítulo 2: Perfil de utilización de carbono de las especies de hongos filamentosos *Fusarium fujikuroi, Penicillium decumbens* y *Acremonium strictum* aislados desde ambientes costeros marinos.

Manuscrito enviado a la revista Mycologia (Mycologia 15-338): Fuentes, M.E., and Quiñones, R.A. Carbon Utilization Profile of the Filamentous Fungus Species *Fusarium fujikuroi, Penicillium decumbens* and *Acremonium strictum* Isolated from Marine Coastal Environments.

Resumen: Los hongos filamentosos marinos han surgido recientemente como un componente funcional en los sistemas marinos costeros, realizando un rol importante en la degradación de materia orgánica. Sin embargo, poco se sabe sobre su rol ecológico y su función en ciclos biogeoquímicos. Se caracteriza el perfil de utilización de carbono de tres especies de hongos filamentosos marinos, *P. decumbens, A. strictum* y *F. fujikuroi*, aislado de la zona de surgencia costera en la zona centro sur de Chile. Se evaluó el consumo de sustratos a través de sus índices metabólicos (asimilación sustrato - crecimiento de hifas), basado en la utilización de sustratos básicos de carbono, con el fin de entender su potencial impacto en la degradación de compuestos, principalmente carbohidratos y aminoácidos. Se encontró que estas especies son versátiles, utilizando una amplia gama de fuentes de carbono, mostrando gran capacidad (57,2 % del total) para la utilización de carbohidratos (monosacáridos, disacáridos, oligosacáridos, polisacáridos), y también aminoácidos (0,99 % del total), sugiriendo el uso de rutas metabólicas que incluyen la glucólisis / gluconeogénesis. La riqueza de sustratos reveló una amplia utilización de fuentes de carbono suministradas experimentalmente, lo que produjo índices Shannon de utilización particular de sustratos de 4.02 para *A. strictum*, 4,01 para *P. decumbes* y 3,91 para *F. fujikuroi*, con diferencias significativas entre 18 de los sustratos utilizados por las tres especies. Los hongos filamentosos deben considerarse parte integral de la comunidad microbiana marina e incluidos en los modelos de ciclos biogeoquímicos de ecosistemas de surgencia.

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Abstract	Facultative marine filamentous fungi have recently emerged as a functional component in coastal marine systems. However, little is known about their ecological role and functions in biogeochemical cycles. P. decumbens, A. strictum and F. fujikuroi were isolated from the coastal upwelling zone off south-central Chile. Their carbon profiles were characterized using Biolog FF MicroPlates. These species used a wide range of carbon sources, mainly carbohydrates, but also amino acids, suggesting the use of metabolic routes that include glycolysis/gluconeogenesis. Substrate richness revealed a great capacity for the utilization of nutritional sources, reflected by the following Shannon Indices of utilization of specific substrates: 4.02 for A. strictum; 4.01 for P. decumbes; and 3.91 for F. fujikuroi. Significant differences were found between 18 substrates utilized by all three species. Results suggest that filamentous fungi should be considered an integral part of the marine microbial community and included in biogeochemical cycling models of upwelling ecosystems.
Associate Editor	Assigned
Keywords	Marine fungi, substrate utilization, Oxidation of substrates, Biolog Filamentous Fungi (FF) microplate, upwelling, Humboldt Current System

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Carbon Utilization Profile of the Filamentous Fungus Species *Fusarium fujikuroi*, *Penicillium decumbens* and *Acremonium strictum* Isolated from Marine Coastal Environments

Running title: Carbon utilization by coastal marine fungi

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Abstract

Marine filamentous fungi have recently emerged as a functional component in coastal marine systems. However, little is known about their ecological role and functions in biogeochemical cycles. *P. decumbens, A. strictum* and *F. fujikuroi* were isolated from the coastal upwelling zone off south-central Chile. Their carbon profiles were characterized using Biolog FF MicroPlates. These species used a wide range of carbon sources, mainly carbohydrates, but also amino acids, suggesting the use of metabolic routes that include glycolysis/gluconeogenesis. Substrate richness revealed a great capacity for the utilization of nutritional sources, reflected by the following Shannon Indices of utilization of specific substrates: 4.02 for *A. strictum;* 4.01 for *P. decumbes;* and 3.91 for *F. fujikuroi*. Significant differences were found between 18 substrates utilized by all three species. Results suggest that filamentous fungi should be considered an integral part of the marine microbial community and included in biogeochemical cycling models of upwelling ecosystems.

Key words: Marine fungi; Biolog Filamentous Fungi (FF) microplate; substrate utilization; oxidation of substrates; upwelling; Humboldt Current System.

Introduction

Scientific knowledge of the structure and function of microorganism communities in the ocean has been constantly evolving since the 1960's (Holmes and Anderson, 1963; Pomeroy, 1974; Azam *et al*., 1983; Biddanda, 1986; Herrndl and Weinbauer, 2003). The study of new highly abundant groups such as viruses and archaea has opened up new questions regarding the ecology and biogeochemistry of the ocean (de Long, 1992; Suttle, 2007; Levipan *et al*., 2012; Breitbart, 2012; Lloyd *et al*., 2013). The first studies to identify and isolate fungi from marine systems were published at the beginning of the 1960's (Kohlmeyer, 1960; Meyers and Reynolds, 1960; Johnson and Sparrow, 1961). Only in recent decades, however, have marine fungi emerged as a functional component now considered a missing link in the marine microbial realm (Jebaraj and Raghukumar, 2010; Gutiérrez *et al*., 2011; Wang *et al*., 2014). At present the ecological role of fungi in marine systems remains poorly understood.

The coastal zone of the Humboldt Current System off south-central Chile (36.5° S) is characterized by seasonal coastal upwelling that leads to high levels of primary productivity (Montero *et al*., 2007). In addition, the Bio-Bio and Itata Rivers deliver an important nutrient load to the coastal zone (Ahuamada, 2002; Pantoja *et al*., 2011; Leniz *et al*., 2012). These processes produce sediments that are rich in predominantly marine organic carbon, affecting the final destination of organic matter (Arnosti, 2011). High rates of primary production generate high availability of organic molecules; the microbial

community that inhabits this zone is responsible for an important fraction of the degradation of photosynthetic carbon products (Troncoso *et al*., 2003; Cuevas *et al*., 2004; Quiñones *et al*., 2010).

According to organic matter degradation models, particulate organic matter (POM) is transformed into dissolved organic matter (DOM) by extracellular enzymes generated by microorganisms that produce low molecular weight compounds. These compounds are taken up through the cell membrane and subsequently respired or used for building cell biomass (Hoppe *et al*., 1988; Arnosti *et al*., 1994; Azam, 1998). The production of small molecules that are susceptible to be incorporated into the cell (e.g. amino acids and sugars) is determined by processes such as hydrolysis of larger molecules (Hoppe *et al*., 1988), dissolution of particles (Nagata *et al*., 2003), sloppy feeding (Moller *et al*., 2003), exudation of organic carbon by plankton (Sharp, 1977), grazing by bacterivorous protozoa (Nagata and Kirchman, 1991) and viral lysis (Fuhrman, 1999).

DOM is one of the major reservoirs of active carbon in the ocean, composed of molecules of varying lability to bacterial degradation (Benner, 2002). The majority of DOM is smaller than 1000 Da; only 1% of known monomers can be used rapidly by microbes in the ocean (Benner, 2002). These substrates generally must be degraded to a size of approximately 600 Da before they can be transported actively through cell wall pores (Weiss *et al*., 1991). In filamentous fungi the active transport of nutrients such as sugars, amino acids, nitrates, ammonium, sulfates and phosphates involves special separation of the ion pumps mostly behind the apex, whereas symport proteins are active close to the tip (Kavanag, 2005). Thus nutrient uptake occurs at the hyphal tip as it

continuously drives into “fresh resource”, and the mitochondria located behind the apex supply ATP to support the ion pump and generate proton motive force (Kavanag, 2005).

Extracellular enzymatic hydrolysis of carbohydrates and proteins has been measured as a proxy for microbial reworking in the coastal upwelling system of the Itata River mouth (Pantoja *et al*., 2011). Rates of hydrolysis of protein substrates were 4 to 7 times higher than those of carbohydrates (glucose and cellulose substrates) in seawater (Pantoja *et al*., 2011), and have been attributed to the preferential mineralization of organic nitrogen with respect to carbon in the marine environment (Lee *et al*., 2004). Moreover, the ratio of the potential activity of protein *vs*. carbohydrate hydrolysis showed a general increase towards the more offshore stations, which was more pronounced during winter (Pantoja *et al*., 2011).

Fungi play a leading role in the initial degradation of polymers such as cellulose and lignin in plant residues (Kjøler and Struwe, 2002; Valdrian and Valásková), given that most bacteria are not capable of attacking these residues because they do not produce the necessary depolymerases (Gadd, 2006). Thus fungi actively participate in the carbon cycle by returning carbon dioxide to the atmosphere (Carlile *et al*., 2001; Hobbie, 2005). The opportunistic strategy of fungal nutrition provides a rapid response to changing substrate conditions (Lindahl *et al*., 2010).

Planktonic fungi are an important microbial component of marine coastal ecosystems, and as with other heterotrophic microplankton they are active in the water column and respond to primary production and organic matter availability (Gutiérrez *et al*.,

2010, 2011; Wang *et al*., 2014). In fact, an increase in metabolic rates of marine filamentous fungi has been found in response to higher levels of nutrient availability (Fuentes *et al*., 2015). Nutrients are the leading determining factor for phenotypic characteristics (e.g. kinetics of respiration and growth), making them a key parameter for assessment (Mchunu *et al*., 2013).

Recent research in the coastal zone of the HCS has shown that the vertical distribution of fungal biomass in the water column is closely linked to phytoplankton biomass and that around 30% of carbon generated photosynthetically may be processed by fungi (Gutiérrez *et al*., 2011). This likely follows a seasonal pattern associated with increases in available organic matter during active upwelling in the photic zone. These filamentous marine fungi were mainly present in the size fraction above 25 μm (i.e. without prokaryotes), a size class showing high rates of extracellular hydrolysis in this ecosystem (Gutiérrez *et al*., 2011).

The main aim of this study was to elucidate the role of fungal species in processing organic matter in a highly productive marine ecosystem. This study characterizes carbon utilization profiles of three species of filamentous marine fungi isolated from the coastal upwelling zone of the Humboldt Current System. The characterization of substrate use assumes the prior utilization of extracellular enzymes in the degradation of large compounds into small molecules, which are then transported through the fungal cell membrane. In the text we refer to the compounds in the culture medium and not the small molecules generated by exoenzyme activity. Particular emphasis was given to the use of

carbohydrates and amino acids because carbohydrates are the major components of organic matter in the ocean and account for 3% to 30% of the bulk dissolved organic carbon (Hung *et al*., 2003; Gueuen *et al*., 2006). Amino acids are the major forms of nitrogen and important components of organic carbon in most marine organisms (Cowie and Hedges, 1992). They also represent significant fractions of organic matter in recent coastal marine sediments and in water column particles (Cowie and Hedges, 1992).

Materials and Methods

Seawater and sediment samples were collected from the coastal zone off south-central Chile on board R/V Kay Kay II (University of Concepción, Chile), at two oceanographic stations near the Itata River mouth (Fig. 1). Field sampling was authorized by Exempt Resolution 244/2013, Undersecretariat of Fisheries, Ministry of Economy, Chile. All samples were cultured with glucose agar and yeast extract for the isolation of marine fungal species (Johnson and Sparrow, 1961). The most abundant colonies were sorted out and cultured in Emerson's YpSs agar and were added to the fungal species bank of the Laboratory of Marine Organic Geochemistry of the University of Concepción. *Penicillium decumbens* was collected from the water column at 30 m depth at station P3-2 (36º 15.1' S, 72º 52.5' W). *Acremonium strictum* and *Fusarium fujikuroi* were collected from surface sediments at station P3-5 (36º 23.6' S, 72º 54.0' W; depth=10 m). For a detailed account of isolation and identification protocols see Gutiérrez *et al*., (2010) and Fuentes *et al*., (2015). A single strain from each species was used in the experiments.

The following steps were taken for the carbon utilization profile. A pure culture of a fungal species was grown on a 2% malt extract agar plate until enough conidia were present to prepare a suspension. The conidia were swabbed from the surface of the agar plate and suspended at a specified density in inoculating fluid (carbon-free). The initial density was the same for all species, measured as the minimum optical density (O.D.) recorded at 450 nm. 100 µl of suspension was pipetted into each well of a Filamentous Fungi (FF)

MicroPlate. The FF MicroPlate was incubated at 20 °C for 192 hours and the MicroPlates were read every 24 h using a spectrophotometer (Elx800, Bio-Tek Instruments) at 490 nm and 750 nm. Each isolate was replicated 3 times.

The Biolog FF MicroPlates test panel contains 95 wells with different carbon-containing compounds and a control well (water). Iodonitrotetrazolium violet (INT) was used as a redox dye to measure colorimetrically the mitochondrial activity (redox value) resulting from oxidation of metabolizable carbon sources. All the wells are colorless when first inoculated. The oxidation of succinate to fumarate in the citric acid cycle, mediated by succinate dehydrogenase and FAD, causes INT to be reduced to a violet-colored formazan dye with peak absorbance at 490 nm (Kubicek *et al*., 2003; Tanzer *et al*., 2003; Bochner, 2009). Reduction of the tetrazolium dye due to increased cell respiration results in formation of a purple color in the well (Tanzer *et al*., 2003). The reduction of INT and production of colored formazan is irreversible, and the accumulation of formazan measured spectrophotometrically reflects quantitatively the oxidation of the test substrate. The 750 nm reading measures turbidity, reflecting mycelial production of the test substrate. Since the absorbance spectrum of hyaline mycelium is essentially level over the range from 490 to 750 nm, a corrected redox value (CRV) for the production of formazan is obtained by subtracting the 750 nm reading (490–750 nm) (Kubicek *et al*., 2003). The CRV of each well was corrected by subtracting the control value. The analyzed substrates were grouped using the criteria applied by Tosiah (2013), in the following manner: amine and amides (A/A), amino acids (AA), carbohydrates (CH), carboxylic acids (CA), miscellaneous (M) and polymers (P).

As a measure of the number of substrates utilized by fungi (substrate richness) and the diversity of the extent of utilization of particular substrates (substrate evenness) at 96 h, the Shannon index (H) was used: $H = -\sum_{i=1}^{N} pi(\ln pi)$, where p_i is the proportion of a microbial activity on substrate *i* of total microbial activity and *N* is the number of substrates on a plate (95 for FF) (Zak *et al*., 1994; Tam *et al*., 2001; Muñiz *et al*., 2014). Here, the value of H describes the ability of the fungal species to degrade more or fewer types of carbon sources, thus acting as an index of physiological diversity. In this case, filamentous fungi that are able to degrade more substrates and/or to degrade substrates with similar efficiency will have higher values of H.

A Joining Cluster Analysis was applied to CRV (as a percentage relative to the total) between species at 96 h, which corresponds to the linear growth phase on the majority of carbon sources. This was carried out using Statistica 6.0 (Stat Soft, Inc.) and was applied to identify the different groups of carbon sources from the experimental data set. The Joining Cluster Analysis is based on Euclidean distance with complete linkage. Out of the 95 compounds included in this study, only amino acids and carbohydrates will be discussed in detail. A one-way ANOVA was used to detect differences in optical density (490 – 750 nm) between species.

The kinetics of reactions were measured using growth rates between days 0 and 2 and between days 2 and 4. Curves of average CRVs were plotted for each substrate and were grouped in 4 subdivisions based on the results of the Joining Cluster Analysis. The metabolic pathways of the substrates producing higher growth were determined using the

Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg), which provides a collection of pathway maps for the metabolism of carbohydrates, amino acids, etc.

Results

The species analyzed displayed great capacity to utilize different substrates, which were grouped according to their range of corrected redox value (CRV): <0 nm; 0-0.2 nm; 0.2-0.4 nm; >0.4 nm. *P. decumbens* possessed the greatest capacity to utilize the greatest amount of substrate with the highest CRV. Growth of *A. strictum* and *F. fujikuroi* (range >0.4 nm) utilized only three substrates each. The substrates that contributed most to the CRVs of all three species were the carbohydrates sucrose, D-fructose, L-arabinose, D-xylose, α-D-glucose and the amino (47.6 ± 4.1 % of the total) acids L-proline and L-alanine (0.99 ± 0.2 % of the total) (Fig. 2). Growth rates of all three species displayed generally similar trends in substrate utilization; the greatest biomass was closely linked to high substrate consumption. Various substrates were capable of generating high CRVs even though the resulting mycelial biomasses were intermediate (<0.3 nm). This was the case of Tween 80 (M), the disaccharide sucrose, monosaccharides (L-arabinose, D-ribose, D-xylose) and α-D-glucose (Fig. 2).

The CRV of *F. fujikuroi* displayed minimal metabolic values in the 0-0.2 nm range, even though this was the species that had the greatest biomass in this range compared to the other species (Fig. 2). The use of substrates (substrate richness) in *A. strictum, P. decumbes* and *F. fujikuroi* was 88, 87 and 81 substrates, respectively. This was reflected by high Shannon indices of substrate consumption in all three species; 4.02, 4.01 and 3.91 for *A. strictum, P. decumbes* and *F. fujikuroi*, respectively. Of the 95 substrates analyzed, 18 presented significant differences between the three species (Fig. 3).

The carbon sources most utilized by the three species were grouped by the cluster analysis in Cluster I (Fig. 3). These produced the fastest growth rates; in most cases growth slowed after 48 h and maximum growth was achieved at 72 h of incubation (Fig. 4). The carbohydrate-based compounds, both single sugars and disaccharides, showed the most efficient CRV profile, with the disaccharide sucrose inducing greatest growth, followed by the sugars D-fructose, D-galactose, L-arabinose, D-ribose, a-D-glucose, D-mannose, D-sorbitol, maltose (disaccharide) and D-xylose (Figs. 3 and 4).

The highest mycelium growth rates were associated with L-proline and D-fructose (*A. strictum*), L-proline and sucrose (*P. decumbens*) and L-proline (*F. fujikuroi*) (Fig. 2). L-proline and L-alanine (0.99 % of the total; Cluster I) promoted the most growth and nutrient utilization, followed by L-glutamic acid and L-serine (Cluster II) (Fig. 3). These results demonstrate the ability of the studied species to grow relatively well on most plant-based compounds.

Cluster II showed moderate growth of mycelia based on the utilization of disaccharides and oligosaccharides (maltotriose, D-raffinose, turanose, gentobiose, D-melezitose, D-trehalose) and m-inositol (monosaccharide) (25.5 ± 0.4 % of the total). Cluster III was made up of a range of carbohydrates from monosaccharides to polysaccharides (Fig. 3). These substrates produced lower CRVs and lower growth rates (Fig. 2). Interestingly, all three fungal species grew well on cellobiose, suggesting that they are able to produce cellulose-hydrolyzing proteins.

Cluster IV was a larger group with very low CRVs and with low associated growth rates (Figs. 2 and 3). Amino acids, amine/amides, carboxylic acids and carbohydrates were all equally represented (Fig. 3). This cluster contained the greatest number of amino acids: L-aspartic acid, L-threonine, L-phenylalanine, L-ornithine, N-acetyl-L-glutamic acid, adenosine and glucuronamide (Fig. 3).

The kinetics of growth displayed mostly hyperbolic curves for the substrates that were most used and sigmoidal curves for most other substrates, with a time lag between 1 d (Cluster I) and 4 d (Cluster IV). Growth rates before the second day were much lower (Figs. 2 and 4), increasing considerably between days 2 and 4, after which the stationary phase occurred where an equilibrium was reached and the growth rate was close to 0 (Fig. 4).

The substrates grouped in Cluster I displayed rapid growth and substrate utilization values, in which the consumption of substrates entered an exponential phase around 24-48 h, reaching a stationary phase at 72 h (Fig. 4). This cluster included most of the substrates used displaying hyperbolic curves, a substrate utilization lag between 1 and 2 days and growth rates increasing rapidly after day 2 (Fig. 2), reaching values of 0.4-0.5 after 72 h (Fig. 4).

The second cluster had high consumption rates, however the kinetics of curves were sigmoidal with a time lag between 24 and 48 h, reaching maximum utilization and growth during the stationary phase at 96 h (Fig. 4). Cluster III contained substrates that became

utilized after 24 to 48 h, reaching the stationary phase at almost 120 h (Fig. 4). In this cluster growth rates increased slightly between days 2 and 4 (Fig. 2).

