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The role of fungi in processing marine organic matter in the upwelling ecosystem off Chile

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Abstract In a study that spanned from March 2007 through November 2009, we report high fungal biomass and over 90% of extracellular enzymatic activity occurring in the size classes dominated by fungi during periods of high autotrophic biomass in surface waters of the upwelling ecosystem off central-southern Chile (36°30.80′S–73°07.70′W). Fungal biomass in the water column was determined by the abundance of hyphae and was positively correlated with the concentration of the fungal biomarker 18:2ω6. High fungal biomass during active upwelling periods was comparable to that of prokaryotes (bacteria plus archaea) and was associated with an increase in phytoplankton biomass and in extracellular enzymatic hydrolysis in waters from the depth of maximum fluorescence. We show fungi as a new microbial component in the coastal upwelling ecosystem of the Humboldt Current System off central Chile. Our results suggest that the temporal pattern in fungal biomass in the water column during a year cycle is a reflection of their capacity to hydrolyze organic polymers and, in consequence, fungal biomass and activity respond to a seasonal cycle of upwelling in this ecosystem.

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Introduction

The Humboldt Current System is one of the most productive ecosystems in the world, with daily primary production >15 g C m^{-2} during active upwelling events (Montero et al. 2007). The microbial community inhabiting the water column of the Humboldt Current System off Chile is responsible for the degradation of a major fraction of the photosynthetic carbon in this ecosystem (González et al. 1998; Troncoso et al. 2003; Cuevas et al. 2004; Montero et al. 2007).

The dissolved organic carbon pool is the largest active marine reservoir of carbon (Hedges 1992), and although most dissolved organic carbon in the ocean is composed of molecules smaller than 1000 D (Benner et al. 1992; Amon and Benner 1994), less than 1% are monomers capable of being directly transferred to microorganisms to be mineralized. Thus, the uptake of organic substrates by heterotrophic microorganisms requires the processing of the polymeric fraction of dissolved organic matter by the hydrolytic activity of extracellular enzymes (Hoppe et al. 2002). In consequence, this process is considered to determine the amount of available substrates for the recycling and mineralization of organic matter (Hoppe et al. 2002; Arnosti 2003).

In seawater, extracellular enzymatic activity is considered best represented in the size fraction dominated by

bacteria 0.2–3 μm (Hoppe 1983; Rosso and Azam 1987; Hoppe et al. 2002). However, significant extracellular hydrolysis has also been found in size classes >3 μm (Hoppe 1983; Unanue et al. 1993; Hoppe et al. 2002), where prokaryote abundance is minimal, suggesting that other microorganisms are also responsible for hydrolytic activity.

Fungi are one of the main organisms responsible for the recycling of plant detritus in terrestrial and freshwater ecosystems and participate actively in the carbon cycle by replenishing carbon dioxide to the atmosphere and releasing inorganic nitrogen and phosphorous into the soil (Carlile et al. 2001; Dighton 2007). The efficiency of fungi at degrading insoluble plant remains is due to their growth mode and metabolism associated with the production of a wide variety of extracellular enzymes, which allow them to utilize a broad range of organic macromolecules (Carlile et al. 2001; Dighton 2007). In addition, the opportunistic strategy of fungal nutrition gives them a rapid response to changing substrate conditions (Lindahl et al. 2010), and the production of resistance and dispersion structures allows them to persist when their growth is limited by adverse environmental conditions (Carlile et al. 2001; Magan 2007).

In the last decade, there has been a growing number of reports of fungi identified in marine environments such as deep-sea sediments (Damare et al. 2006), hypersaline waters (Buchalo et al. 2000; Kis-Papo et al. 2003), methane hydrates (Lai et al. 2007), oxygen deficient zones (Jebaraj and Raghukumar 2009), and in hydrothermal vents (Le Calvez et al. 2009). Nevertheless, the study of the biogeochemical and ecological roles of fungi in the marine ecosystem has been mainly limited to tropical ecotones under a strong influence of terrestrial detritus (Fell and Newell 1998; Hyde et al. 1998; Raghukumar 2005; Clipson et al. 2006), and their distribution and role in ocean processes is less known when compared with terrestrial environments (Fell and Newell 1998; Clipson et al. 2006). In particular, the presence of fungi as active degraders of marine-derived organic matter has been largely underestimated. The major problem for mycologists to study fungi in the marine ecosystem is the availability of appropriate methodological approaches to estimate their activity and spatial and temporal distribution in the environment.