Lastly, the CRVs of Cluster IV showed substrate utilization and growth curves with low rates until 72-96 h, after which there was a slight increase, reaching maximum rates at 144 h, in addition to reaching maximum values of O.D. that did not exceed 0.2 nm (Fig. 4). This cluster included intermediates of the TCA-cycle: quinic acid, D-gluconic acid, g-amino-butyric acid, succinic acid, D-glucoronic acid, 2-keto-D-gluconic acid, D-galacturonic acid, bromosuccinic acid and succinamic acid.

Discussion

The analysis of substrate utilization for *P. decumbens*, *A. strictum* and *F. fujikuroi* indicated the versatility of these organisms, which utilize a wide variety of carbon sources including carbohydrates (monosaccharides, disaccharides, oligosaccharides and polysaccharides), amino acids, carboxylic acids, amines, amides and polymers. Extracellular enzymes break down many of these compounds prior to their utilization as carbon and energy sources. Fungi possess different modes of uptake by the plasma membrane: free diffusion, facilitated diffusion, diffusion channels and active transport (Kavanagh, 2011). Sugars and amino acids in filamentous fungi use active transport of nutrients (Kavanagh, 2011). Filamentous fungi are well known for having several unique biochemical pathways that assimilate a broad range of simple and complex substrates and produce a wide variety of metabolites (Singh, 2009; Kavanagh, 2011).

The carbohydrates which produced the greatest growth rates and CRVs were grouped in Cluster I. As observed in other studies, this principal cluster (greatest metabolic contribution) was almost exclusively made up of carbohydrates (Druzhinina *et al*., 2006; Seidl *et al*., 2006; Mohammad *et al*., 2012; Zheng *et al*., 2012). However, there were amino acids such as L-proline and L-alanine in this cluster, with maximum values for metabolic activity. Proline can be used as a source of carbon and nitrogen, as seen for example in *Aspergillus nidulans* (Bailey and Arst, 1975; González *et al*., 1997; Cuber *et al*., 2000). Alanine (similar to proline) can be transformed into glucose (gluconeogenesis) and can be

used in the TCA cycle to supply the cell with energy, and thus can be used by fungi to complement their supply of nitrogen and energy (Bechem, 2012). Other gluconeogenic amino acids used included L-serine, L-glutamic acid (Cluster II), L-asparagine (Cluster III), L-aspartic acid, L-threonine, L-phenylalanine, L-ornithine, N-acetyl-L-glutamic acid, adenosine and glucuronamide (Cluster IV) (Fig. 3).

Sixty-one substrates have been described for marine, coastal or estuarine environments as dissolved compounds in the water column or as products of microalgal degradation liberated into the marine environment. Moreover, of the 28 substrates that made up the principal Clusters (I and II) (Cantino, 1949; Jannash, 1949; Liebezeit *et al*., 1980; Ittekkot *et al*., 1981; Sakugawa and Hanta, 1985; Fuhrman and Ferguson, 1986; Paanagiotopoulos and Sempere, 2005; Poretsky *et al*., 2010; Yang *et al*., 2010; Hassler *et al*., 2011; Arnosti and Steen, 2013), only 3 (salicin, turanose, amigdalin) are not found in the ocean.

Carbon from autochthonous primary production (i.e. phytoplankton) and allochthonous production (terrestrially derived material) is found in high concentrations near the coast off south-central Chile (Daneri *et al*., 2000; Montero *et al*., 2007; Salamanca and Pantoja, 2009; Iriarte *et al*., 2010), which promotes mycoplankton abundance and diversity (Gutiérrez *et al*., 2011). A similar effect has been observed in other systems such as the Pacific Warm Pool (from Honolulu, Hawaii to Brisbane, Australia) (Wang and Johnson, 2009), in Hawaiian coastal waters (Gao *et al*., 2010), and from direct observations

of the parasitic relationship of fungi on phytoplankton off Southern India (Raghukumar, 2006).

There are large populations of planktonic fungi in coastal and oceanic waters, including filamentous free-living forms, yeasts, and forms associated with planktonic organisms (Raghukumar, 2006; Wang and Johnson, 2009; Gao *et al*., 2010; Gutiérrez *et al*., 2011). These fungi are clearly an important component of the microbial community, along with other microplanktonic heterotrophic organisms which are active in the water column and respond to primary production (Gutiérrez *et al*., 2011; Wang *et al*., 2014). The biochemical composition of the main algal taxa (diatoms, primnesiophytes, prasinophytes, chlorophytes, eustigmatophytes, cryptomonads and rhodophytes), indicates that the sugar content is considerably variable across these groups. Glucose is the predominant sugar, varying between 21-87%, followed by galactose, mannose and arabinose; fucose, ribose and xylose; all in varying proportions (0-17%) (Hellebust, 1974; Brown *et al*., 1997). All components were highly assimilated (>0.29 nm) by the filamentous fungi in this study (except for fucose, which had a lower CRV), which implies that these microalgae are a good source of nutrition for our species isolated from the coastal upwelling zone.

The amino acid content of microalgae (diatoms, primnesiophyces, prasinophytes, chlorophytes, eustigmatophytes, cryptomonads and rhodophytes) is surprisingly similar regardless of the algal taxon, which suggests that the quality of proteins is also similar (Hellebust, 1974; Volkman *et al*., 1993; Brown *et al*., 1997). Among the most common amino acids produced by microalgae are the proteinogenic L-aspartic acid and L-glutamic

acid (Brown and Jeffrey, 1992). These amino acids are utilized by all three species of fungi included in this study, but displayed low growth and CRVs, with O.D. values below 0.2 nm. Other important sources of carbohydrates and amino acids are available in costal environments such as vascular plant tissues (angiosperms and gymnosperms, woody and nonwoody), marine macrophytes, phytoplankton and zooplankton (Cowie and Hedges, 1992).

In terms of terrestrial carbon, organic detritus serves as a source of nutrients for fungi in coastal marine ecosystems (Steele, 1967; Raghukumar, 2004). This renders these systems suitable for the development of diverse mycoplankton communities (Lindahl *et al*., 2010). Fungi play a key role in the initial degradation of polymers such as cellulose and lignin in plant residues (Kjøller and Struwe, 2002; Baldrian and Valásková, 2008). In the microorganisms that produce cellulase, cellulose is a common indicator of cellulase formation (Knowles *et al*., 1987). Given that cellulose is insoluble and impermeable, oligosaccharides such as D-cellobiose and a-D-lactose –used by all three species in this study – can be considered direct indicators of cellulases (Knowles *et al*., 1987; El-Gogary *et al*., 1989; Gong *et al*., 19879). Of these oligosaccharides, cellobiose is a common product formed from cellulose via exo-cellobiohydrolase and is considered a natural inductor (Mandels and Reese, 1960; Stemberg and mandels, 1979). Thus species of fungi isolated from coastal areas may also be contributing to the degradation of large plant polymers.

It has been suggested that the main biochemical pathways used by the species included in this study are related to hexose (e.g. glucose, fructose, galactose and mannose) and disaccharide (e.g. maltose or sucrose) metabolism, similar to what has been observed in

Saccharomyces cerevisiae (Pronk *et al*., 1996). These metabolic pathways are made up of intermediaries of glycolysis/gluconeogenesis (e.g. amino acids, glycerol), the tricarboxylic acid cycle (TCA) and the pentose phosphate pathway (Pronk *et al*., 1996; Rodrigues el al., 2006). Moreover, they can use catabolic alternatives such as the glyoxylate shunt and oxidative phosphorylation (Mohammad *et al*., 2012).

Filamentous fungi isolated from the coastal zone of south-central Chile utilize a wide range of substrates, which provide them with a great capacity for assimilating plant detritus and phytoplankton biomass, among other sources of carbon. Filamentous fungi should be considered an integral part of the microbial community, as put forward by Gutiérrez *et al*., (2011) and Wang *et al*., (2014), and should be included in models of microbial ecosystems and biogeochemical cycles. Metabolic activity in marine fungi, in addition to the activity of prokaryotic microbes, should be highlighted in relation to the assimilation of large molecules that are difficult to degrade, such as cellobiose. These assimilation/degradation processes play an important role in the conversion of POM to DOM in marine coastal systems, as well as in processing a great and varied quantity of carbon-based molecules.

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Figure legends

Figure 1. Map of the study area.

Figure 2. Corrected redox value (□ 490–750 nm) and growth rate for *A. strictum*, *Penicillium decumbens* and *F. fujikuroi*. Δ Growth rate measured at 750 nm (day 4 – day2) and Δ Growth rate measured at 750 nm (day 2 – day1).

Figure 3. Joining cluster analysis and Heatmap applied to 95 carbon sources.
Analysis based on utilization of carbon sources and growth rate for *A. strictum*, *P. decumbes* and *F. fujikuroi* measured at 490 nm and 750 nm. + Significant differences between species ($p<0.05$).

Figure 4. Growth and consumption kinetics for three species isolated from the coastal zone, grouped according to clusters generated in Fig. 2. Colored curves indicate the substrates that display significant differences between species ($p>0.05$).

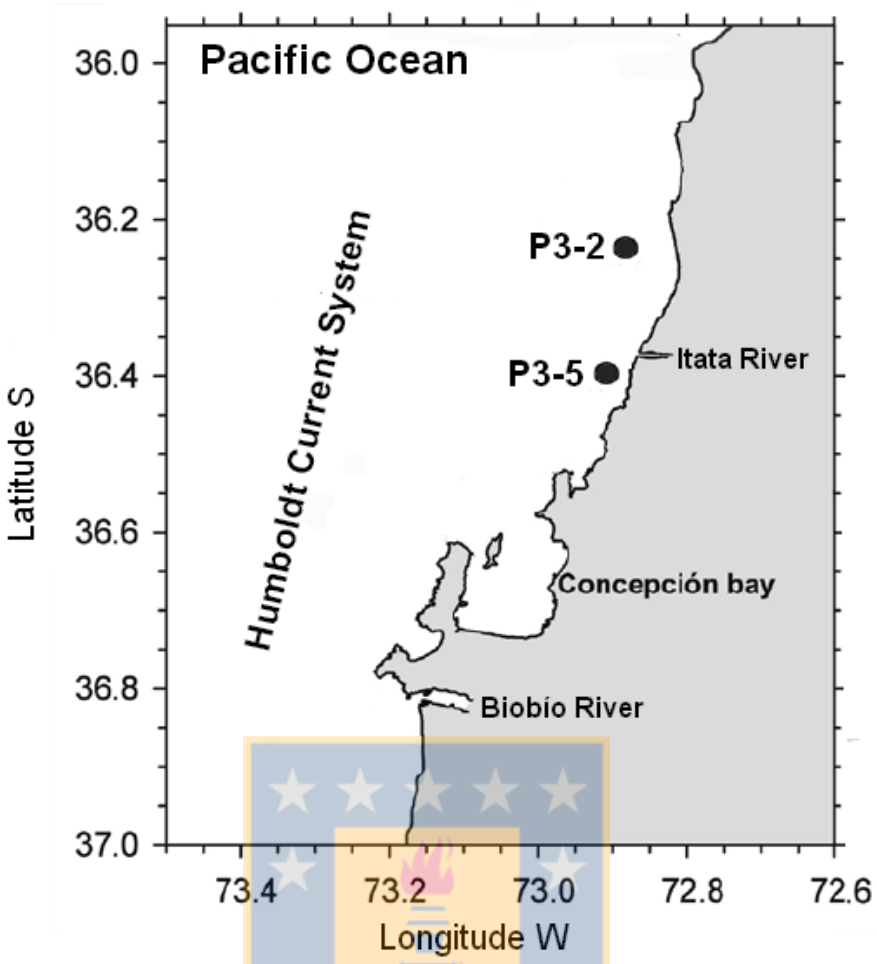

Figure 1. Map of the study area.

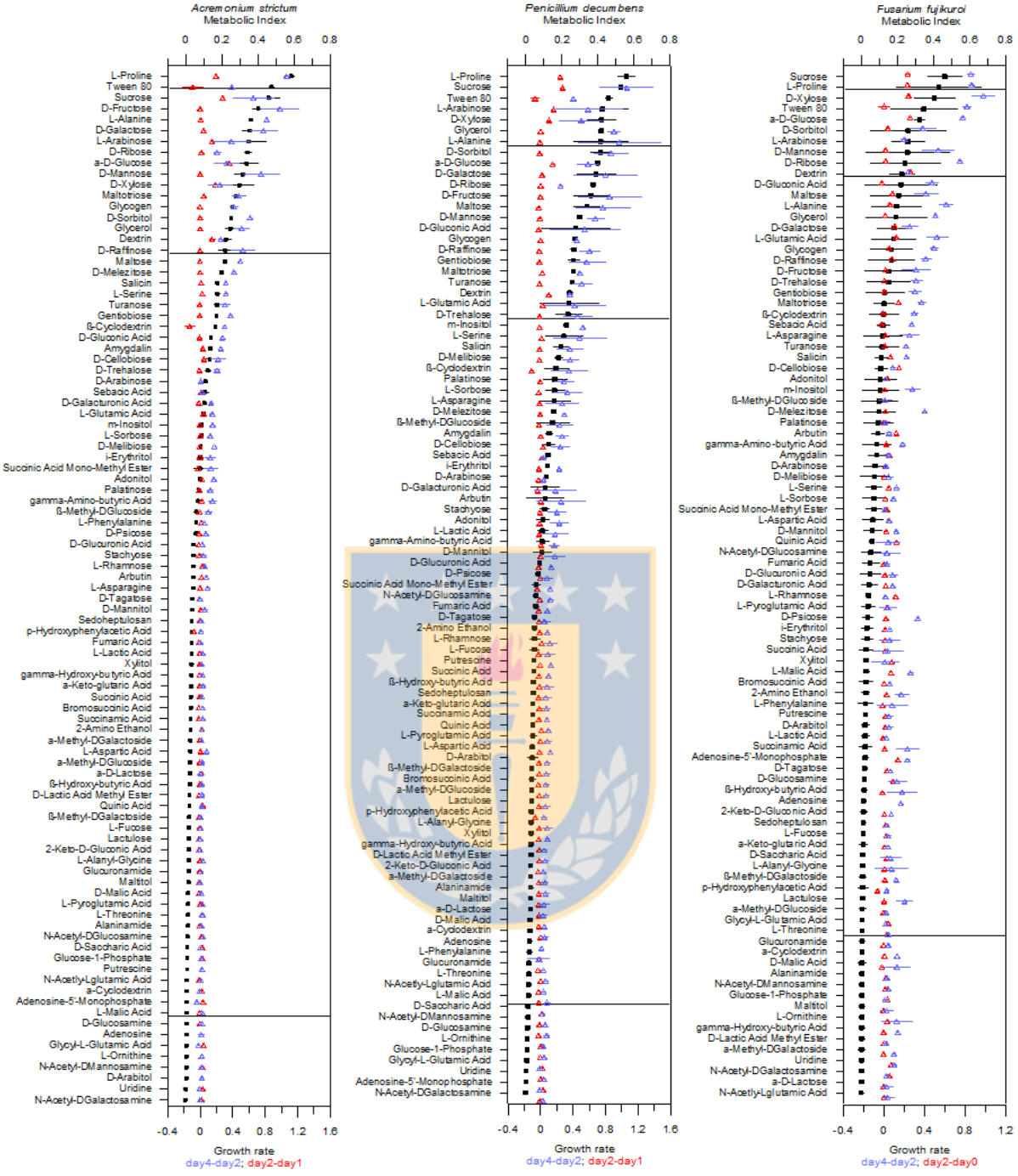

Figure 2. Metabolic index (□ 490-750 nm) and growth rate for *A. strictum, Penicillium decumbens* and *F. fujikuroi*. Δ Growth rate measured at 750 nm (day 4 – day2) and Δ Growth rate measured at 750 nm (day 2 – day1).

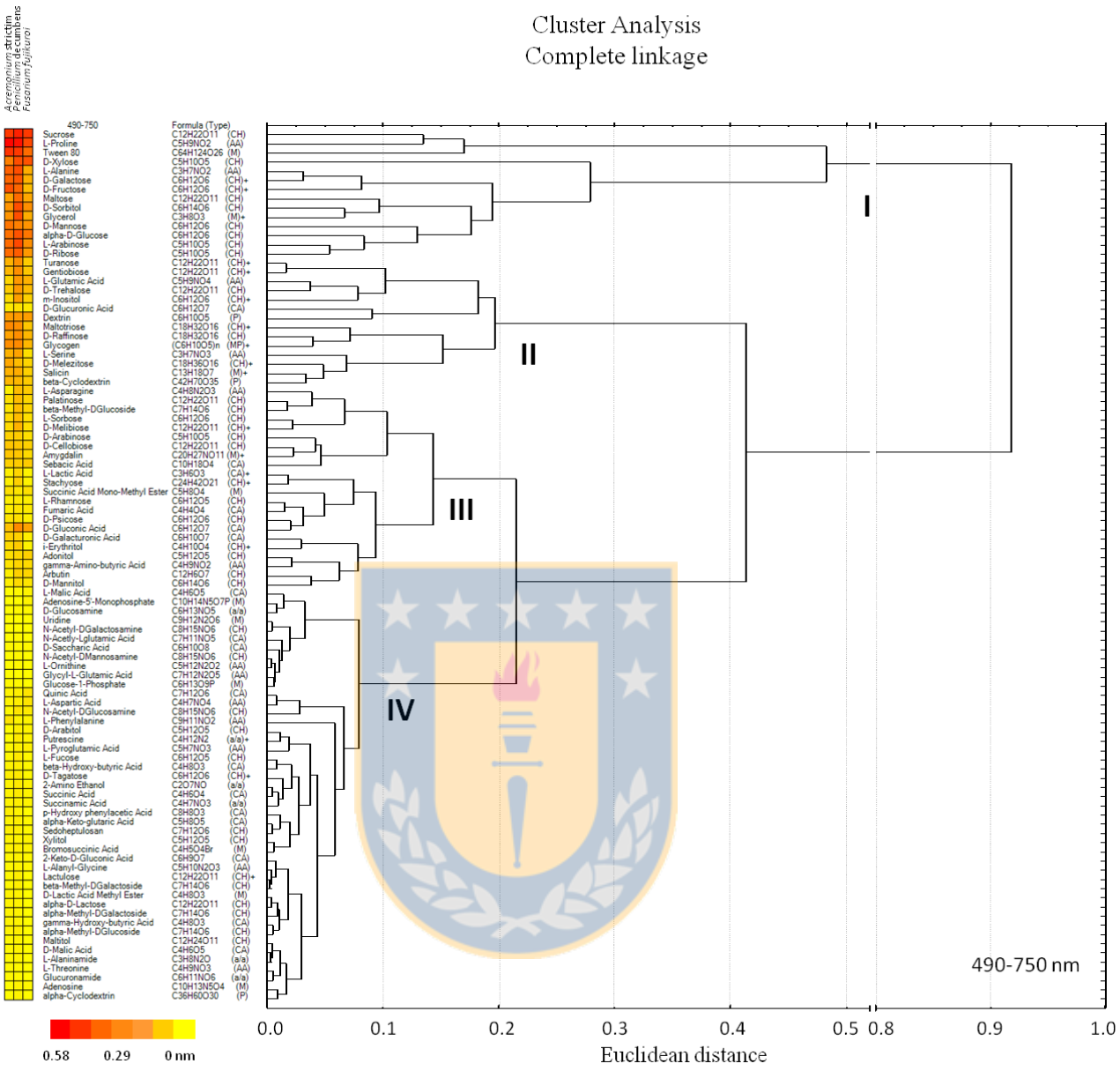

Figure 3. Joining cluster analysis and Heatmap applied to 95 carbon sources. Analysis based on their assimilation, utilization of carbon sources and growth rate for *A. strictum, P. decumbes* and *F. fujikuroi* measured at 490 nm and 750 nm. A/A: Amine and amides, AA: amino acids, CH: Carbohydrates, CA: Carboxylic acids, M: Miscellaneous and P: Polymers. + Significant differences between species ($p<0.05$).

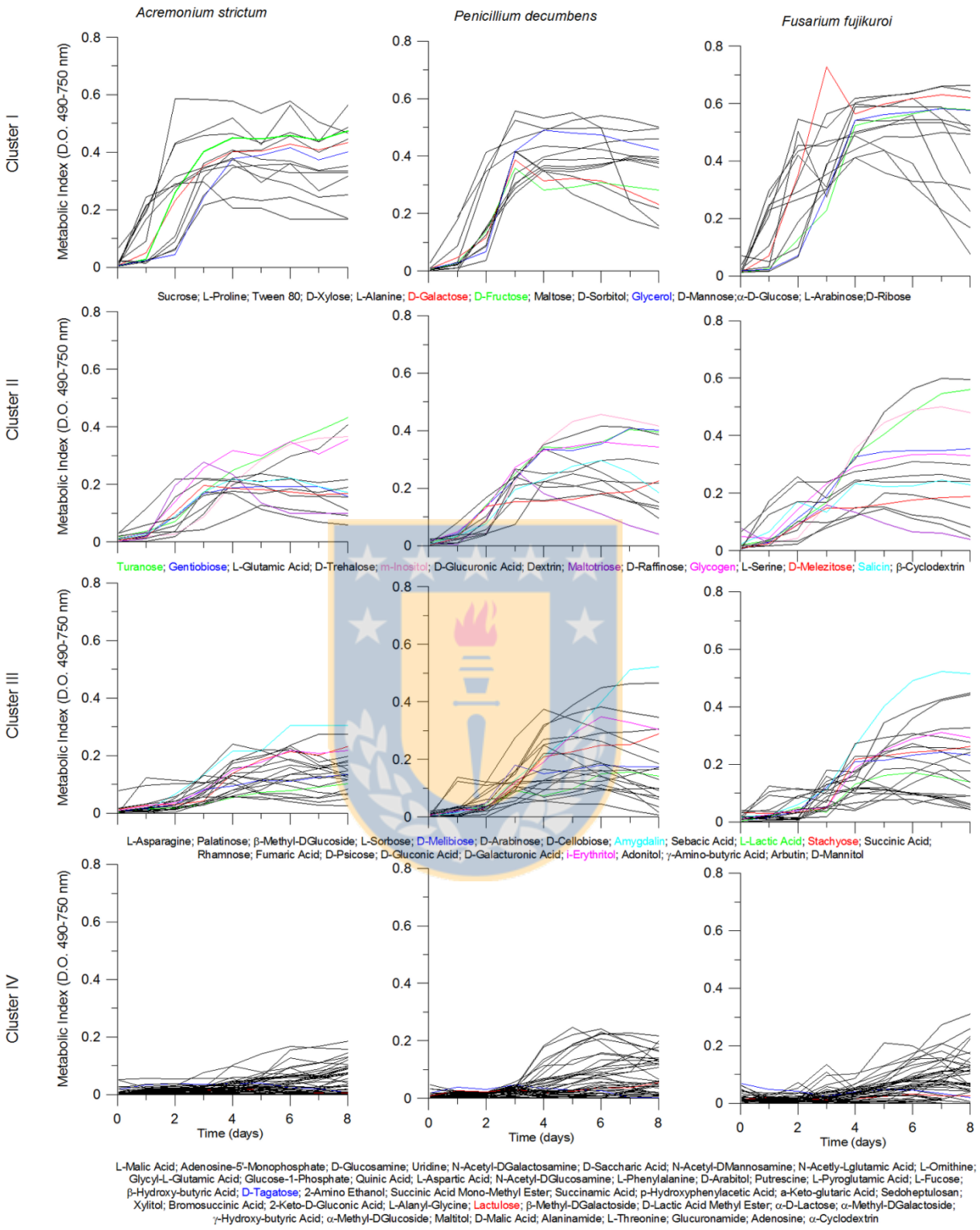

Figure 4. Growth and consumption kinetics for three species isolated from the coastal zone, grouped according to clusters generated in Fig. 3. Colored curves indicate the substrates that display significant differences between species (p>0.05).

4.3. Capítulo 3: Utilización de sustratos de Nitrógeno, Fósforo y Azufre por hongos filamentosos aislados de la zona costera de surgencia frente a Chile centro sur.

Manuscrito enviado a la revista Fungal Biology (FUNBIO-D-15-00398): Fuentes, M.E. and Quiñones, R.A. Substrate utilization of Nitrogen, Phosphorus and Sulphur by filamentous fungi isolated from the coastal zone of upwelling south-central Chile.

Resumen: El crecimiento de tres especies de hongos filamentosos *Penicillium decumbens*, *Acremonium strictum* y *Fusarium fujikuroi* aislados de la zona de surgencia costera frente a Chile centro sur fueron analizados en base a los efectos de distintos sustratos orgánicos e inorgánicos de nitrógeno, fósforo y azufre a través del uso de microplacas Biolog Phenotype microarrays. Estas especies mostraron altos índices de utilización de sustratos para las tres fuentes de sustratos, en donde los componentes orgánicos proporcionaron mayor crecimiento, siendo los L-amino ácidos, aminas y nucleótidos/nucleósidos las fuentes de nitrógeno preferenciales, sugiriendo el uso de la vías metabólicas de la arginina y prolina, la vías del aspartato y glutamato y/o el metabolismo de las Purinas. Otros compuestos como urea, alantoína y ácido úrico registraron crecimiento moderado. Destaca el uso de ribonucleótidos de adenosina y guanosina, como principal fuente de sustratos para la utilización de fósforo, y del uso de amino ácidos sulfatados de cisteína y metionina como principal aporte de azufre al crecimiento de estas especies. Además se observa crecimiento con variadas fuentes de sustratos inorgánicos como nitrato, nitrito, tiofosfatos, tetrationato, etc. de los cuales se discuten las posibles rutas metabólicas. Este trabajo se considera como el primer acercamiento al uso de sustratos de nitrógeno, fósforo y azufre por hongos filamentosos en una zona de surgencia costera, con alta disponibilidad de materia orgánica y la presencia de una zona de mínimo oxígeno, en donde la alta disponibilidad de nutrientes otorgan muchas posibilidades para el crecimiento y la asimilación de sustratos.

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Utilization of nitrogen, phosphorous and sulfur substrates by facultative marine filamentous fungi isolated from a coastal upwelling zone off central-south Chile.

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Abstract

The growth of three species of fungi, *Penicillium decumbens*, *Acremonium strictum* and *Fusarium fujikuroi* isolated from the coastal upwelling off central-south Chile was analyzed with different organic and inorganic nitrogen, phosphorous and sulfur substrates, using Biolog phenotype microarrays. These species were found to have high indices of utilization of all three substrate types. Organic components produced most growth. L-amino acids were the preferred nitrogen sources. The ribonucleotides adenosine and guanosine were the main sources of phosphorous, while the amino acids cysteine and methionine were the main sources of sulfur for the growth. All three species also grew with a variety of inorganic substrates, such as nitrate, nitrite, thiophosphates, etc. This study is the first to treat the use of nitrogen, phosphorous and sulfur substrates by filamentous fungi in this highly productive zone, which offers a wide range of possible substrates for assimilation, as well as the presence of an oxygen minimum zone.

Key words: Facultative marine fungi; Fungal growth; Nitrogen; Phosphorous; Sulfur, Coastal upwelling.

Introduction

The coastal zone of the Humboldt Current System off south-central Chile (36.5° S) is characterized by seasonal coastal upwelling that leads to high levels of primary productivity (Daneri *et al.*, 2000; Montero *et al*., 2007). In addition, the Bio-Bio and Itata Rivers deliver an important nutrient load to the coastal zone (Pantoja *et al*., 2011; Leniz *et al.*, 2012). This zone is also influenced by the presence of a sub-surface/mid-water eastern South Pacific oxygen minimum zone (Wyrtki 1962, Helly and Levin 2004). High rates of primary production generate high availability of organic molecules; the microbial community that inhabits this area is responsible for an important proportion of the degradation activity of photosynthetic carbon products (Troncoso *et al*., 2003; Cuevas *et al*., 2004; Quiñones *et al*., 2010). Also, a large fraction of the organic material produced in the coastal zone is buried in the area, thereby producing sediments rich in carbon, nitrogen and phosphorous (Ruttenberg, 1993; Howarth *et al.*, 1995; Muñoz *et al.*, 2004; González *et al*., 2006).

Associated with the high productivity and availability of organic material in this area, there is an active community of marine fungi in the water column and the sediments (Gutiérrez *et al*., 2010, 2011). The ecological role of the marine fungi is mostly related to the decomposition of organic material of plant and animal origin, and as pathogens of other organisms and mutualistic associations with animals, plants, algae, cyanobacteria, etc. (Burford *et al* 2003; Braissant *et al* 2004; Gadd, 2004; Fomina *et al*., 2005; Gutiérrez *et al*., 2011; Damare *et al*., 2013; Wang *et al*., 2014). They also participate in the cycling and/or transformation of organic compounds and biomass into elements: carbon, hydrogen, oxygen, nitrogen, phosphorous, sulfur, metals, etc.; the production of extracellular enzymes; the production of inorganic and organic metabolites (i.e. carbon dioxide, organic acids); the degradation of fossil fuels; and the degradation of xenobiotics (e.g. polynuclear aromatic hydrocarbons) (Gadd, 2001; Raghukumar, 2006; Gutiérrez *et al*., 2011; Jones and Pang, 2012).

These filamentous fungi play a leading role in the initial degradation of polymers (such as cellulose and lignin) in plant residues (Kjoller and Struwe, 2002; Baldrian and

Valášková, 2008) and are closely related to the biomass of phytoplankton (Gutiérrez *et al*., 2011; Wang *et al*., 2014). These filamentous marine fungi, mainly in the size fraction above 25 μm, are associated with high rates of extracellular hydrolysis, without representation of the prokaryotic community (0.2-3 μm) (Gutiérrez *et al*., 2011). Also, the species analyzed in this study area reveal that the carbon sources utilized are based mainly on carbohydrates and amino acids, which are mainly involved in the glycolysis/gluconeogenesis pathway and the amino acid metabolism (Fuentes and Quiñones, 2015). The nitrogen requirements of fungi are generally provided by organic sources or ammonia; also, their sulfur requirements are met by cysteine and other sulfur-containing organic compounds (Kulkarni and Nielsen, 1986).

There is little information on the participation of marine fungi in phosphorus and sulfur cycles. In soils, phosphorous is processed by the degradation of organic material via decomposition, immobilization and mineralization (Walbridge, 1991; Bolan, 1991; Voss *et al*., 2011; Posada *et al*., 2012). Phosphorous is a key element in marine biogeochemistry, both in the form of organic (P-esters, P-diesters, phosphonates) and inorganic components (orthophosphate, pyrophosphate, polyphosphate and phosphate-containing minerals) (Bjerrum and Canfield, 2002). The sulfur in the ocean represents a major reservoir of sulfur on Earth (e.g. dissolved sulfate and sedimentary minerals). However, at any given time only a small fraction is bound in biomass. Sulfur makes up about 1% of the dry weight of organisms, where it occurs mainly as a constituent of protein (Sievert *et al*., 2007). It is interesting to note that fungi have lyase enzymes that cleave compounds, such as dimethylsulfopropionate (DMSP), which is the biological precursor of dimethylsulfide (DMS), a key natural oceanic source of sulfur (Simó, 2001).

Oxygen minimum zones are globally significant sites of marine nitrate loss (Hamersley *et al*., 2007; Kuypers *et al*., 2005; Ward *et al*., 2009); marine fungi have been shown to be active participants in this process (Shoun and Tanimoto, 1991, Tsuruta *et al*., 1998, Shown *et al*., 2011). When the O_2 supply falls or its demand increases, marked changes occur in the dominant metabolic pathways of benthic microbial processes in the sediments. These changes affect the balance between nitrogen processes, basically organic nitrogen mineralization, nitrification and nitrate reduction, such as denitrification

(Hamersley *et al*., 2007; Farias *et al*., 2009; Ward *et al*., 2009), determining the form of the N species being exported from the sediments to the water column (Risgaard-Petersen *et al*., 1994; Rysgaard *et al*., 1994).

The main objective of this study is to characterize the utilization of preferential substrates of nitrogen, phosphorous and sulfur (with glucose as carbon source) by marine fungal species, using growth measures (growth rates, area under the curve) to determine the role of different species of filamentous fungi in the processing of organic material in a highly productive ecosystem. These are evaluated by using substrates of the potential metabolic pathways that species isolated from the coastal zone may be using, and thus elucidate the ecological role of the mycoplankton community and its participation in crucial nutrient cycles in the ocean in an upwelling area off the central-south Chilean coast.

Methodology

Seawater and sediment samples were collected from the coastal zone off central-south Chile on board R/V Kay Kay II (University of Concepción, Chile), at two oceanographic stations near the Itata River mouth. *Penicillium decumbens* was collected from the water column at 30 m depth at station P3-2 (36º 15.1' S, 72º 52.5' W). *Acremonium strictum* and *Fusarium fujikuroi* were collected from surface sediments at station P3-5 (36º 23.6' S, 72º 54.0' W; depth=10 m). For a detailed account of isolation and identification protocols see Gutiérrez *et al*., (2010) and Fuentes *et al*., (2015). A unique strain from each species was used in the experiments.

For the nitrogen, phosphorous and sulfur growth profiles the following steps were taken: A pure culture of a fungal species was grown on a 2% malt extract agar plate until enough conidiation was present to prepare a suspension. The conidia were swabbed from the surface of the agar plate and suspended to a specified density in an inoculating fluid. The initial density was the same for all species, measured as the minimum optical density (O.D.) at 450 nm (Tanzer *et al*., 2003; Bochner, 2009). Each spore suspension was poured into a sterile filling reservoir and PMs were inoculated by pipetting 100 μL per well, using glucose as a carbon source. PM3 and PM4 were incubated at 20 °C for 120 hours and read every 24 h using a spectrophotometer (Elx800, Bio-Tek Instruments) at 750 nm. Each isolate was replicated 3 times. The Biolog PM3 microplate test panel comprises 96 wells with different nitrogen sources (A-1 control well); the PM4 microplate test panel includes 60 wells with phosphorous sources (A-1 control well) and 36 wells with sulfur sources (F-1 control well) (Bochner, 2009; Tanzer *et al*., 2003).

A Joining Cluster Analysis was applied to growth values (as a percentage relative to the total) at 96 h, which corresponds to the linear growth phase for the majority of substrate sources. This was carried out using Statistica 6.0 (Stat Soft, Inc.) and was applied to identify the groups of nitrogen, phosphorous and sulfur sources (from the experimental data set), which produced similar growth. The Joining Cluster Analysis is based on Euclidean distance with complete linkage. A three-way ANOVA (Factors: growth, species and days)

was used to detect differences in substrate utilization between species. The kinetics of reactions were measured using growth rates between days 0 and 2, and between days 2 and 4, and compared to the value of the integrated area under the curve over the 5 days of culture.

The potential metabolic pathways of the substrates producing higher growth were determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg), which provides a collection of pathway maps for the metabolism of carbohydrates, amino acids, nucleotides, etc.

As a measure of the number of substrates utilized by fungi (substrate richness) and the diversity of the extent of utilization of particular substrates (substrate evenness) at 96 h, the Shannon Index (H) was used: $H=-\sum pi\ (\ln pi)$, where p_i is the proportion of a microbial activity on substrate *i* of total microbial activity and *N* is the number of substrates on a plate (95 for nitrogen, 59 for phosphorous and 35 for sulfur) (Zak *et al*., 1994; Tam *et al*., 2001; Wang *et al*., 2011; Muñiz *et al*., 2014). Here, H describes the ability of the fungal species to use a greater or lesser number of nitrogen, phosphorous or sulfur sources, thus acting as an index of physiological diversity. In this case, filamentous fungi that are able to degrade more substrates, and/or to degrade them with similar efficiency, would have higher values of H.

Results

Nitrogen

The species analyzed possessed high indices of utilization of particular substrates, with Shannon Index values of 4.2, 4.3 and 4.2 for the species *Acremonium strictum*, *Penicillium decumbens* and *Fusarium fujikuroi*, respectively. The three species analyzed were capable of growing in all the nitrogen substrates tested; growth presented significant differences between the 41 substrates studied, with species-specific responses in substrate use (Figure 1).

Substrates that produced the greatest increase in mycelia growth were compounds of the amino acid metabolism of the arginine and proline pathway: L-arginine, L-proline, glutamine, L-ornithine, putrescine (An), and urea (ON). L-asparagine (aspartate and glutamate pathway), agmatine (An), inosine and L-guanosine also produced high growth in the studied species (Figure 2). There were significant differences in growth among species in 9 of the 10 substrates in Cluster I, where most of the L-amino acids (L-AA) are represented (Figure 2). *Acremonium strictum* showed more growth than the other two species with these substrates (Figure 1). The biomass began to show differences with the control, beginning on day 2 with a sigmoid growth curve (data not shown), and increasing considerably on day 4. The areas under the curve calculated were closely related to biomass on day 4 ($r^2 > 0.89$ for the three species) (Figure 1).

The second group of nitrogen substrates that promote growth of studied species included L-citrulline, γ-amino-N-butyric acid, D-alanine, nitrate, L-glutamic acid, L-aspartic acid, N-acetyl-D-glucosamine, ethanolamine, adenosine, formamide, L-lysine, uric acid, adenine, glycine, allantoin and L-pyroglutamic acid. This group contained mainly L-AA, followed by nucleotides/nucleosides (N/N), using the amino acid and purine metabolisms (Figure 2). This group also showed moderate growth with nitrate (IN) (Figures 1 and 2). Those substrates grouped in Cluster IIa produced growth with clear differences between the biomass formed between days 2 and 4 (Figure 1). A group formed mainly by L-AA and dipeptides (di AA), as well as ammonia and IN, made up the second part of

Cluster II (Figure 2). This Cluster IIb was composed of ammonia, tyramine, L-alanine, L-valine, L-isoleucine, L-phenylalanine, xanthine, gly-met, met-ala, L-methionine, L-serine, gly-gln, L-histidine, ala-glu, gly-asn, ala-leu, ala-gln, L-threonine, L-leucine, ala-gly, L-tyrosine, ala-thr, nitrite, xanthosine, L-tryptophan and ala-asp. There were significant differences between the growth of all three species in 50% of the substrates of Cluster II (Figure 2).

Cluster III contained various nitrogenous substrates, dominated by amines (An), as well as di AA, fatty acids (FA) and D-amino acids (D-AA), among others. The substrates (16) that made up Cluster IIIa showed sigmoid curves of low growth, with small differences in growth at days 2 and 4 (Figure 2). The substrates in this cluster were D-glucosamine, methylamine, guanine, D,L-α-amino-N-butyric acid, β-phenylethyl-amine, ethylamine, ala-his, histamine, gly-glu, L-homoserine, cytidine, D-asparagine, parabanic acid, α-amino-N-valeric acid, N-acetyl-D-mannosamine and δ-amino-N-valeric acid (Figures 1 and 2). The substrates in Cluster IIIb produced very little growth; biomass at days 2 and 4 did not show any marked difference (Figure 1). Only two of these substrates produced significant differences in growth rate among the studied species (Figure 2). The substrates in Cluster IIIb included cytosine, L-cysteine, D,L-α-amino-caprylic acid, D-serine, glucuronamide, D-mannosamine, N-butylamine, N-acetyl-L-glutamic acid, D,L-lactamide, acetamide, N-amylamine, D-valine, ε-amino-N-caproic acid, D-glutamic acid, uridine, N-phthaloyl-L-glutamic acid, alloxan, D-aspartic acid, thymine, ethylenediamine, D-galactosamine, N-acetyl-D-galactosamine, hydroxylamine, D-lysine, uracil, biuret and thymidine.

Phosphorous

The number of substrates utilized was high, with values of 58, 59 and 54 (of a total of 59) for *Acremonium strictum*, *Penicillium decumbens* and *Fusarium fujikuroi*, respectively. The utilization of particular substrates showed high values: 3.98 (*A. strictum*), 4.0 (*P. decumbens*) and 3.98 (*F. fujikuroi*), with species-specific responses in substrate use (Figure 3).

Cluster I was composed exclusively of ribonucleotides adenosine and guanosine (guanosine-2'-monophosphate, adenosine-5'-monophosphate, guanosine-2',3'-cyclic-monophosphate, guanosine-5'-monophosphate, adenosine-2'-monophosphate, guanosine-3'-monophosphate, adenosine-2',3'-cyclic monophosphate, adenosine-3'-monophosphate, adenosine-3',5'-cyclic-monophosphate and guanosine-3',5'-cyclic monophosphate) (Figure 4). These substrates were equally utilized by all three species studied, which did not display any significant differences in growth (Figure 4). Cluster I included substrates that produced high biomass in the species studied, with notable increases after two days and reaching maximum values at day 4 (Figure 3). The analysis of metabolic pathways suggests that the ribonucleotides adenine and guanine participate in the purine metabolism, for the formation of nucleic acids; the formation of xanthine to connect with the urea metabolism; and the formation of more energetic compounds, such as ATP and GTP (www.genome.jp/kegg). *Penicillium decumbens* showed the greatest growth, followed by *Fusarium fujikuroi* and *Acremonium strictum*, in which growth on day 2 was closely related to the area under the curve (r^2>0.84) (Figure 3).