Utilizing a combination of methods based on direct detection and counting of fungal structures by microscopy, the presence of phospholipid fatty acid biomarkers, and size-fractionated extracellular enzyme activity assays, we studied fungal abundance and its relationship with the degradation of model protein and carbohydrate polymers during a year-long cycle in a coastal upwelling ecosystem of the Humboldt Current System off the coast of Chile.

Materials and methods

Study area and sampling

The study area was located in the coastal upwelling ecosystem off central-south Chile, at the Coastal Time Series Station of the COPAS Center (Station 18, http://copas.udec.cl/eng/research/serie/, Online resource 1). Station 18 is one of the fixed time series stations that is routinely sampled by the Center for Oceanographic Research in the eastern South Pacific (FONDAP COPAS Center) since 2002. Sampling was carried out between March 2007 and November 2009 (21 sampling dates, Online resource 2) onboard R/V Kay-Kay II (University of Concepción). Temperature, salinity, oxygen, and fluorescence data were obtained using CTDO casts (Seabird 19 plus). Water column samples were collected with Niskin bottles.

Water samples from either the surface (0–1 m) or the depth of maximum fluorescence were collected to estimate fungal and prokaryote abundance, for biomarker analysis, and for extracellular enzymatic activity measurements (Online resource 2). Additionally, the depth distribution of hydrolytic activity and fungal and prokaryotic biomass in the water column were determined by carrying out experiments and measurements at several depths at Station 18 (Online resource 2).

All water and sediment samples were stored in sterile containers at in situ temperature and protected from direct sunlight until processing back in the laboratory. Water subsamples were filtered through combusted (4 h, 450°C) 0.7-μm glass fiber filters and stored at −20°C for biomarkers analysis. Aliquots of 50 and 15 mL were preserved with formaldehyde (final concentrations 3 and 2%, respectively) and stored at 4°C under dark for fungi and prokaryote counting.

Seawater samples, stored in sterile polycarbonate flasks, were kept at the in situ temperature for less than 5 h for hydrolysis experiments.

Extracellular enzymatic activity experiments

Duplicate 5-mL aliquots of seawater were incubated in darkness with l-leucine-4-methylcoumarinyl-7-amide (MCA-Leu), methylumbelliferyl-β-D-cellobiose (MUF-Cel) and 4-methylumbelliferyl-β-D-glucoside (MUF-Glu) at 10 μM final concentrations (Hoppe 1983).

Controls were run with seawater samples previously boiled for 10 min. Fluorescence was measured on subsamples removed every ~2 h using a Turner BiosSystem Inc. fluorometer (TBS-380 Mini) at excitation of 365 nm and emission 455 nm (Meyer-Reil 1987). Calibration curves were constructed by measuring the fluorescence in

seawater with hydrolysis products MCA and MUF at concentrations ranging between 0.03 and 0.5 μM. The initial time course (less than 10%) was used for obtaining the hydrolysis rates to avoid interferences during longer incubations (Pantoja and Lee 1994). Coefficient of variation of replicates was <17%.

Size-fractionated extracellular enzymatic activity experiments

Four hydrolysis experiments were carried out with unfiltered seawater and six size fractions using sequential sieving and filtration (Isopore filters) of 100 mL seawater from the depth of maximum fluorescence (Fig. 1) collected on March 4, April 15, June 24 and November 26 2009. The resulting size fractions were >90 μm, 90–25 μm, 25–8 μm, 8–5 μm, 5–3 μm and 3–0.2 μm.

Duplicate 5-mL aliquots of each size fraction, unfiltered seawater, and 0.2 μm filtered seawater (blank) were incubated with 20 μL MCA-Leu, MUF-Cel and MUF-Glu solutions at 10 μM final concentrations. Subsamples were removed every ca. 2 h and fluorescence read as before. The estimated potential hydrolysis rates of each fraction were corrected by the activity in the size fraction <0.2 μm used for re-suspending. Rates were calculated as described by Pantoja and Lee (1994). Subsamples from each size fraction were formaldehyde-preserved and stored for fungi and prokaryote counts as described in the next section.

Fungal and prokaryote abundance

The abundance of fungal hyphae in seawater was estimated by epifluorescense microscopy using an adaptation of the Calcofluor White stain method described previously for detecting fungi in marine sediments (Damare and Raghukumar 2008; Jebaraj and Raghukumar 2009) and lakes (Rasconi et al. 2009). Duplicate aliquots from 5 to 30 mL of seawater were filtered on 0.22-μm mesh, black, 25-mm-diameter polycarbonate filters (Millipore Corp.). Filters with the retained material were stained directly with 600 μL of aqueous 0.1% Calcofluor White, making sure to cover the entire area of the filter.

The entire effective area of the filters was examined under an epifluorescence microscope (Axioscop 2 Plus, Carl Zeiss Ltd.) equipped with Filter set 49 (Carl Zeiss Ltd., 365-nm excitation and 445- to 450-nm emission band pass). To ensure that filaments were from a fungal source, we quantified only the septate hyphae. Septa give a segmented aspect to the hyphae and make them clearly and readily distinguishable from other filamentous materials such as detritus. All hyphae identified on the filters were counted at 1000× and their length and width recorded. Considering the tubular shape of hyphae, a cylinder volume was used as a morphological approximation to estimate the biovolume of fungal filaments (Jebaraj and Raghukumar 2009). Fungal carbon was estimated using the conversion factor 1 $\mu m^3 = 1$ pg C (van Veen and Paul 1979). The staining method for detecting fungi in seawater samples was further validated using a fungal suspension prepared with a strain isolated from the study area (laboratory collection) in filtered seawater. The coefficient of variation for fungal carbon estimated for replicate samples was less than 20%.

For prokaryote abundance, aliquots (2–5 mL) of formaldehyde-preserved samples were stained with 100 μL DAPI (4, 6-diamidino-2-phenylindole, Sigma D-1388) per mL sample for 10 min and collected on black polycarbonate filters (0.2 μm pore size, 25 mm diameter; Millipore Corp.) (Porter and Feig 1980). Isolated water samples of the size fractions ≥90 and 90–25 μm used for incubations were sonicated three times for 30 s, at 35 kHz to release any attached prokaryotic cells from particles before DAPI staining. Samples with and without sonication showed no differences in prokaryote cell counts. Ten random fields and at least 400 cells were counted at 1000× with an epifluorescence microscope Axioscop 2 Plus, Carl Zeiss Ltd. Prokaryote carbon was calculated using a conversion factor of 22 pg C per prokaryote cell (Cuevas et al. 2004).

Determination of organic biomarkers of phytoplankton and fungi

Chlorophyll-*a* was determined by fluorometry (Parsons et al. 1984) in a Turner Designs® fluorometer, and phytoplankton carbon was estimated using a conversion factor of 57 g C (g Chl-*a*)$^{-1}$ (Eppley et al. 1992; Chavez et al. 1991).

Fungal fatty acid biomarker 18:2ω6 was obtained from total lipid extracts, and the identification and quantification was carried out by gas chromatography coupled with a mass selective detector (GC–MS, Agilent 5975) according to Christie (1998). Lipids were extracted with a dichloromethane/methanol mix (Bligh and Dyer 1959) from suspended material (5–20 L of seawater) retained on 0.7-μm glass fiber filters (Whatman GF/F). Water and hexane were added to the extract, and the volume of the organic extract was reduced in a rotary evaporator. The extract was saponified with 15 mL KOH:MeOH 0.5 N (Christie 1989), and polar lipids were converted into fatty acid methyl esters by the addition of 1 mL 10% BF_3/MeOH (Christie 1989; Tolosa et al. 2004; Méjanelle and Laureillard 2008). The saponified fractions were injected into a GC–MS equipped with a capillary column HP5-MS (30 m, 0.25 mm, 0.25 μm film thickness; Agilent Technologies),