The second group of substrates (Cluster II) had intermediate growth, with biomass values on day 4 that were lower than those of Cluster I (Figure 3). The compounds in this cluster included cytidine-3'-monophosphate, O-phospho-L-threonine, D-3-phospho-glyceric acid, O-phospho-L-tyrosine, cytidine-2',3'-cyclic monophosphate, O-phosphoryl-ethanolamine, phospho-L-arginine, cytidine-2'-monophosphate, carbamyl phosphate, phosphoryl choline, cytidine-5'-monophosphate, O-phospho-L-serine, D-2-phospho-glyceric acid, uridine-3'-monophosphate, D-glucosamine-6-phosphate, phosphoenol pyruvate, cytidine- 3',5'-cyclic monophosphate, O-phospho-D-serine, uridine-2'-monophosphate, D-mannose-6-phosphate, uridine-5'-monophosphate, 2-deoxy-D-glucose 6-phosphate, uridine-2',3'-cyclic monophosphate, thymidine- 3'-monophosphate, 6-phospho-gluconic acid and uridine-3',5'-cyclic monophosphate. These compounds are mainly phospho amino acids (PAA) (that may participate in the metabolism of phosphonates and phosphinates, glycine metabolism and amino acid metabolism), cytidine and uridine ribonucleotides (RN) (pyrimidine metabolism), carbohydrates (CH) (used in the metabolism of amino sugars, and fructose and mannose metabolism), among others

(Figures 3 and 4). None of the substrates produced significantly different growth in the three species (Figure 4).

Cluster III included carbohydrates (e.g. Glycolysis/gluconeogenesis, pentose phosphate pathway), D-glucose-1-phosphate, D-mannose-1-phosphate, D-glucose-6-phosphate, D,L-α-glycerol phosphate and inositol hexaphosphate (the principal storage form of phosphorus in many plant tissues), as well as the inorganic substrates tripoly-phosphate, thiophosphate and phosphate, and other substrates: thymidine- 5'-monophosphate, trimeta-phosphate, β-glycerol phosphate, phosphocreatine, thymidine 3',5'- cyclic monophosphate, methylene diphosphonic acid, dithiophosphate, phospho-glycolic acid and 2-aminoethyl phosphonic acid (Figure 4). These compounds produced low growth, showing little differences between the biomass generated at days 2 and 4.

Cluster IV included miscellaneous substrates, which produced little or no growth; some were in the range of the control. These compounds are: O-phospho-D-tyrosine, cysteamine-S-phosphate, pyrophosphate, triethyl phosphate, phosphono acetic acid and hypophosphite.

Sulfur

The species studied showed high utilization values of particular substrates, with values of 3.22, 3.46 and 3.17 for *Acremonium strictum*, *Penicillium decumbens* and *Fusarium fujikuroi*, respectively. They also used a large number of substrates, 24, 32 and 30 (of a total of 35), respectively, with species-specific responses in substrate use (Figure 5).

Cysteine, methionine and their derivatives were the substrates that produced the greatest growth in all studied species (Figure 6). This suggests that the use of the amino acid metabolism, especially the cysteine and methionine metabolism. These substrates were grouped in Cluster I, and included L-cysteine sulfinic acid, L-methionine sulfone, hypotaurine, N-acetyl-D-L-methionine, S-methyl-L-cysteine, tetramethylene sulfone and L-methionine sulfoxide (Figure 6). *Fusarium fujikuroi* showed greater growth with the substrates of the first cluster compared with *Penicilllium decumbens* and *Acremonium*

strictum. There was a close relationship between biomass at day 4 and the area under the curve (r^2: 0.4-0.92). The mycelia in this cluster showed considerable growth at day 2, reaching maximum values at day 4 (Figure 5).

The members of Cluster II were a combination of various substrates, such as amino acids, inorganic compounds, etc., which produced little growth, with minimal differences between the biomass at days 2 and 4 (Figures 5 and 6). This cluster included the inorganic substrates tetrathionate, thiophosphate and dithiophosphate, sulfate, which participate in the cysteine and methionine metabolism, and the taurine and hypotaurine metabolism, as well as organic compounds, amino acids, etc. (1-thio-beta-D-glucose, taurine, D-methionine, L-methionine, butane sulfonic acid, cystathionine, D-cysteine, p-amino benzene sulfonic acid, L-cysteine and taurocholic acid) (Figure 6).

The compounds of Cluster III produced a minimum growth increment, only *Penicillium decumbens* showed differences in biomass in the experiments (Figure 5). The members of this cluster were mostly derived from amino acids, and included methane sulfonic acid, glutathione, 2-hydroxyethane sulfonic acid, L-djenkolic acid, lanthionine, thiosulfate, cysteamine, L-cysteinyl-glycine, glycyl-L-methionine and L-cysteic acid (Figure 6).

The substrates in Cluster IV had growth within the range of control experiments. Cluster IV was composed of thiourea, N-acetyl-L-cysteine, D,L-ethionine and D,L-lipoamide.

Discussion

The three species of filamentous fungi isolated from the coastal zone off central Chile have a great capacity to use a wide variety of complementary substrates of nitrogen, phosphorous and sulfur (with glucose as a source of carbon). Organic substrates produced greater mycelial growth compared to inorganic substrates. Carbohydrates and amino acids were the preferential substrates for all three species, producing the greatest growth (Fuentes *et al*., 2015). Our results are consistent with previous findings, that the coastal upwelling zone off south-central Chile provides high availability of organic material and phytoplankton biomass, capable of sustaining high levels of fungal biomass in the water column (Gutiérrez *et al*., 2011).

All experiments were performed in aerobic conditions, which benefitted the metabolic pathways that use oxygen as an electron acceptor; however, possible alternative pathways are discussed for the use of studied substrates, due to the presence of an oxygen minimum zone in the study area that could benefit certain metabolic pathways in marine fungi, such as denitrification (Tsuruta *et al*., 1998; Shoun *et al*., 1992; Jebaraj and Raghukumar, 2009), and sulfur oxidation (Slautghter, 1989).

Nitrogen substrate-based growth

The nitrogenous compounds that produced the greatest growth in species studied included L-amino acids, amines and nucleotides/nucleosides, which suggest that they are assimilated by means of amino acid metabolism, in particular that of arginine and proline and the aspartate and glutamate pathway, as well as purine metabolism. Previous observations in these species showed that the preferred carbon sources include the amino acids L-proline and L-alanine, which may be used as sources of carbon and nitrogen in the glycolysis/gluconeogenesis pathway (Fuentes and Quiñones, 2015). The L-amino acids arginine, asparagine, ornithine, proline and glutamine produced high mycelia in all three studied species. This group of compounds (L-amino acids) was also the main component of the growth of fungal hyphae in the second group of substrates analyzed (Figures 1 and 2).

Compounds, such as urea, allantoine and uric acid, are processed by the purine metabolism, in which catabolic process have two very different pathways in two different groups of fungi. The first is that of the Euascomycetidae, such as *Neurospora crassa* and *Aspergillus nidulans*, which use all the N in adenine and guanine, and carry out the direct decomposition of urea to ammonia CO_2 using the enzyme urease (Srb and Horowitz, 1944). The second pathway is found in *Saccharomyces cerevisiae*, which does not use the N of the purine nucleus directly (Wiame *et al*., 1985); it uses intermediates of purine catabolism, such as allantoine and allantoic acid. This fungus has a different method for the conversion of urea to ammonia since it does not possess urease (Wiame *et al*., 1985). *Candida albicans*, which also lacks urease (Odds, 1988), grows well with urea as its only carbon source (Dastidar *et al*., 1967) by using urea amidolyase, which hydrolyzes urea (Ghosh *et al*, 2009).

Although the nutrition of marine filamentous fungi was promoted mainly by organic substrates, studies have shown the presence of nitrate reductase (Na-R) in more than 80 strains of marine fungi comprising all major systematic groups in 54 species (1 Phycomycete, 15 Ascomycetes, 1 Basidiomycete, 21 Deuteromycetes, 6 ascosporous yeasts, 8 basidiosporous yeasts and 3 asporogenous yeasts); only a few showed low Na-R activity (Rau and Molitoris, 2001). Although it had been thought that denitrification was only present in bacteria, many fungi also show different denitrifying activity (Shoun and Tanimoto, 1991, Tsuruta *et al*., 1998, Shown *et al*., 2011). The fungal denitrification system includes NirK (copper-containing nitrite reductase) and P450nor (a cytochrome P450 nitric oxide (NO) reductase (Nor)) that reduces nitrite to nitrous oxide (N_2O) (Nakahara *et al*., 1993; Shown *et al*., 2011). This system is located in the mitochondria that function during anaerobic respiration. Some fungal systems contain and use dissimilatory and assimilatory nitrate reductases to denitrify nitrate (Averill, 1996; Shown *et al*., 2011).

The components of the nitrogen-nitrate-nitrite-ammonia system produced moderate growth in filamentous fungi in the second group of substrates (Figures 1 and 2). The assimilation of ammonia into glutamate and glutamine plays a central role in the nitrogen metabolism of all organisms (Meti, *et al*., 2011). Glutamate dehydrogenase, glutamate synthase and glutamine synthetase are key enzymes involved in ammonia assimilation in

fungi (Meti *et al*., 2011). In marine environments, NO_3^- is the major form of inorganic nitrogen used to support primary production, despite the importance of NO_3^- assimilation in controlling the rate of new production in the ocean (Dugdale *et al*., 1990). Assimilatory nitrate reductase catalyzes the first step of NOS- assimilation in plants and fungi (Beevers and Hageman, 1980). The nitrate assimilation pathway in *Aspergillus nidulans* involved in nitrate uptake includes three genes that code for a product required for the absorption of nitrate and the enzymes nitrite reductase and nitrate reductase (Johnstone *et al*., 1990; Crawford and Arst, 1993).

Most fungi grow under aerobic conditions by generating ATP through the respiration of oxygen. However, they alternatively express two pathways of dissimilatory nitrate reduction in response to environmental oxygen stress when the oxygen supply is insufficient. The fungus *Fusarium oxysporum* expressed the pathway of respiratory nitrate denitrification that is catalyzed by the sequential reactions of nitrate reductase and nitrite reductase (Takaya, 2002). These enzymes are coupled with ATP generation through the respiratory chain and produce nitric oxide (Takaya, 2002). Denitrifying fungi have been recently found in marine environments, where it was found that the main groups of fungi are capable of the denitrification process, although predominantly only producing nitrous oxide (Tsuruta *et al*., 1998; Shoun *et al*., 1992; Jebaraj and Raghukumar, 2009). In fungal denitrifying activity requires a minimal amount of oxygen for induction, which is prepressed by excess O_2 (Zhou *et al*., 2001). This contrasts with bacterial denitrification, which occurs in the complete absence of oxygen (Zhou *et al*., 2001). The optimal oxygen supply differed between the denitrification substrates: 690 μmol O_2 h^{-1} (g dry cell wt.)$^{-1}$ for nitrate and about 250 μmO_2 h^{-1} (g dry cell wt.)$^{-1}$ for nitrite (Zhou *et al*., 2001). Fungi are known to stop at the formation of N_2O and fungal denitrifiers do not reportedly produce N_2 as the end-product (Bleakley and Tiedje 1982, Shoun *et al*., 2012). Because of this incomplete reaction, denitrification by fungi leads to an increase in greenhouse gases (Shoun *et al*., 1998).

Phosphorus substrate-based growth

Coastal environments and the continental platform are responsible for the sedimentation of up to 70-90% of organic material and reactive phosphorus (Berner, 1982; Ruttenberg., 1993; Howarth *et al*, 1995). Reactive phosphorous is present in three forms in the water column: particulate organic phosphorous in the form of marine biomass or biogenic detritus; biogenic calcium phosphate in the hard parts of fish; and soluble reactive phosphorous, including dissolved organic and inorganic phosphorous (Slomp and Van Cappellen, 2007; Paytan, and McLaughlin, 2007).

The substrates that produced the greatest mycelial growth in all three studied species were the ribonucleotides of adenosine and guanosine. Fungi have specific transporters in their plasma membranes for the assimilation of purine bases (Scazzocchio *et al*., 1982; Pantazopoulou and Diallinas, 2007). The phospho-amino acids and the pyrimidine ribonucleotides cytidine and uridine produced moderate growth rates; however, the latter cannot be used by fungi as a nitrogen source, but only in nucleic acid synthesis (Vlanti and Diallinas, 2008). Some organisms have developed mechanisms to recycle extracellular nucleotides and use phosphorous (mainly in conditions of inanition) (Kennedy et al., 2005). The hydrolysis of AMP to adenosine and GMP to guanine (by the action of nucleotidases) forms the extracellular arm of the rescue of purines and phosphate; adenosine and guanine can cross the cell membrane by nucleoside transporters, and be used as substrates for the production of purine nucleotides in the cell (Zimmermann et al., 1992). Products of yeast genes (nucleotide phosphatase phosphodisterase 1 and 2 (Npp1 and Npp2)) have recently been implicated as extracellular nucleotidases; they are members of an alkaline phosphatase superfamily that also include extracellular 5'-nucleotidases with ATPase and ADPase (Kennedy et al., 2005). The phosphate produced enters the cell through protein transporters (Yompakdee et al., 1996; Van Belle y André, 2001).

Finally, inorganic substrates, such as thiophosphate and phosphate, produced moderate hypha growth, in which inorganic polyphosphates play an important role in the energy metabolism (Vagabov *et al*., 1998). It has been suggested that under unfavorable

conditions and stress, the hydrolysis of polyphosphates provides additional energy to maintain cell processes (Kulaev and Kulakovskaya, 2000).

Sulfur substrate-based growth

The substrates that promoted most growth were mainly amino acids containing sulfur (cysteine and methionine) and their derivatives. Sulfur makes up about 1% of the dry weight of organisms, where it is mainly a constituent of protein (primarily the S-containing amino acids cysteine and methionine) (Kulkarni and Nielsen, 1986; Sievert *et al*., 2007).

Finally, inorganic substrates, such as sulfate, thiophosphate, tetrathionate, dithiophosphate made a slight contribution to growth. Sulfur is also an essential element for all organisms including fungi (Slautghter, 1989; Jennings, 1995). Some fungal species are also capable of oxidizing inorganic sulfur (Wainwright, 1989) and producing compounds like dimethyl sulfide (Slautghter, 1989), which is important in the cycling of sulfur in marine environments (Gadd, 2006).

The coastal upwelling zone off central-south Chile provides high availability of photosynthetic biomass and organic material (Daneri *et al*., 2000; Montero *et al*., 2007; Sanchez *et al*., 2009); it is a heterogeneous environment with temporal changes in the quality and availability of organic material. This sustains a wide community of heterotrophs and bacteria (Troncoso *et al.* 2003, Cuevas *et al.* 2004), archaea (Levipán *et al.* 2007, Quiñones *et al.* 2009) and filamentous fungi. Fungi are well-represented in the >25µm size fraction, with high activity of extracellular enzymes capable of degrading large polymers (Gutiérrez *et al*., 2011). The coastal upwelling zone studied here contains waters with low oxygen content (Equatorial Subsurface Water) (Schneider et al., 2006), where metabolic routes such as denitrification may be favored, as has been observed in the fungal species *Fusarium oxyporum*, *Cylindrocapon tonkinense* and *Aspergillus oryzea* (Shoun and Tanimoto, 1991; Usuda et al., 1995; Jebaraj and Raghukumar, 2009; Shoun *et al*., 2012). This information reinforces the concept that filamentous fungi must be considered as an integral component of the microbial community in coastal marine environments, as has been suggested by authors, such as Gutiérrez *et al*., (2011; 2015), Jones and Pang (2012) and Wang *et al*., (2014), and should be included in microbial models and biogeochemical

cycles, due to their utilization of a wide variety of organic and inorganic nitrogen, phosphorous and sulfur compounds.

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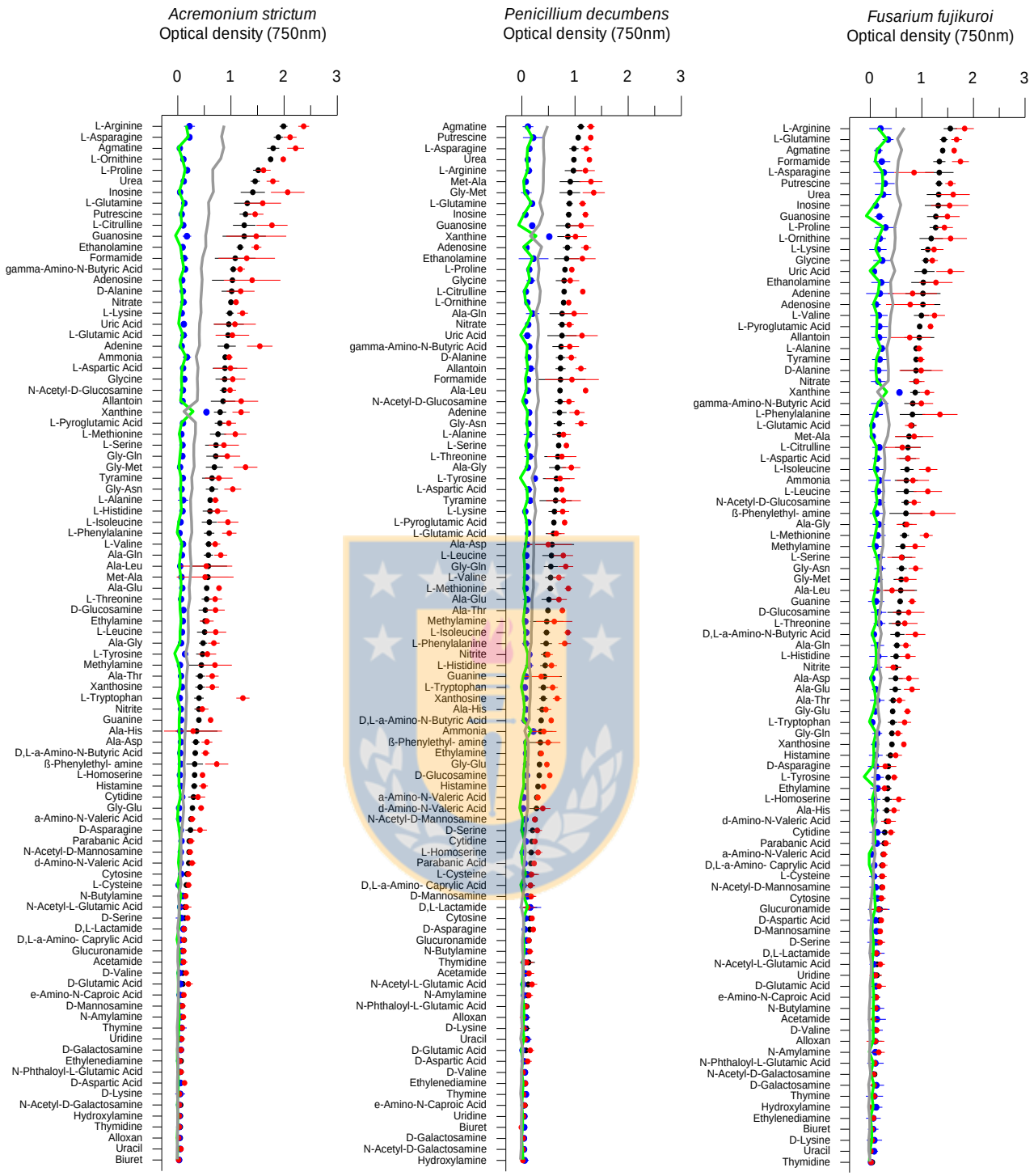

Figure 1. Consumption of nitrogen substrates measured as mycelium biomass (O.D.750 nm) for the species *A. strictum*, *P. decumbens* y *F.fujikuroi*. ● Mycelium biomass at day 2, ● Mycelium biomass at day 4 and ● Area under the curve for growth kinetics during 5 days, growth rates between days 0 and 2 (green line), growth rates between days 2 and 4 (gray line). The horizontal lines represent one standard deviation.

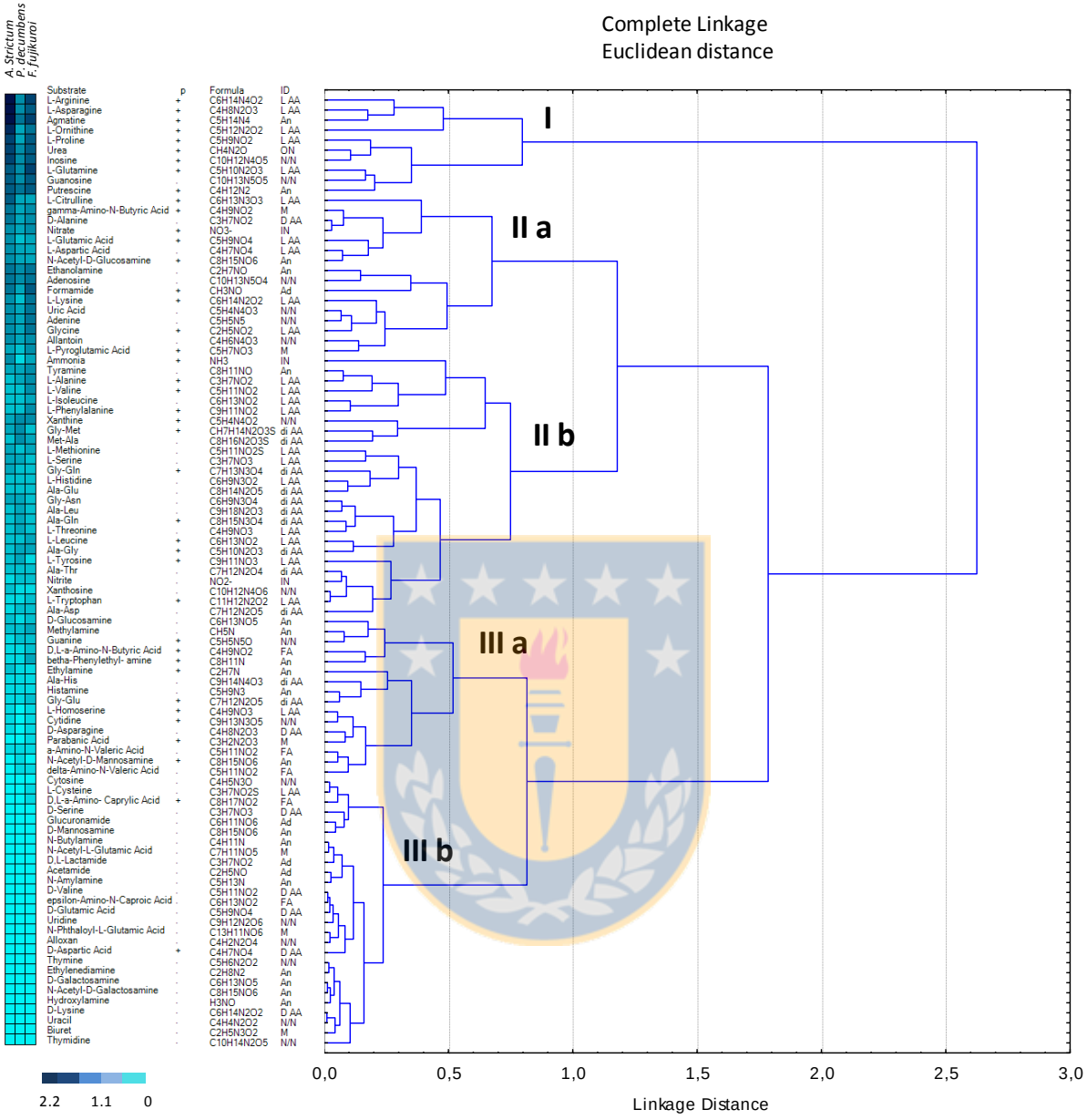

Figure 2. Cluster analysis and Heatmap of the growth of filamentous fungi under the effect of different nitrogen sources. N/N: Nucleotides and Nucleosides, IN: Inorganic Nitrogen, An: Amines, Ad: Amides, D-AA: D-Amino Acids, L-AA: L- Amino Acids, Di AA: Di peptides, FA: Fatty Acids, and M: miscellaneous. p-value + represents significant difference among the three species.

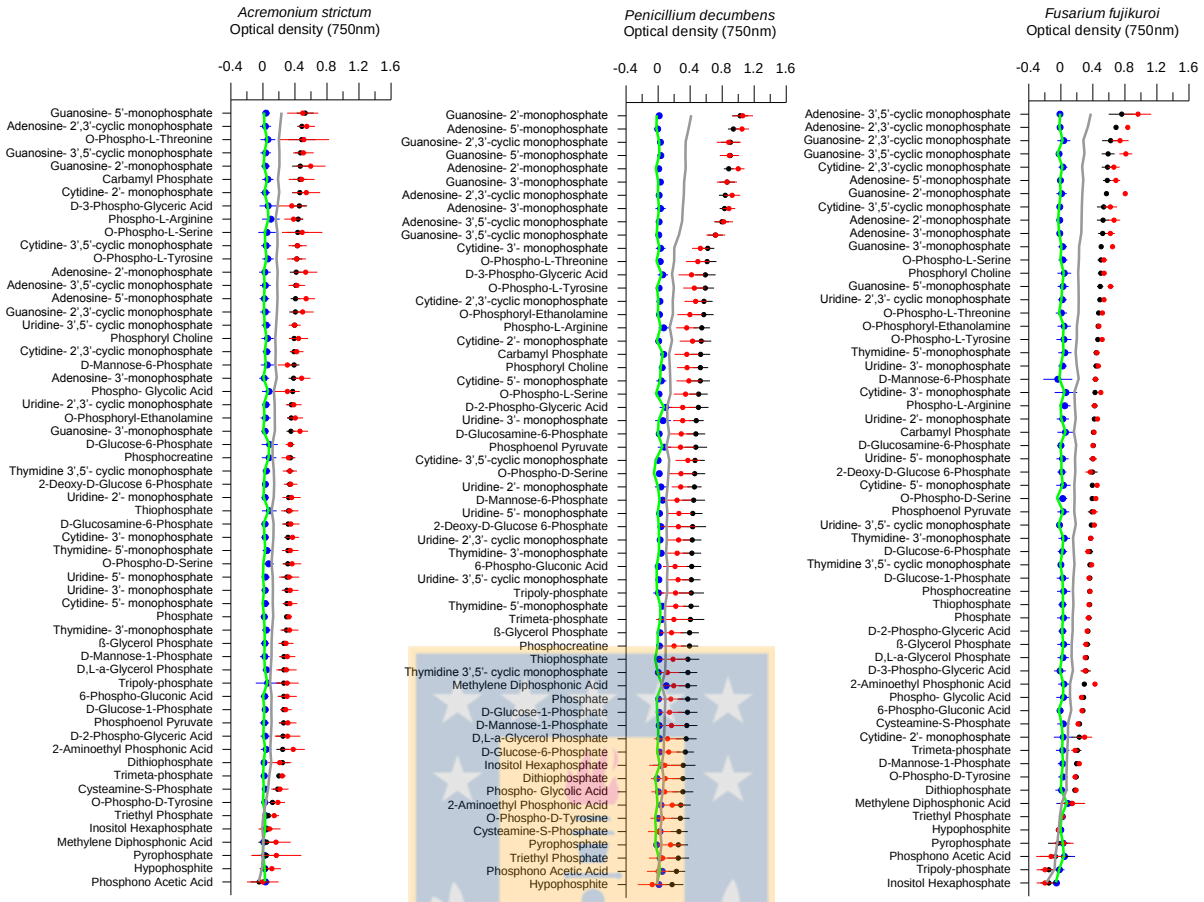

Figure 3. Substrate consumption of phosphorus substrata as ● Mycelium biomass at day 2, ● Mycelium biomass at day 4 and ● Area under the curve for growth kinetics during 5 days, growth rates between days 0 and 2 (green line), growth rates between days 2 and 4 (gray line). The horizontal lines represent one standard deviation.

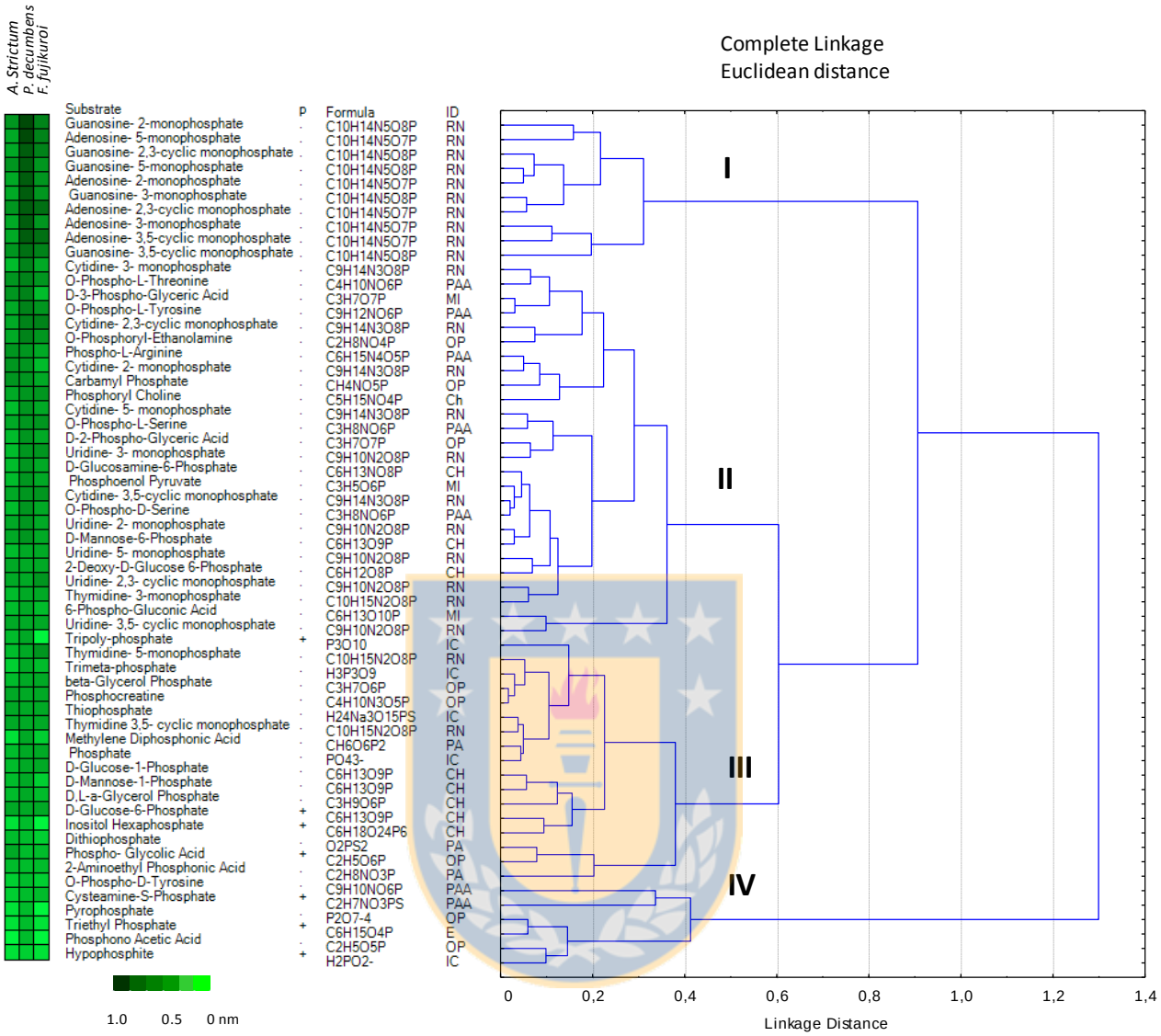

Figure 3. Cluster analysis by phosphorus substrate read at 750 nm at day 4. RN: Ribonucleotide, PAA: Phospho amino acid, OP: Organic phosphorous, Ch: Cholines, CH: Carbohydrates, MI: Metabolic intermediate, IC: Inorganic compound, PA: Phosphonic acid.

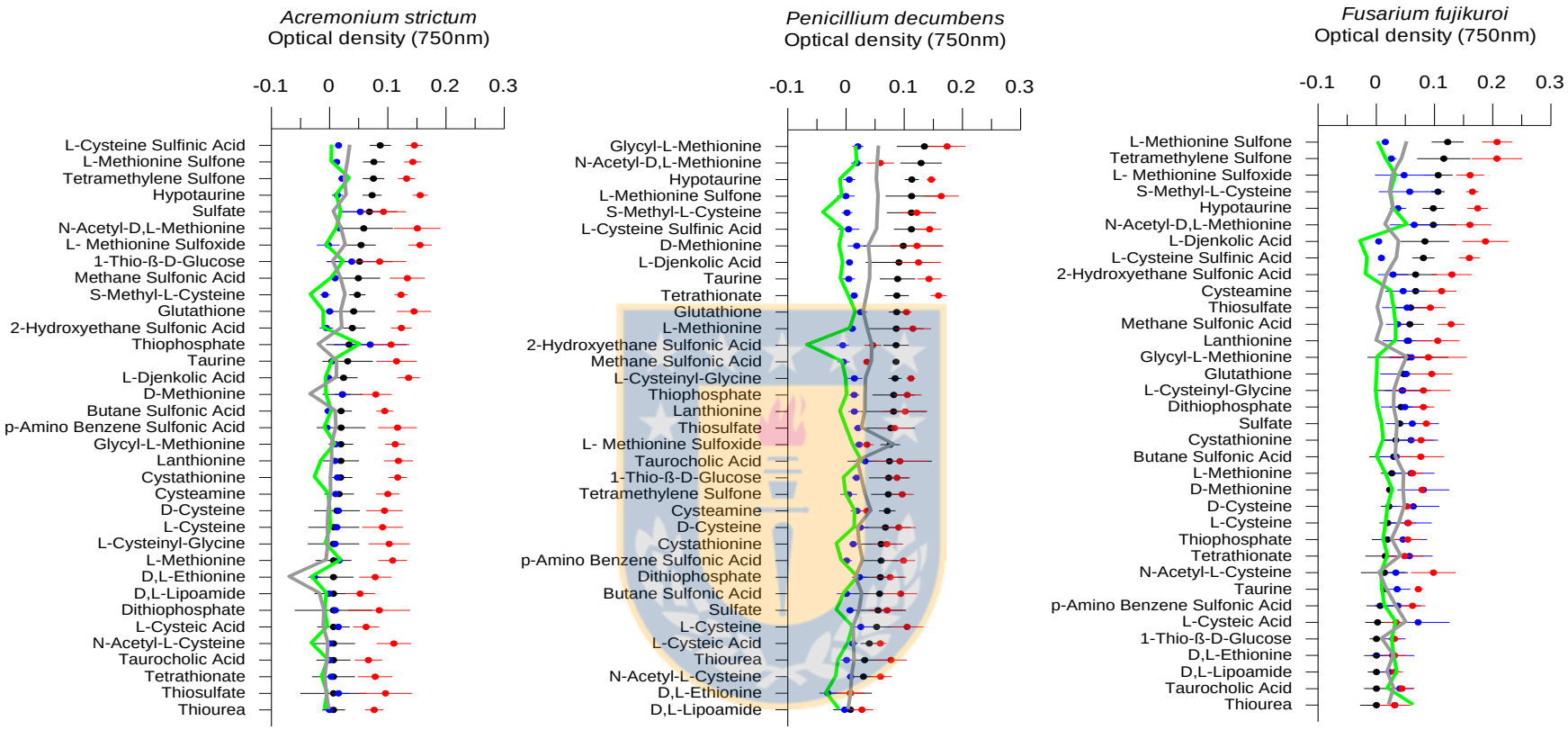

Figure 6. Substrate consumption of Sulfur substrata as ● Mycelium biomass at day 2, ● Mycelium biomass at day 4 and ● Area under the curve for growth kinetics during 5 days, growth rates between days 0 and 2 (green line), growth rates between days 2 and 4 (gray line). The horizontal lines represent one standard deviation.

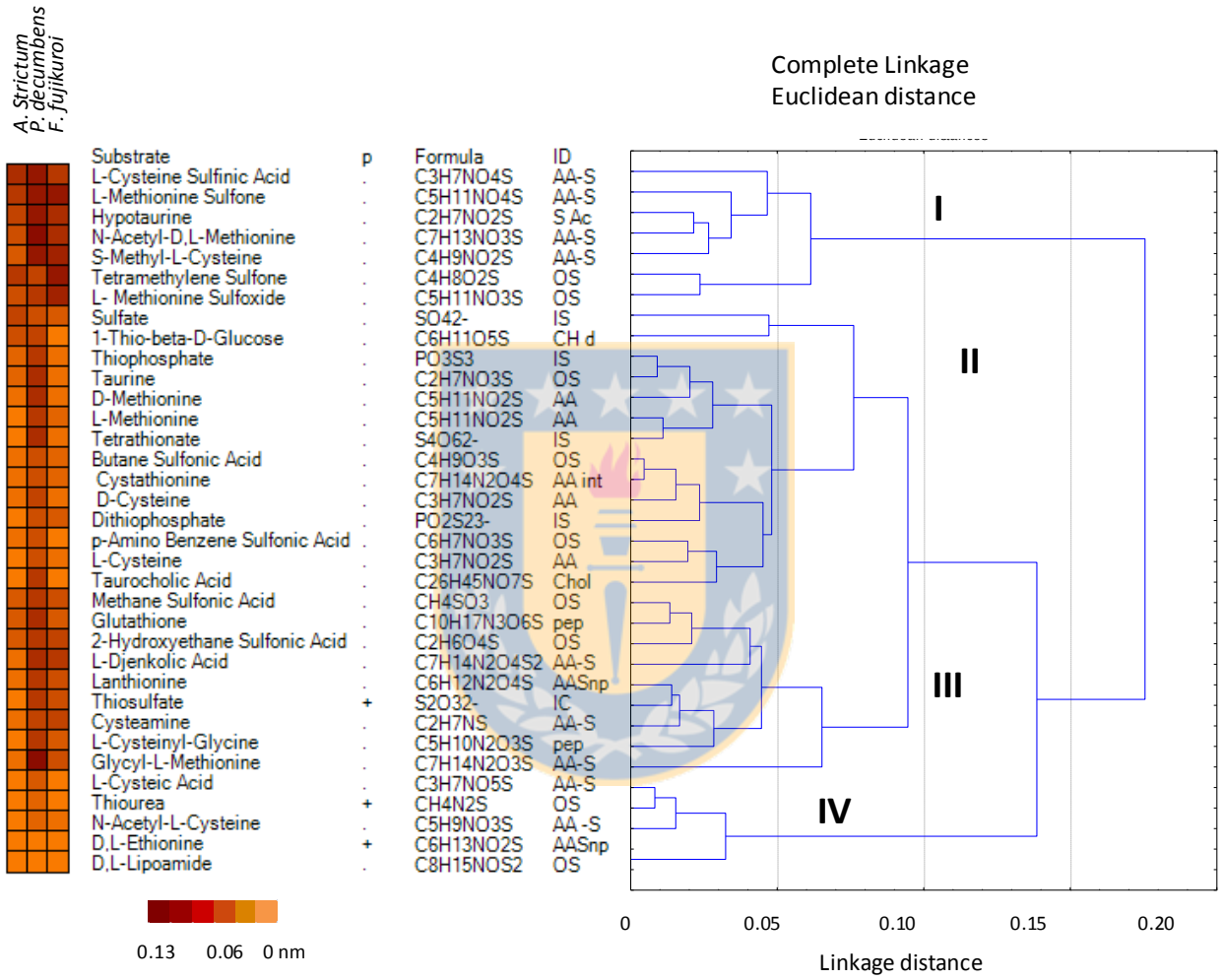

Figure 5. Cluster analysis by sulfur substrata read at 750 nm at day 4. AA: amino acids, AA-S: Amino acids derivate, AASnp: non-protein amino acids, OS: organic sulfur, IS: Inorganic sulfur, CH-der: Carbohydrate derivate, Chol: Cholic acid, Acid: sulfinic acid, pep: di and tripeptides.