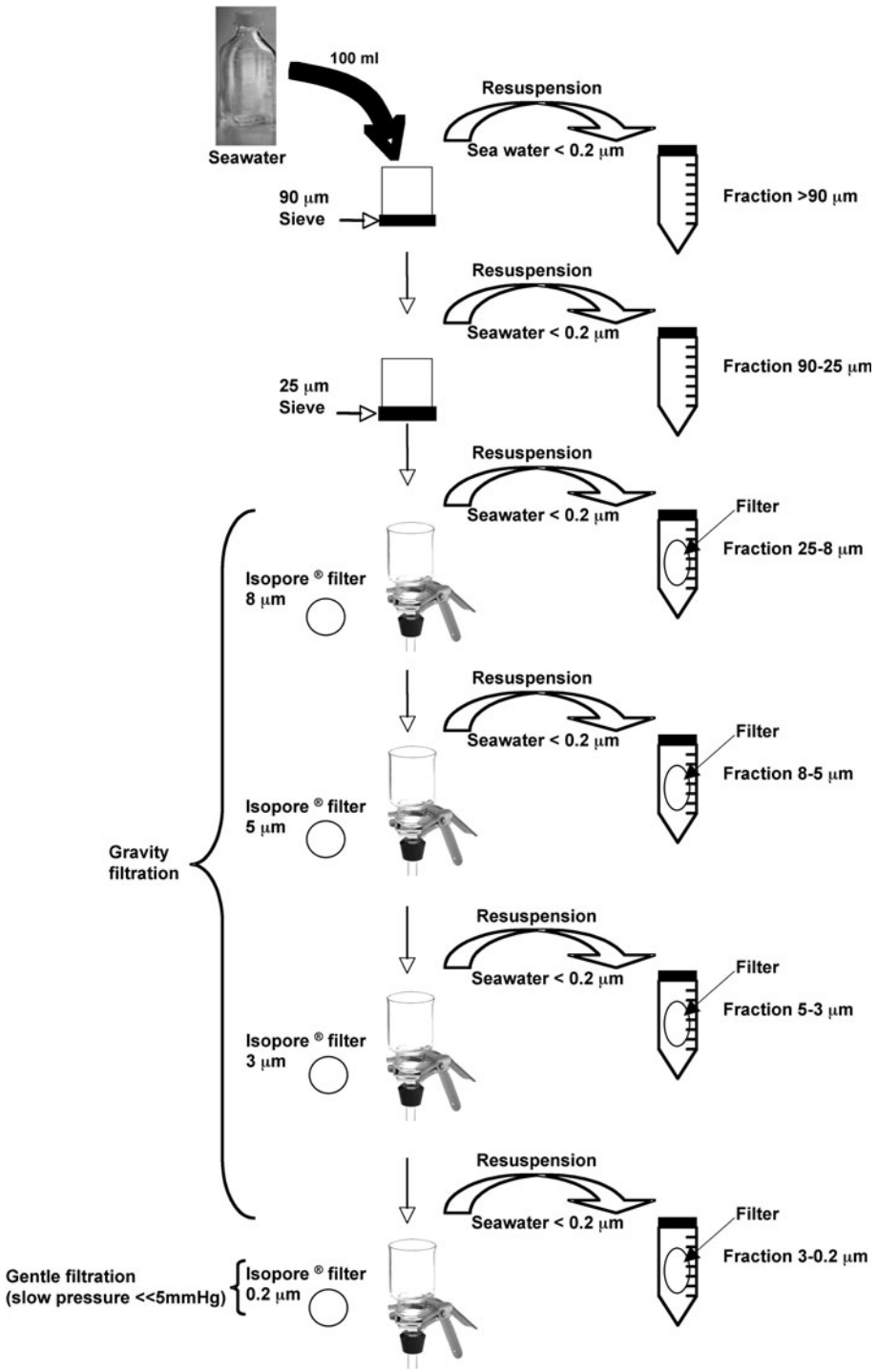

Fig. 1 Methodological approach utilized to separate size classes for fractionated hydrolysis incubations

and the fatty acid identified using the internal library of the equipment and the electronic database www.lipidlibrary.co.uk/ms/arch_me/index.htm. Quantification of the fatty acid was carried out using a calibration curve with serial dilutions of fatty acids standard mix (FAME mix, Supelco Analytical). The coefficient of variation for the method was 14%.

Scanning electron microscopy

Seawater samples were filtered on 0.22-μm cellulose ester filters, dried and processed for scanning electron microscopy (SEM) as described by Anderson (1951). SEM samples were observed under an ETEC microscope (Autoscan), and fungal structures were photographed.