5. DISCUSIÓN

La temperatura en la zona costera frente a Chile centro sur sigue un ciclo anual asociado con un calentamiento superficial, en el cual temperaturas de 11,5 a 12,5 son características de aguas superficiales (0-30 m) en el invierno austral, las cuales corresponden a Aguas de Transición del Pacífico Sur-Oriental (Eastern South Pacific Transition Water (ESPTW)), las cuales se forman en base al Agua Superficial Subantartica (SASW) (Sobarzo *et al*., 2007). De esta misma forma, durante marzo la temperatura superficial alcanza cerca de los 18 °C, similar a períodos de relajación de la surgencia (Parada, 2001; Sobarzo *et al*., 2007). Cerca del fondo de la plataforma continental la presencia del Agua Ecuatorial Subsuperficial (ESSW) presenta temperaturas menores a 11°C durante los meses de primavera (Parada *et al.*, 2001; Grob *et al*., 2003; Sobarzo *et al*., 2007). Como se demostró en el capitulo 1, esta alta variabilidad térmica, la cual se considera uno de los factores fundamentales que regulan el crecimiento de hongos (Madan y Thind, 1998), pueden producir considerables efectos sobre el crecimiento de hongos filamentosos. Es así que a bajas temperaturas y durante períodos de surgencia las tasas de crecimiento serían bajas (21-51 μgC hifas día^{-1}), mientras que cuando la temperatura incrementa, conducen a considerables aumentos en la tasa de crecimiento, los cuales potencialmente podrían alcanzar valores de hasta 153 μgC hifas día^{-1}. De esta misma forma, la temperatura afecta la tasa de consumo de oxígeno, la cual registra un rango de valores de Q_{10} que va desde 3,9 a 6,85 en las especies de hongos filamentosos estudiadas (excepto *P. decumbens*), lo cual es considerablemente alto en relación a otros organismos poiquilotermos. Valores de Q_{10} cercanos a 2 han sido reportados para tasas metabólicas de microorganismos como procariotas (White *et al.*, 1991), protistas (Choi y Peters, 1992), fitoplancton (Eppley, 1972) y zooplancton (Ikeda *et al.*, 2001). Estos altos valores reportados en nuestra investigación se encuentran cercanos a los reportados para zooplancton del Ártico (Q_{10} = 6,51; Alcaraz *et al.*, 2013), los cuales son altamente dependientes del tamaño del corporal (Ikeda, 1985), por lo que cualquier covarianza entre la

temperatura y la biomasa individual generaría un sesgo en la respuesta metabólica en función de la temperatura (Alcaraz *et al.,* 2013).

Condiciones ideales para el crecimiento de hongos filamentosos marinos se generarían durante períodos con alta biomasa autotrófica en aguas superficiales en la zona costera (Gutiérrez *et al.,* 2011). Esto se encuentra estrechamente relacionado al hecho que el fitoplancton produce exopolímeros basados en glucosa y almacena polisacáridos (Biersmith y Benner, 1998; Aluwihare y Repeta, 1999; Janse *et al.*, 1999). Además, esta zona se encuentra influenciada por la presencia de los ríos Itata y Biobío, los cuales descargan al océano altos niveles de materia orgánica (Sánchez *et al.,* 2008; Iriarte *et al.*, 2012).

Las cinco especies estudiadas muestran incrementos en el crecimiento y en la tasa de respiración a altas concentraciones de glucosa (5 g L^{-1}; medio líquido para experimentación estándar), registrando tasas de respiración sobre los 200 μmol O_2 $L^{-1}h^{-1}$, además de producir altas biomasas. Lo que nos demuestra que la concentración de nutrientes en el agua afecta la actividad fúngica, tanto el consumo de oxígeno (Gulis *et al*., 2004), como también procesos fundamentales para la reproducción de hongos como la esporulación (Suberkropp, 1998; Ferreira *et al*., 2006).

El crecimiento de *P. decumbens*, *P. chrysogenum* y *F. fujikuroi* es claramente temperatura-dependiente; sin embargo, sus tasas de respiración dependen de la temperatura sólo a bajas concentraciones de glucosa, es decir, que su tasa respiratoria se mantiene constante cuando la disponibilidad de glucosa es alta, independiente de las variaciones de temperatura. Se ha observado que las tasas de consumo de oxígeno de hongos ascomicetes mesófilos en suelos son independientes de los cambios de temperatura en un rango de 15 a 40 °C, o que solo producen un efecto de menor grado (Maheshwari *et al*., 2000). La base bioquímica de esto no está clara, pero puede estar relacionado con la cadena respiratoria ramificada como función tanto del sistema citocromo o rutas alternativas en hongos (Joseph-Horne y Hollomon, 2000; Joseph-Horne *et al*., 2001). Este comportamiento puede ser significativo en relación a su crecimiento en la naturaleza, en donde se espera que los hongos que mantienen una tasa metabólica óptima en un amplio rango de temperaturas

tengan ventajas competitivas sobre los que carecen de esta habilidad (Prasad *et al*., 1979). Esto sugiere que el metabolismo (crecimiento y respiración) de hongos en la zona costera frente a Chile centro sur esta altamente relacionado a la variabilidad de la temperatura y concentración de sustratos en el medio.

Las biomasas fúngicas más altas reportadas en el área de estudio son de 40 μg C L^{-1}, localizadas en el máximo de clorofila (ca. 20 m), asociadas a temperaturas entre 12 y 13 °C (Gutiérrez *et al*., 2011). Durante los experimentos realizados en la presente Tesis, a concentraciones de glucosa bajo 0,01 g L^{-1} la biomasa de hongos alcanzó valores de 290 μg C L^{-1}. Sin embargo, bajo condiciones naturales, la productividad de hongos puede estar limitada por interacciones ecológicas como competencia con bacterias (Mille-Lindblom *et al*., 2006; Frey-Klett *et al*., 2011), predación (McMahon *et al*., 1974; Kagami *et al*., 2004; Kagami *et al*., 2007; Lepère *et al*., 2007), y procesos físicos como enterramiento (Janssen y Walker, 1999; Cornut *et al*., 2010). Estas limitaciones ecológicas y físicas, permitirían a las especies de hongos filamentosos alcanzar, en el ambiente, solo 13% de la biomasa registrada en laboratorio.

El papel de la respiración en la bomba biológica del océano ha sido un punto de controversia por muchos años debido al debate sobre fuentes y sumideros de carbono en el océano (e.g.Williams, 1998; del Giorgio y Williams, 2005; Laruelle, 2010). El conocimiento de la respiración a nivel comunitario es limitado por la falta de conocimiento de la fisiología de muchas especies y de cómo estas se ven afectadas por las condiciones ambientales (Jin y Bethke, 2003; Suberkropp *et al*., 2010). Desde los estudios de Rubner (1883), se ha definido una relación entre las tasas metabólicas y la masa corporal de los organismos a través de una ecuación potencial que es válida para la gran mayoría de los organismos, donde la tasa metabólica es igual a una constante multiplicada por la masa corporal elevada a un exponente de ¾ (Kleiber, 1961; West *et al*., 2002). En este estudio, la tasa de consumo de oxígeno de las 5 especies de hongos filamentoso registró una pendiente de 0,76 (equivalente al exponente de ¾) y está dentro de la media típica de exponentes que explica la variabilidad alométrica en el metabolismo desde

organismos unicelulares a metazoos (Peters, 1983; Prothero, 1986). Por otra parte, tasas metabólicas masa-específica y la mayoría de las tasas biológicas, tales como la frecuencia respiratoria y frecuencia cardiaca, están en una escala de $M^{-1/4}$, con M como masa corporal (Lindstedt y Calder, 1981; McMahon y Bonner, 1983; Peters, 1983; Calder, 1984; Schmidt y Nielsen, 1984), las que coinciden con los valores reportados en este estudio de -0,26 (-1/4) entre la tasa respiratoria-específica y la talla corporal de organismos desde esporas a micelios al día 5 de crecimiento. Este exponente (-1/4) puede estar relacionado con las características de crecimiento de los hongos filamentosos, los cuales a medida que crecen, van ramificando sus hifas, formando asociaciones con sus propias hifas y con las de otros individuos, creando un micelio cuya área geométrica específica disponible es inversamente proporcional a su tamaño (Žnidaršič y Pavko, 2001). Esto generaría una superficie menor y, por lo tanto, una disminución en el intercambio de gases, materia y calor con el medio ambiente. En consecuencia, se espera que a medida que la biomasa (filamentos) aumenta, debe tener lugar una disminución en la tasa de respiración específica.

La respiración es un indicador importante de la actividad fisiológica de una comunidad, dado que los organismos heterótrofos adquieren su energía de la oxidación de compuestos de carbono (Varó *et al*., 1993; Ikeda *et al*., 2001; Irwin *et al*, 2007). La respiración a nivel celular es una reacción exergónica, donde parte de la energía contenida (~42%) en las moléculas de sustratos nutricionales es utilizada por las células para sintetizar ATP (Campbell, 1997). El primer paso de la respiración celular es la glucólisis, en donde la ganancia neta es de dos moléculas de ATP, y dos de NADH por cada molécula de glucosa (Campbell, 1997). Los resultados de los perfiles metabólicos de carbono, muestran que los principales sustratos consumidos por hongos filamentosos aislados desde la zona costera, fueron azucares simples y disacáridos como la sucrosa, fructosa, galactosa, arabinosa, ribosa, y glucosa. Estos azucares junto con el glucógeno y el almidón, pueden ingresar en la glucólisis una vez convertidos en glucosa 6-fosfato, generando una energía acumulada en la glucolisis de 61 kJ mol^{-1} (Campbell, 1997). En presencia de oxígeno, la etapa siguiente es la oxidación escalonada del ácido pirúvico a dióxido de carbono y agua (Campbell, 1997). La respiración aeróbica se cumple en dos etapas: (i) el ciclo de Krebs y (ii) el transporte de electrones y fosforilación oxidativa (estos dos últimos procesos transcurren acopladamente) (Campbell, 1997). Durante periodos de inanición de

carbohidratos, el catabolismo de amino ácidos es la fuente principal de piruvato y oxaloacetato para ser convertidos en glucosa a través de la gluconeogénesis (proceso anabólico) (Campbell, 1997). Todos los aminoácidos, excepto la leucina y la lisina, pueden suministrar carbono para la síntesis de glucosa. Las altas tasas de crecimiento y utilización de sustratos registradas por las especies de hongos estudiadas en esta Tesis con el uso de prolina y alanina pueden ser explicadas debido a que estos amino ácidos pueden ser usados como fuente de carbono y nitrógeno, como se ha observado en *Aspergillus nidulans* (Bailey y Arst, 1975; González *et al*., 1997; Cubero *et al*., 2000), los cuales utilizan la vía de la gluconeogénesis para ser transformados en glucosa y, de esta forma, pueden ser usados en el ciclo del ácido cítrico para suplir a la célula con energía (Bechem, 2012). Los aminoácidos pueden ser oxidados por tres vías: transaminación, desaminación oxidativa y descarboxilación (Campbell, 1997). Los derivados de estas oxidaciones pueden entrar en el ciclo de Krebs y en la cadena respiratoria (Campbell, 1997). Los detalles de algunos de estos procesos se describen en la Figura 5.1.

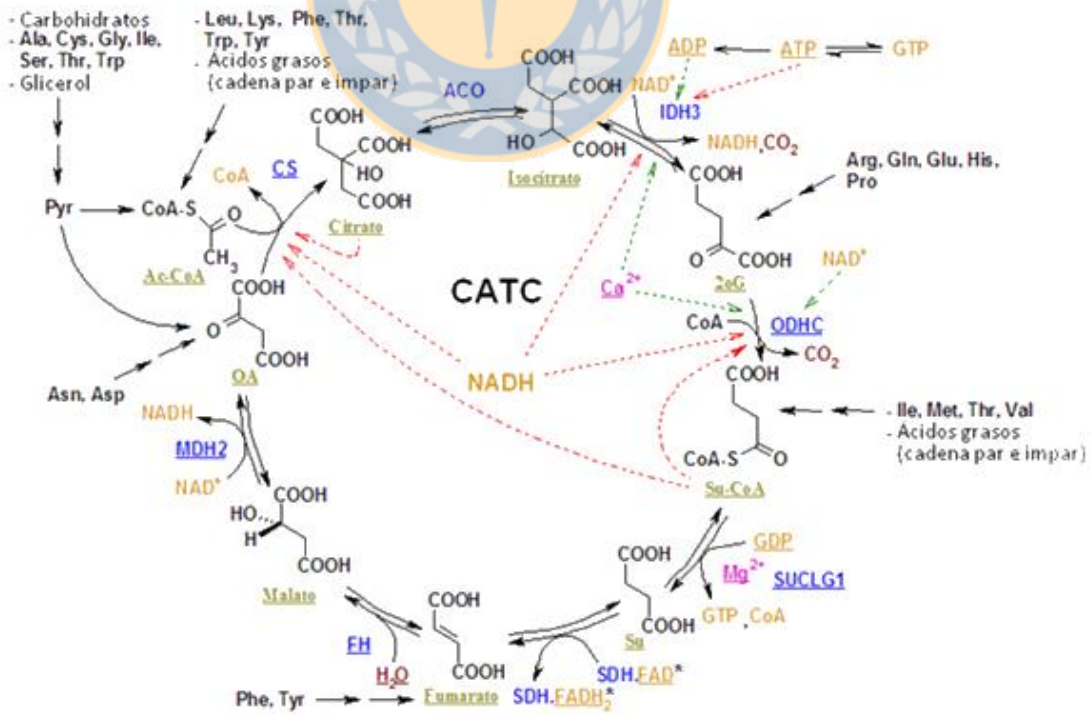

Figura 5.1. Ciclo de los ácidos tricarboxílicos (CATC), señalando los principales componentes que participan, y la posición de entrada de amino ácidos (abreviación internacional de tres letras) y carbohidratos. El esquema de color es el siguiente: enzimas: (CS) Citrato sintasa, (ACO) aconitasa, (IDH) isocitrato deshidrogenasa, (ODHC) complejo 2-oxoglutarato deshidrogenasa, (SUCLG) Succinato-CoA ligasa, (SDH) Succinato deshidrogenasa, (FH) Fumarato hidratasa (o fumarasa) y (MDH) Malato deshidrogenasa; coenzimas; componentes principales; iones metálicos; moléculas inorgánicas; --- inhibición y --- estimulación (modificado de Meléndez-Hevia *et al*., 1996).

En la gluconeogénesis el grupo amino se separa convirtiéndose en urea (Campbell, 1997), con un coste extra de la gluconeogénesis de 4 moléculas de alto potencial de transferencia de grupos fosforilo (2 ATP y 2 GTP). La energía del ATP y GTP se usa para convertir una reacción energéticamente desfavorable como es la reacción inversa de la glucolisis (Campbell, 1997). Considerando que la reacción general de oxidación de la glucosa es:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O + 686\ \text{Kcal mol}^{-1}\ (2870\ \text{KJ mol}^{-1}) \qquad \text{(ec. 5.3)}$$

Esto explica que por cada mol de oxígeno absorbido (32 g) se producen 114 Kcal de energía. Por lo tanto, al considerar las tasas respiratorias máximas de 400 μmol O_2 L^{-1} producidas por las especies de hongos filamentosos estudiadas, equivales a 12,8 mg de O_2, podemos calcular la utilización de 12 mg de glucosa, para la producción de 0,19 KJ de energía.

Los análisis de utilización de substratos para *P. decumbens, A. strictum* y *F. fujikuroi* confirman la versatilidad de estos organismos, los cuales utilizan una amplia variedad de fuentes de carbono, incluyendo monosacáridos, disacáridos, oligosacáridos y polisacáridos, además de amino ácidos, ácidos carboxílicos, aminas, amidas y polímeros. La mayor parte de estos compuestos son hidrolizados por enzimas extracelularmente, previo a la incorporación de monómeros de carbono a través de la membrana plasmática (Kavanagh, 2011). Los hongos poseen variados modos de asimilación a través de la membrana plasmática: difusión simple, difusión facilitada, canales de difusión y transporte activo, donde los azucares y amino ácidos son incorporados a la célula a través de transporte activo (Kavanagh, 2011). Los hongos filamentosos son conocidos por la utilización de variadas rutas bioquímicas para asimilar una amplia variedad de sustratos simples y complejos y por producir una extensa variedad de metabolitos (Singh, 2009;

Kavanagh, 2011). Las tasas metabolicas más altas determinadas para las especies analizadas se produjeron por la utilización principalmente de carbohidratos, además de amino ácidos como la L-prolina y L-alanina. Otros estudios muestran que los grupos principales de sustratos utilizados por especies de hongos corresponden casi exclusivamente a carbohidratos (Druzhinina *et al*., 2006; Seidl *et al*., 2006; Mohammad *et al*., 2012; Zheng *et al*., 2012).

La zona centro sur de Chile presenta alta productividad primaria autóctona, además de registrar el ingreso de altas concentraciones de materia orgánica de origen alóctona (terrígeno) cerca de la costa (Daneri *et al*., 2000; Montero *et al*., 2007; Iriarte *et al*., 2012). La alta producción primaria estaría sustentando a la comunidad micoplanctónica presente en esta zona (Gutiérrez *et al*., 2011), de la misma forma que se ha observado en otros sistemas, como en la zona de acumulación cálida del Pacífico (Pacífic Warm Pool) desde Honolulu (Hawaii) a Brisbane (Australia) (Wang y Johnson, 2009), en las aguas costeras de Hawai (Gao *et al*., 2011), y a partir de observaciones directas de relaciones parasitarias de hongos sobre fitoplancton frente a la zona sur de India (Raghukumar, 2006).

La composición bioquímica de los principales taxones de algas (diatomeas, primnesiophytes, prasinofitas, clorofitos, eustigmatophytes, cryptomonadas y rhodophytes) contiene una amplia variedad de azúcares (considerablemente variable entre estos grupos), en donde la glucosa es el azúcar predominante, (21-87%), seguido por galactosa, manosa y arabinosa; y en menor cantidad fucosa, ribosa y xilosa (Hellebust, 1974; Brown *et al*, 1997). Estas moléculas fueron altamente utilizados (> 0,29 nm) por los hongos filamentosos (excepto la fucosa) estudiados en la presente Tesis, lo que sugiere que estas microalgas son una buena fuente de nutrición para especies de hongos filamentosos de la zona de surgencia costera. En cuanto al contenido de aminoácidos de las microalgas mencionadas anteriormente es muy similar, independientemente del taxón de algas, lo que sugiere que la calidad de las proteínas es también similar (Hellebust, 1974; Volkman *et al*, 1995; Brown *et al*, 1997). Entre los aminoácidos más comunes producidos por las microalgas son el ácido L-aspártico y ácido L-glutámico proteinogénico (Brown y Jeffrey, 1992).

Estos aminoácidos son utilizados por las tres especies de hongos incluidas en este estudio, *A. strictum*, *P. decumbens* y *F. fujikuroi*, pero muestran índices de crecimiento y metabólicos bajos, con valores de densidad óptica bajo 0,2 nm. Otras fuentes Importantes de carbohidratos y amino ácidos disponibles en ambientes costeros son: tejidos de plantas vasculares (angiospermas y gimnospermas, leñosas y no leñosas), macrófitas marinas, fitoplancton y zooplancton (Cowie y Hedges, 1992). El la Figura 5.2 se puede apreciar un esquema conceptual de la degradación de la materia orgánica desde POC a DOC, señalando las principales fuentes de sustratos (carbohidratos y amino ácidos) para la asimilación de carbono, nitrógeno, fósforo y azufre.

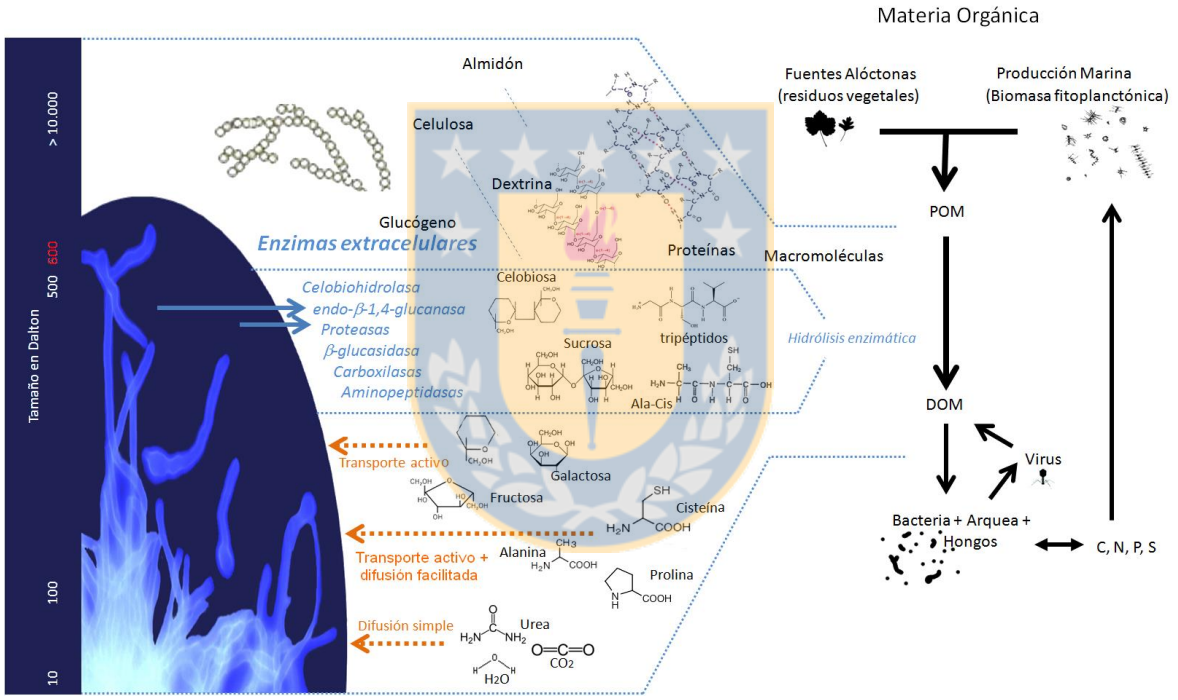

Figura 5.2. Esquema conceptual de la degradación de materia orgánica particulada a compuestos de menor tamaño (DOM), mostrando algunas de las enzimas involucradas en la degradación de polímeros de gran tamaño y los componentes del anillo microbiano que estarían participando principalmente en el proceso de degradación.

En términos del carbono terrestre, el detritus orgánico sirve como fuente de nutrientes para los hongos en los ecosistemas marinos costeros (Steele, 1967; Raghukumar, 2004). Esto hace que estos sistemas sean adecuados para el desarrollo de diversas comunidades de micoplancton (Lindahl *et al*., 2010). Los hongos juegan un papel clave en la degradación inicial de polímeros tales como la celulosa y la lignina en residuos de

plantas (Kjøller y Struwe, 2002; Baldrian y Valášková, 2008; Gutiérrez *et al*., 2011) (Figura 5.2). En los microorganismos que producen celulasa, la degradación de celobiosa es un indicador común de la formación de celulasa (Knowles *et al*., 1987). Dado que la celulosa es insoluble e impermeable, oligosacáridos tales como D-celobiosa y D-lactosa, los cuales son utilizados por las tres especies analizadas en este estudio, pueden ser considerados indicadores directos de celulasas (Gong, 1979; El-Gogary *et al*, 1989). De estos carbohidratos, la celobiosa es un producto común formado a partir de la celulosa a través de exo-celobiohidrolasa, y se considera un inductor natural (Mandels y Reese, 1960; Sternberg y Mandels, 1979; Lynd *et al*., 2002). De este modo, las especies de hongos aislados de la zona costera en el presente estudio han demostrado ser capaces de contribuir a la degradación de polímeros vegetales de gran tamaño.

Prácticamente todos los compuestos orgánicos naturales pueden ser degradados por una o más especies de hongos gracias a la producción de una variedad de enzimas tales como amilasas, lipasas, proteasas y que les permiten utilizar sustratos tales como almidones, grasas, y proteínas. Un número más limitado de especies puede usar pectinas, celulosa, hemicelulosa como fuentes de carbono (Anastasi *et al*., 2013). Las cinco especies estudiadas, han sido identificadas como saprótrofos y patógenos de plantas (Lysková, 2007, Summerbell *et al*., 2011; Wiśniewska *et al*., 2011; Herron *et al*., 2015). P. decumbens, por ejemplo, se ha utilizado para la producción de celulasas a escala industrial (Fang *et al*., 2010).

Algunos hongos son los principales microorganismos degradadores de polímeros naturales especialmente complejos y resistentes al ataque microbiano, tales como la queratina, quitina y lignina (Anastasi *et al*., 2013). Debido a la gran inespecíficidad de las enzimas implicadas en la degradación de la lignina, los hongos de madera pueden atacar a numerosos compuestos xenobióticos aromáticos y alifáticos, incluidos contaminantes ambientales, como hidrocarburos aromáticos policíclicos (PAHs), bifenilos policlorados (PCB), pesticidas y herbicidas. Estas

capacidades hacen de estos organismos de gran interés para su posible uso en biorremediación ambiental (Anastasi *et al*., 2013).

Biomasa lignocelulósica, como por ejemplo, partes no comestibles de plantas, cultivos leñosos o residuos agrícolas, están ganando un interés creciente como fuente para la producción de biocombustibles de segunda generación. Sin embargo, el cuello de botella actual son los complejos costos y la energía que consume el convertir materia vegetal rica en lignina a azúcares simples o productos químicos como ácido itacónico o glicerol (Margeot *et al*., 2009; Jäger y Buchs, 2012). La lignina actúa como un compuesto de unión entre los componentes de celulosa y hemicelulosa y por tanto, necesita ser degradado para poder tener acceso a los hidratos de carbono de alto valor para la posterior fermentación a etanol (Feldbrügge *et al*., 2013). Esto permitiría una producción sostenible y económica de biocombustibles (Sánchez, 2009; Chundawat *et al*., 2011).

A partir de los resultados obtenidos, se sugiere que las principales vías bioquímicas utilizadas por las especies incluidas en este estudio están relacionadas con el metabolismo de las hexosas (por ejemplo, glucosa, fructosa, galactosa y manosa) y disacáridos (por ejemplo, maltosa o sacarosa) (Figura 5.3), de la misma manera que se ha observado en *Saccharomyces cerevisiae* (Pronk *et al*., 1994). Estas rutas metabólicas se componen de los intermediarios de la glucólisis / gluconeogénesis (por ejemplo, aminoácidos, glicerol), el ciclo del ácido tricarboxílico (TCA), y la vía de las pentosas fosfato (Pronk *et al*., 1994; Rodrigues *et al*., 2006). Además, se podrían utilizar vías catabólicas alternativas tales como la derivación del glioxilato y la fosforilación oxidativa (Mohammad *et al*., 2012).

En los análisis de utilización de sustratos de nitrógeno, fósforo y azufre (con glucosa como fuente de carbono), se determinó que las especies *P. decumbens, A. strictum* y *F. fujikuroi*, asimilan una amplia gama de sustratos, lo que confirma la versatilidad de estas especies para crecer utilizando una extensa variedad de compuestos. En términos generales, se favorece la utilización de sustratos orgánicos por sobre los inorgánicos, en donde los primeros producen mayores tasas de crecimiento. Las tres especies muestran diferencias significativas ($p<0,05$) en el crecimiento dependiendo de los sustratos utilizados (capítulo 2).

Entre los sustratos de nitrógeno que producen mayor crecimiento en hongos filamentosos, encontramos amino ácidos (arginina, asparagina, ornitina, prolina, glutamina), aminas, nucleótidos/nucleósidos, los cuales se sugiere podrían ser asimilados principalmente a través del metabolismo de la urea, el metabolismo de amino ácidos (ruta de la arginina y prolina, aspartato y glutamato), y el metabolismo de las purinas (Figura 5.3). Además, podrían estar siendo usados como fuente de carbono y nitrógeno, por la vía de la glucolisis/gluconeogénesis (Fuentes y Quiñones, 2015).

Compuestos orgánicos como la urea, alantoína y ácido úrico, pueden ser procesados por el metabolismo de las purinas, en donde el proceso catabólico tiene dos vías muy diferentes entre los hongos. La primera en la cual se encuentran los Euascomicetidae, como *Neurospora crassa* y *Aspergillus nidulans*, los cuales utilizan por completo el N de la adenina y guanina, y la descomposición directa de la urea en amonio y CO_2 por la ureasa (Srb y Horowitz. 1944). Una segunda, que se encuentra en *Saccharomyces cerevisiae*, la cual no utiliza el anillo de N del núcleo de la purina directamente (Wiame *et al*., 1985), sino que utiliza los intermediarios de catabolismo de las purinas, como la alantoína y ácido alantoico. Este hongo tiene un método diferente para la conversión de urea a amonio al no poseer ureasa (Wiame *et al*., 1985). Otras especies como *Candida albicans* que también carece de ureasa (Odds, 1988), es capaz de crecer bien con urea como única fuente de carbono (Dastidar *et al*., 1967) a través del uso de urea amidoliasa la cual hidroliza urea (Ghosh *et al*, 2009).

Se determinó que los sustratos fundamentales del ciclo del nitrógeno como: amonio, nitrato y nitrito produjeron crecimiento moderado en las tres especies estudiadas (Figura 5.3). La asimilación de amonio en glutamato y glutamina juega un rol central en el metabolismo del nitrógeno de todos los organismos (Meti *et al*., 2011). Estos compuestos son asimilados por hongos a través de la actividad de las enzimas glutamato deshidrogenasa, glutamato sintasa, y glutamina sintetasa (Meti *et al*., 2011), son compuestos preferenciales para el consumo de nitógeno en hongos terrestres y cuando estas no están presentes, el nitrato, junto con amino ácidos,

amidas y purinas son consumidos (Marzluf, 1997; Finlay *et al*., 2006; Itoo y Reshi, 2014).

Las cadenas laterales de los amino ácidos les otorgan diferencias en reactividad, las cuales presentan diferencias al ser asimilados a través de proteínas de membrana, donde moléculas más pequeñas y más hidrofílicas difunden más rápidamente a través de proteínas transportadoras (Mishra *et al*., 2014). No hay una respuesta específica en base a los grupos radicales de estas cadenas laterales, sin embargo, se observa una relación general en base a la solubilidad, donde compuestos más hidrofílicos como los negativamente cargados (Asp y Glu), positivamente cargados (Lys, Arg y His) y polares sin carga (Ser, Thre, Cys, Pro, Asp, Gln) producen mayores biomasas de micelios en los hongos filamentosos estudiados (Figura 5.3).

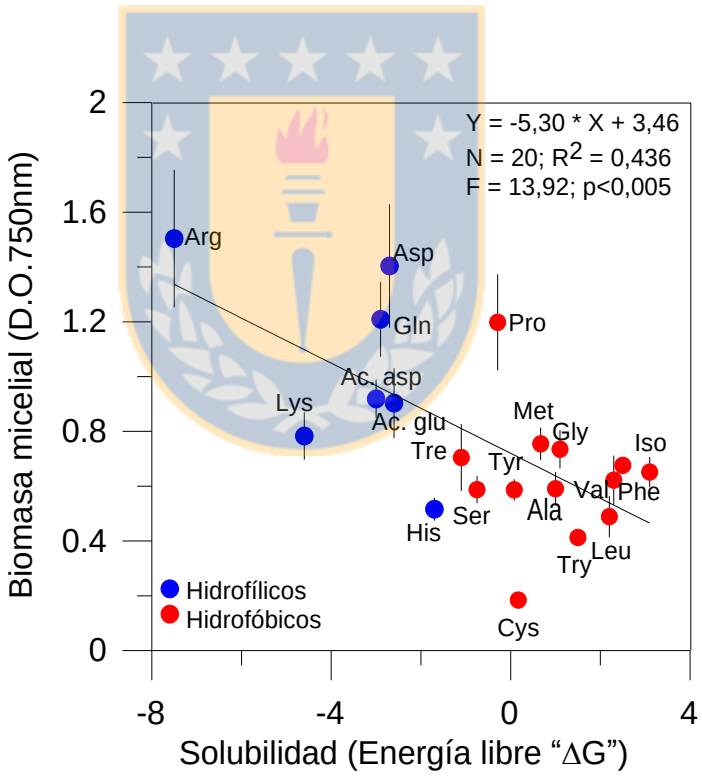

Figura 5.3. Utilización de amino ácidos en base a su solubilidad.

Todos los experimentos se realizaron en condiciones aeróbicas y por ende se benefician las rutas metabólicas que utilizan oxígeno como aceptor de electrones, sin embargo discutiremos posibles rutas alternativas para el uso de los sustratos utilizados,

debido a la presencia de una zona de mínimo oxígeno en la zona estudiada. Esta zona de bajo contenido de oxígeno podría beneficiar ciertas vías metabolicas presentes en especies de hongos marinos, tales como la denitrificación (Tsuruta *et al*., 1998; Shoun *et al*., 1992; Jebaraj y Raghukumar, 2010), o la oxidación de azufre inorgánico (Slautghter, 1989).

Los hongos pueden expresar dos vías de reducción desasimilatoria de nitrato en respuesta a la tensión de oxígeno ambiental cuando el suministro de oxígeno es insuficiente. El hongo *Fusarium oxysporum* expresa la vía de la denitrificación de nitrato que es catalizada por las reacciones secuenciales de la nitrato reductasa y la nitrito reductasa (Takaya, 2002). Estas enzimas se acoplan con la generación de ATP a través de la cadena respiratoria y producen óxido nítrico (Takaya, 2002). Se ha demostrado la existencia de hongos capaces de hacer denitrificiación en ambientes marinos, aunque predominantemente forman sólo óxido nitroso (Tsuruta *et al*., 1998; Shoun *et al*., 1992; Jebaraj y Raghukumar, 2010). La denitrificación fungica requiere un aporte mínimo de oxígeno para la inducción (el cual es reprimido por exceso de O_2), lo cual marca una diferencia con la denitrificación bacteriana que ocurre en completa ausencia de oxígeno (Zhou *et al*., 2001). El aporte óptimo de O_2 difiere entre los substratos para la denitrificación: 690 μmol O_2h^{-1} (g pesos seco)$^{-1}$ para nitrato (NO_3^-) y cerca de 250 μmol O_2h^{-1} (g pesos seco)$^{-1}$ para nitrito (NO_2^-) (Zhou *et al*., 2001). Debido a este sistema incompleto, la denitrificación por hongos provoca un aumento de los gases de efecto invernadero y conduce a efectos perjudiciales sobre el clima global (Shoun *et al*., 1998). Se ha determinado la presencia de nitrato reductasa (Na- R) en más de 80 cepas de hongos marinos repartidos en todos los grupos sistemáticos, representados por 54 especies (1 Ficomicete, 15 Ascomicetes, 1 Basidiomicete, 21 Deuteromicetes, 6 levaduras con ascosporas, 8 levaduras con basidiosporas y 3 levaduras asporógenas) (Rau y Molitoris, 2001). El sistema de denitrificación hongos, comprende NirK (nitrito reductasa que contiene cobre) y P450nor (una citocromo P450 oxido nítrico reductasa (Nor)) para reducir el nitrito a óxido nitroso (N_2O) (Nakahara *et al*., 1993; Shown *et al*., 2011).

Los ambientes costeros y de plataforma continental son responsables del enterramiento de hasta el 70-90% de materia orgánica y fósforo reactivo (Berner, 1982; Ruttenberg, 1993; Howarth *et al.*, 1995). El fósforo reactivo está presente en tres formas en la columna de agua: (i) fósforo orgánico particulado como biomasa marina o detritus biogénico, (ii) fosfato de calcio biogénico en partes duras de peces, y (iii) fósforo reactivo soluble que comprende fosforo disuelto orgánicos e inorgánicos (Slomp y Van Cappellen, 2007; Paytan y McLaughlin, 2007). Los sustratos con fósforo que produjeron mayor crecimiento de micelios en las tres especies estudiadas fueron ribonucleotidos de adenosina y guanosina (Figura 5.3). Los hongos poseen transportadores específicos en la membrana plasmática para la asimilación de bases púricas (Scazzocchio *et al*., 1982; Pantazopoulou y Diallinas, 2007). Los fosfo-amino ácidos, ribonucleótidos de pirimidinas como la citidina y uridina promueven tasas de crecimiento moderadas, sin embargo estas últimas no pueden ser usadas como fuente de nitrógeno en hongos, solo pueden ser usadas para la síntesis de ácidos nucleicos (Vlanti y Diallinas, 2008). Algunos organismos han desarrollado mecanismos para reciclar nucleótidos extracelulares y usar el fósforo (principalmente en condiciones de inanición) (Kennedy *et al*., 2005). La hidrólisis de AMP a adenosina y GMP a guanina (por la acción de nucleotidasas) forma el brazo extracelular del rescate de purinas y fosfato, de tal manera que la adenosina y guanina puedan cruzar la membrana celular a través de transportadores de nucleósidos y ser utilizados como sustratos para la producción de nucleótidos de purina en la célula (Zimmermann *et al*., 1992). Productos de genes de levadura (Nucleótido Fosfatasa Fosfodiesterasa 1 y 2 (Npp1 y Npp2)) han sido recientemente implicados como nucleotidasas extracelulares, que son miembros de una superfamilia de fosfatasa alcalina que también incluyen 5'-nucleotidasas extracelulares con ATPasa y la actividad ADPasa (Kennedy *et al*., 2005). Finalmente el fosfato producido ingresa a la célula a través de proteínas transportadoras (Yompakdee *et al*., 1996; Van Belle y André, 2001).

Por último, sustratos inorgánicos como, por ejemplo, tiofosfato, y fosfato, muestran crecimiento de hifas moderado, en donde polifosfatos inorgánicos juegan un importante rol en el metabolismo energético (Vagabov *et al*., 1998). Se supone que en condiciones desfavorables y de estrés, la hidrólisis de polifosfatos proporciona energía adicional para el mantenimiento de procesos celulares (Kulaev y Kulakovskaya, 2000).

Por otra parte, se determinó que los sustratos que complementan de mejor manera las necesidades de asimilación de azufre de las especies estudiadas fueron principalmente compuestos con cisteína y metionina (Figura 5.3). El azufre compone cerca del 1 % del peso seco de los organismos, los cuales se encuentran mayoritariamente en proteínas (Sievert *et al*., 2007). Además, sustratos inorgánicos como sulfato, tiofosfato, tetrationato, ditiofosfato mostraron un leve aporte al crecimiento. El azufre es un elemento esencial en todos los organismos, incluyendo a los hongos (Slautghter, 1989; Jennings, 1995), siendo incluso, algunas especies de hongos, capaces de oxidar azufre inorgánico y de producir compuestos como el dimetil sulfuro (Slautghter, 1989). Este último es un importante compuesto en el ciclo del azufre en ambientes marinos y posee una importante participación en el clima de la Tierra (Gadd, 2006).

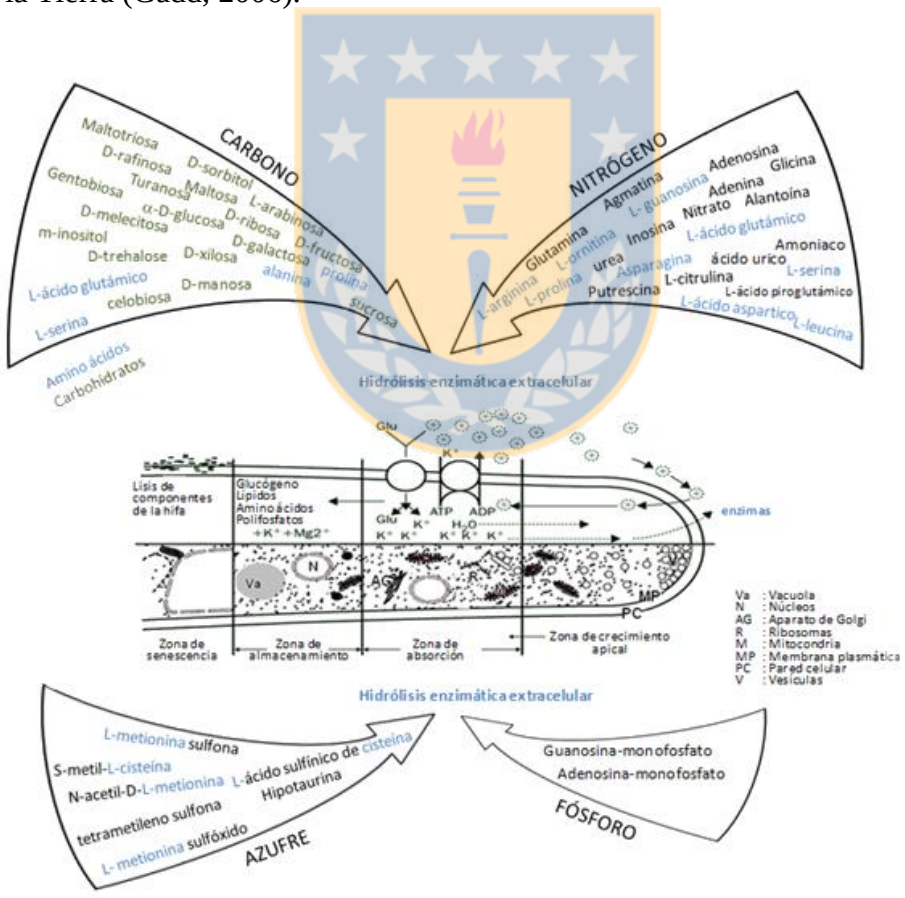

Figura 5.4. Sustratos principales de carbono, nitrógeno, fósforo y azufre utilizados por hongos filamentosos aislados de la zona costera frente a Chile centro sur. Detalle de las

zonas de la hifa donde se producen los procesos de crecimiento, absorción, reserva y senescencia (modificado de Mennink-Kersten *et al*., 2004).

La zona costera de surgencia frente a Chile centro sur, presenta alta disponibilidad de biomasa fotosintética y de materia orgánica (Daneri *et al*., 2000; Montero *et al*., 2007; Sánchez *et al*., 2009), presentando un ambiente heterogéneo con cambios temporales en la temperatura, calidad y disponibilidad de materia orgánica. Esto permite sostener a una amplia comunidad de heterótrofos como bacterias (Troncoso *et al.* 2003; Cuevas *et al.* 2004), arqueas (Levipán *et al.* 2007; Quiñones *et al.* 2009), y hongos filamentosos (Gutierrez *et al*., 2010, 2011), donde estos últimos se ven bien representados en la fracción de tamaño >25um, presentando una alta actividad de enzimas extracelulares capaces de degradar polímeros de gran tamaño (Gutiérrez *et al*., 2011) como la celulosa. El crecimiento y la respiración de hongos filamentosos aislados de la zona costera pueden estar fuertemente influenciados por la presencia de altas concentraciones de sustratos nutricionales y los cambios en la temperatura del agua, mostrando respuestas oportunistas con efectos especie-específicos que se observan tanto en las tasas de crecimiento, como en las de respiración (Fuentes *et al*., 2015). En base a la alta biomasa de micelios observada en terreno en algunos periodos del año, se ha planteado que estos podrían competir fuertemente con la biomasa bacteriana (Gutierrez et al., 2011), por lo que la respiración microplanctonica, en algunos períodos, podría estar dominada por la comunidad fungal.

Las especies estudiadas en esta Tesis doctoral mostraron ser versátiles al utilizar una amplia gama de compuestos (principalmente orgánicos) para su crecimiento, especialmente carbohidratos (desde azucares simples hasta polisacáridos) y amino ácidos. Esto demuestra sus capacidades para la degradación y utilización de detritus vegetal y biomasa de fitoplancton, con las cuales cubren sus necesidades de carbono, nitrógeno, fósforo y azufre, participando en importantes ciclos biogeoquímicos de nutrientes y energía. La influencia en la degradación de materia orgánica por parte de los hongos, junto a la de bacterias y arqueas, es fundamental en el funcionamiento de la bomba biológica, potenciando el retorno de carbono a la atmosfera y limitando la exportación de éste hacia el fondo del océano.

Características de la zona costera de surgencia, como la presencia de una zona de mínimo oxígeno, podrían generar ambientes propicios para la participación de hongos filamentosos en procesos como la denitrificación, la cual ha sido ampliamente estudiada en otros ecosistemas (Shoun y Tanimoto, 1991; Usuda *et al*., 1995; Jebaraj y Raghukumar, 2010; Shoun *et al*., 2012), o la oxidación de azufre inorgánico (Wainwright, 1989). Los hongos, por su rol heterótrofo activo y su importancia en la degradación de materia orgánica, deben ser considerados como un componente integral de la comunidad microbiana en ambientes marinos costeros, como ya ha sido sugerido por autores como Gutiérrez *et al*., (2011), Jones y Pang (2012) y Wang *et al*., (2014). Además, los hongos deben ser considerados como un eslabon fundamental en la degradación inicial de polímeros de gran tamaño y compuestos difíciles de descomponer y en ciclos biogeoquímicos, por su utilización de una amplia variedad de compuestos orgánicos e inorgánicos de carbono, nitrógeno, fósforo y azufre.

6. CONCLUSIÓN

- Tres métodos para la determinación de biomasa en hongos filamentosos (*Acremonium strictum, Penicillium decumbens, P. chrysogenum, Fusarium fijikuroi* y *F. sporotrichioides*) fueron testeados, encontrando relaciones altamente significativas, en donde el mejor método para el trabajo en laboratorio fue la Densidad Óptica (método rápido, confiable y económico) y las determinaciones de ATP para muestras de muy baja biomasa fúngica. Para determinación de biomasa fúngica en terreno se recomienda el uso de Microscopía de Epifluorescencia.

- El crecimiento y respiración de hongos filamentosos bajo distintas temperaturas en el rango ambiental de la zona costera frente a Chile centro sur (9, 13 y 20 °C), mostró el crecimiento de las especies estudiadas fue significativamente estimulado por el aumento de temperatura, registrando respuestas especie-específicas con tasas de crecimiento que alcanzan valores de entre 66 y 153 μg C hifa día^{-1} a 20 °C. (**Objetivo 1: Se acepta hipótesis 1**)

- La biomasa fúngica incrementa a medida que la concentración de glucosa aumenta, con valores de biomasa de hifas entre 0,42 y 0,65 mg C a incubaciones de 0,1 g L^{-1} de glucosa; mientras que las incubaciones con 1 g L^{-1} de glucosa se asociaron con altos incrementos de biomasa principalmente en las especies *F. sporotrichioides* (1,39 mg C) y *P. chrysogenum* (1,07 mg C) desde al cuarto día en adelante. (**Objetivo 1: Se acepta hipótesis 2**)

- Las diferencias en las tasas de crecimiento causadas por la interacción entre la temperatura y la concentración de glucosa fueron significativos para las 5 especies estudiadas. Sin embargo, en el caso de *A. strictum* y *P. decumbens* las diferencias de crecimiento observados se debieron principalmente al efecto de la concentración de glucosa.

- Las tasas de respiración de las 5 especies de hongos presentaron altos valores, los cuales superan los 200 μmol O_2 L^{-1} en incubaciones con 5 g L^{-1} de glucosa en el medio. A menores concentraciones, las tasas de consumo de oxígeno registraron valores de 107,9 y 150,6 μmol O_2 h^{-1} en incubaciones a 0,01 y 0,1 mg L^{-1} de glucosa en el medio.

- Valores de Q_{10} variaron desde 2,2 hasta 6,7 demostrando que la tasa de respiración tiene una fuerte dependencia de la temperatura, especialmente en *A. strictum* y *F. sporotrichioides.*

- La tasa de consumo de oxígeno mostró aumentos relacionados a la biomasa de hifas en los cultivos, lo que se traduce en una relación alométrica positiva de la biomasa fúngica con un exponente de 0,75.

- El registro de las tasas de crecimiento específica de hongos (tasa de consumo de oxígeno por unidad de masa), mostró que organismos más pequeños y jóvenes (desde esporas) registraron mayores tasas de respiración específica, las que disminuyen a medida que los organismos incrementan en tamaño y se hacen mayores, formando micelios definidos desde el día 3-4 en adelante dependiendo de la especie. Esta relación alométrica responde a la biomasa de micelios con un exponente de -0,26.

- Las especies estudiadas (*Acremonium strictum, Penicillium decumbens* y *Fusarium fijikuroi*) poseen la capacidad de utilizar una amplia variedad de compuestos orgánicos e inorgánicos del ciclo del carbono, nitrógeno, fósforo y azufre, donde se observa una marcada preferencia en la utilización de compuestos orgánicos (como carbohidratos y amino ácidos). Esta capacidad sugiere que hongos filamentosos pueden jugar un papel significativo en la degradación de materia orgánica y participar activamente en ciclos biogeoquímicos de la zona costera del Sistema de la Corriente de Humboldt.

- Los sustratos más utilizados por las especies estudiadas (*Acremonium strictum, Penicillium decumbens* y *Fusarium fijikuroi*) fueron el disacárido sacarosa, seguido por los azúcares D-fructosa, D-galactosa, L-arabinosa, D-ribosa, α-D-glucosa, D-manosa, D-sorbitol, maltosa (disacárido) y D-xilosa y los aminoácidos L-prolina y L-alanina. Sin embrago, la gran capacidad de degradación y la numerosa cantidad de enzimas extracelulares descritas para los hongos hacen posible que estos organismos sean capaces de crecer bien en sustratos de gran tamaño $(C_6H_{10}O_5)n$ como glucógeno con 12 a 18 unidades de α-glucosa, dextrina con

6 a 9 unidades de glucosa, ciclodextrina con 7 unidades de glucosa. El consumo de carbohidratos no presenta relación con el tamaño (peso molecular) de los sutratos (F=0,26; p=0,613) (**Objetivo 2: Se rechaza la Hipótesis 3**). En consecuencia, la capacidad de asimilar sustratos dependerá más de la producción de enzimas para degradar compuestos que del tamaño de los sustratos presentes en el medio.

- Los índices de utilización de sustratos (riqueza sustrato) en *A. strictum, P. decumbes* y *F. fujikuroi* fue de 88, 87 y 81 sustratos respectivamente. Registrando además, altos valores del índice de Shannon en las tres especies, con valores de 4.02 (*A. strictum*), 4.01 (*P. decumbes*) y 3.91 (*F. fujikuroi*). De los 95 sustratos analizados, 18 presentaron diferencias significativas entre las tres especies estudiadas. Se sugiere que la principal ruta metabólica utilizada sería la glucolisis/gluconeogénesis.

- Es interesante destacar que las tres especies de hongos crecieron en celobiosa, lo que sugiere que son capaces de producir enzimas para hidrolizar celulosa.

- Altos índices de utilización particular de sustratos de nitrógeno se registraron, donde altos valores del índice de Shannon de 4.2, 4.3 y 4.2 son determinados para las especies *A. strictum, P. decumbens* y *F. fujikuroi,* respectivamente.

- Los sustratos de nitrógeno que más incrementaron el crecimiento de micelios corresponden a compuestos del metabolismo de amino ácidos en las vías de la arginina y prolina como la L-arginina, L-prolina, glutamina, L-ornitina, putrescina (amina) y urea.

- Entre los amino ácidos que produjeron mayor crecimiento micelial, no se observa una relación con el peso molecular de los sustratos (F=1,28; p=0,27), ni con la reactividad de un grupo R en particular (p>0,05). Sin embargo, el crecimiento de las especies estudiadas presenta una relación significativa con la solubilidad de los amino ácidos (F=1153,01; p=0,0001), siendo los amino ácidos más hidrofílicos los más utilizados (**Objetivo 2: se acepta la hipótesis 4**). Además, casi la totalidad de los sustratos presentes en el cluster I

forman parte de la vía catabólica de la urea y amino acidos (e.g. Vía del aspartato y glutamato).

- Compuestos orgánicos como L-asparagina (Vía del aspartato y glutamato), agmatina (amina), inosina, y L- guanosina también produjeron alto crecimiento en las especies estudiadas. Un segundo grupo conformado por L-amino ácidos, nucleótidos/nucleósidos (N/N), seguidos de dipéptidos, produjeron crecimiento moderado de las especies estudiadas (**Objetivo 3: Se acepta hipótesis 5**).

- Entre los sustratos de nitrógeno inorgánico que produjeron bajo crecimiento, destacan el nitrato (Cluster 2a), además de compuestos como amonio y nitrito (Cluster 2b), los cuales, principalmente el amonio, pueden ser importantes aportes de nitrógeno en hongos terrestres (**Objetivo 3: Se rechaza hipótesis 6**).

- Los sustratos preferidos por hongos filamentosos para suplir las necesidades de fósforo, y que producen mayor crecimiento, se componen exclusivamente de los ribonucleótidos de adenosina y guanosina. Se sugiere que la ruta metabólica de estos ribonucleotidos sería el metabolismo de las purinas, ya sea para la formación de ácidos nucleícos, formación de xantina (para pasar al metabolismo de la urea) o para formar compuestos más energéticos como ATP y GTP. Un segundo grupo de sustratos preferidos se compone de fosfo amino ácidos y ribonucleotidos de citidina y uridina.

- Los principales sustratos con azufre que promueven el crecimiento *A. strictum*, *P. decumbens* y *F. fujikuroi* son aquellos derivados de cisteína y metionina. Por otra parte, estas especies presentan crecimiento moderado con tetrationato, tiofosfato, ditiofosfato, sulfato, taurina, hipotaurina, amino ácidos, entre otros compuestos orgánicos.

- Los hongos filamentosos que habitan en la zona de surgencia deben ser considerados como un componente integral de la comunidad microbiana en ambientes marinos costeros. En este sentido, debieran ser incluidos en modelos de degradación de materia orgánica y como componentes de ciclos biogeoquímicos, por su importancia en la respiración, degradación

de materia orgánica y utilización de una amplia variedad de compuestos orgánicos e inorgánicos de carbono, nitrógeno, fósforo y azufre, los cuales afectan el destino de la materia orgánica en el océano.

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ANEXO 1

Paneles de Identificación de Hongos BIOLOG

Hongos Filamentosos (Filamentous Fungi (FF) MicroPlate™)

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetly-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

Figura A-1. Fuentes de carbon en la placa FF Microplate.

Microarreglos Fenotípicos (Phenotype Microarray (PM). PM3 B MicroPlate^{TM}) para fuentes de Nitrógeno.

A1 Negative Control	A2 Ammonia	A3 Nitrite	A4 Nitrate	A5 Urea	A6 Biuret	A7 L-Alanine	A8 L-Arginine	A9 L-Asparagine	A10 L-Aspartic Acid	A11 L-Cysteine	A12 L-Glutamic Acid
B1 L-Glutamine	B2 Glycine	B3 L-Histidine	B4 L-Isoleucine	B5 L-Leucine	B6 L-Lysine	B7 L-Methionine	B8 L-Phenylalanine	B9 L-Proline	B10 L-Serine	B11 L-Threonine	B12 L-Tryptophan
C1 L-Tyrosine	C2 L-Valine	C3 D-Alanine	C4 D-Asparagine	C5 D-Aspartic Acid	C6 D-Glutamic Acid	C7 D-Lysine	C8 D-Serine	C9 D-Valine	C10 L-Citrulline	C11 L-Homoserine	C12 L-Ornithine
D-1 N-Acetyl-D,L-Glutamic Acid	D2 N-Phthaloyl-L-Glutamic Acid	D3 L-Pyroglutamic Acid	D4 Hydroxylamine	D5 Methylamine	D6 N-Amylamine	D7 N-Butylamine	D8 Ethylamine	D9 Ethanolamine	D10 Ethylenediamine	D11 Putrescine	D12 Agmatine
E1 Histamine	E2 β-Phenylethyl-amine	E3 Tyramine	E4 Acetamide	E5 Formamide	E6 Glucuronamide	E7 D,L-Lactamide	E8 D-Glucosamine	E9 D-Galactosamine	E10 D-Mannosamine	E11 N-Acetyl-D-Glucosamine	E12 N-Acetyl-D-Galactosamine
F1 N-Acetyl-D-Mannosamine	F2 Adenine	F3 Adenosine	F4 Cytidine	F5 Cytosine	F6 Guanine	F7 Guanosine	F8 Thymine	F9 Thymidine	F10 Uracil	F11 Uridine	F12 Inosine
G1 Xanthine	G2 Xanthosine	G3 Uric Acid	G4 Alloxan	G5 Allantoin	G6 Parabanic Acid	G7 D,L-α-Amino-N-Butyric Acid	G8 γ-Amino-N-Butyric Acid	G9 ε-Amino-N-Caproic Acid	G10 D,L-α-Amino-Caprylic Acid	G11 δ-Amino-N-Valeric Acid	G12 α-Amino-N-Valeric Acid
H1 Ala-Asp	H2 Ala-Gln	H3 Ala-Glu	H4 Ala-Gly	H5 Ala-His	H6 Ala-Leu	H7 Ala-Thr	H8 Gly-Asn	H9 Gly-Gln	H10 Gly-Glu	H11 Gly-Met	H12 Met-Ala

Figura 2. Fuentes de Nitrógeno en la placa PM3

Microarreglos Fenotípicos (Phenotype Microarray (PM). PM4 A) MicroPlate^{TM}) para fuentes de Fósforo y Azufre.

A1 Negative Control	A2 Phosphate	A3 Pyrophosphate	A4 Trimeta-phosphate	A5 Tripoly-phosphate	A6 Triethyl Phosphate	A7 Hypophosphite	A8 Adenosine- 2'-monophosphate	A9 Adenosine- 3'-monophosphate	A10 Adenosine- 5'-monophosphate	A11 Adenosine- 2',3'-cyclic monophosphate	A12 Adenosine- 3',5'-cyclic monophosphate
B1 Thiophosphate	B2 Dithiophosphate	B3 D,L-α-Glycerol Phosphate	B4 β-Glycerol Phosphate	B5 Carbamyl Phosphate	B6 D-2-Phospho-Glyceric Acid	B7 D-3-Phospho-Glyceric Acid	B8 Guanosine- 2'-monophosphate	B9 Guanosine- 3'-monophosphate	B10 Guanosine- 5'-monophosphate	B11 Guanosine- 2',3'-cyclic monophosphate	B12 Guanosine- 3',5'-cyclic monophosphate
C1 Phosphoenol Pyruvate	C2 Phospho-Glycolic Acid	C3 D-Glucose-1-Phosphate	C4 D-Glucose-6-Phosphate	C5 2-Deoxy-D-Glucose 6-Phosphate	C6 D-Glucosamine-6-Phosphate	C7 6-Phospho-Gluconic Acid	C8 Cytidine- 2'-monophosphate	C9 Cytidine- 3'-monophosphate	C10 Cytidine- 5'-monophosphate	C11 Cytidine- 2',3'-cyclic monophosphate	C12 Cytidine- 3',5'-cyclic monophosphate
D1 D-Mannose-1-Phosphate	D2 D-Mannose-6-Phosphate	D3 Cysteamine-S-Phosphate	D4 Phospho-L-Arginine	D5 O-Phospho-D-Serine	D6 O-Phospho-L-Serine	D7 O-Phospho-L-Threonine	D8 Uridine- 2'-monophosphate	D9 Uridine- 3'-monophosphate	D10 Uridine- 5'-monophosphate	D11 Uridine- 2',3'-cyclic monophosphate	D12 Uridine- 3',5'-cyclic monophosphate
E1 O-Phospho-D-Tyrosine	E2 O-Phospho-L-Tyrosine	E3 Phosphocreatine	E4 Phosphoryl Choline	E5 O-Phosphoryl-Ethanolamine	E6 Phosphono Acetic Acid	E7 2-Aminoethyl Phosphonic Acid	E8 Methylene Diphosphonic Acid	E9 Thymidine- 3'-monophosphate	E10 Thymidine- 5'-monophosphate	E11 Inositol Hexaphosphate	E12 Thymidine 3',5'-cyclic monophosphate
F1 Negative Control	F2 Sulfate	F3 Thiosulfate	F4 Tetrathionate	F5 Thiophosphate	F6 Dithiophosphate	F7 L-Cysteine	F8 D-Cysteine	F9 L-Cysteinyl-Glycine	F10 L-Cysteic Acid	F11 Cysteamine	F12 L-Cysteine Sulfinic Acid
G1 N-Acetyl-L-Cysteine	G2 S-Methyl-L-Cysteine	G3 Cystathionine	G4 Lanthionine	G5 Glutathione	G6 D,L-Ethionine	G7 L-Methionine	G8 D-Methionine	G9 Glycyl-L-Methionine	G10 N-Acetyl-D,L-Methionine	G11 L- Methionine Sulfoxide	G12 L-Methionine Sulfone
H1 L-Djenkolic Acid	H2 Thiourea	H3 1-Thio-β-D-Glucose	H4 D,L-Lipoamide	H5 Taurocholic Acid	H6 Taurine	H7 Hypotaurine	H8 p-Amino Benzene Sulfonic Acid	H9 Butane Sulfonic Acid	H10 2-Hydroxyethane Sulfonic Acid	H11 Methane Sulfonic Acid	H12 Tetramethylene Sulfone

Figure 3. Fuentes de Fósforo y Azufre en la placa PM4.