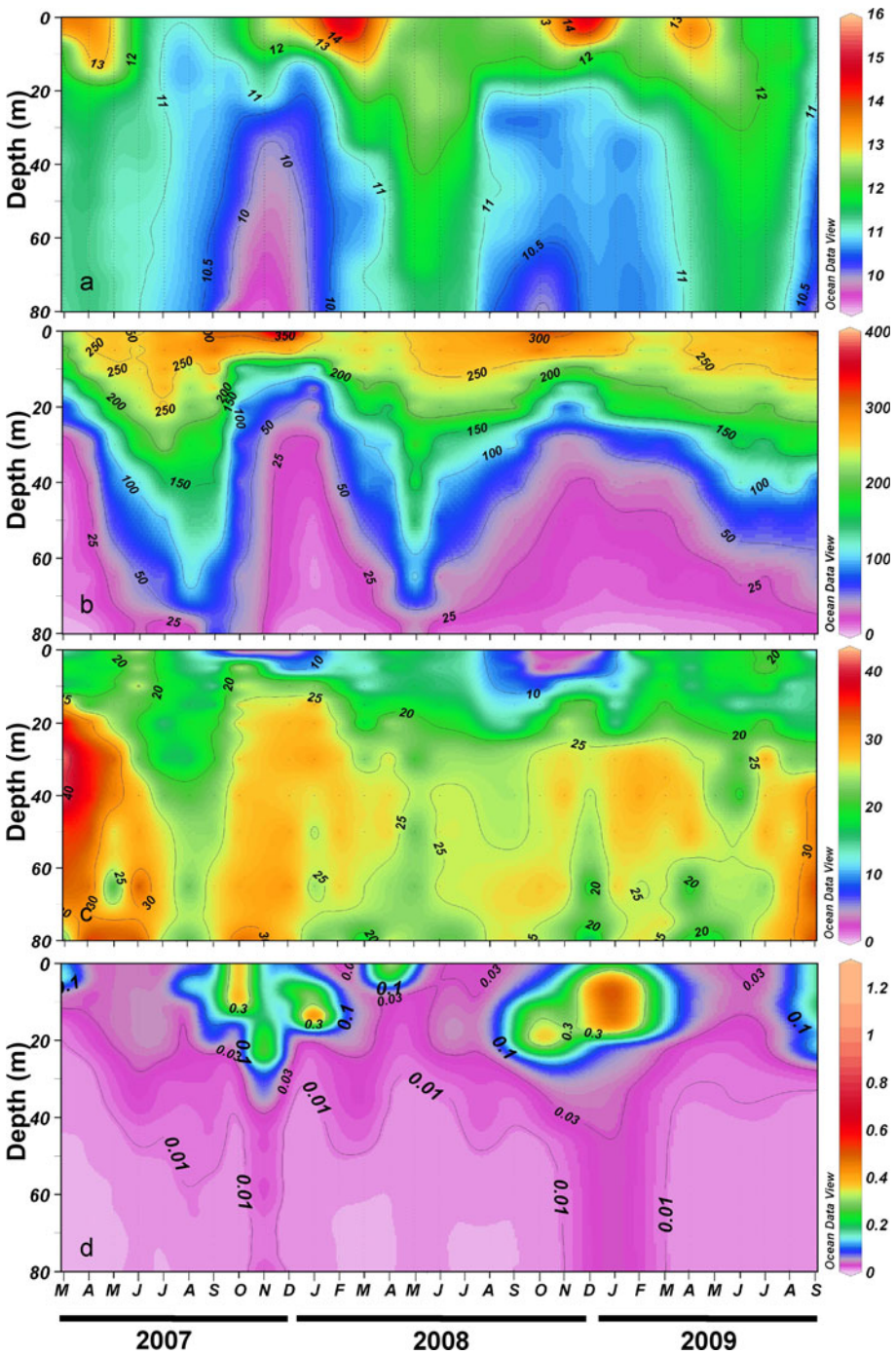

Fig. 2 Temporal variability in hydrographic characteristics of the water column during the study period. **a** Temperature (°C), **b** oxygen (μM), **c** nitrate (μM) and **d** fluorescence

Results

Hydrographic variability in an annual cycle in the water column

During the study period (March 2007–November 2009), the water column was characterized by upwelling events during spring and summer (September to March). The upwelling periods are evidenced by the intrusion of cold waters with low oxygen and high nutrient concentrations toward the surface in Station 18 (Fig. 2). In surface water between 0 and 20 m (Fig. 2a), temperature was influenced by seasonal changes with higher values during spring and summer (12–15°C) than winter (<12°C). During upwelling periods (austral spring and summer), waters colder than 11°C reached about 15 m depth (Fig. 2a).

Seasonal variability in oxygen concentrations was observed. The whole water column showed O_2 concentrations >100 μM during autumn and winter (April to September), whereas during spring and summer, suboxic

waters (<22 μM) upwelled to the surface reaching ca. 20 m depth, thus restricting the oxygenated conditions to the top 10 m (Fig. 2b).

A strong gradient in nitrate concentration associated with upwelling events was observed during spring and summer, with waters with ~25 μM nitrate reaching 10 m depth and values of ca. 5 μM NO_3^- in the top 5 m. In contrast, during winter (June to September) nutrients were more homogeneously distributed along the whole water column and nitrate concentration in the range 15–25 μM was observed (Fig. 2c).

Chlorophyll-*a* fluorescence intensity (Fig. 2d) showed high values (>0.1) in surface waters associated with periods of active upwelling during spring and summer (September to March), while during winter (April to August) fluorescence intensity fell to values lower than 0.003, denoting the marked seasonality in phytoplanktonic biomass in this ecosystem. Thus, during spring and summer the depth of maximum fluorescence was clearly identified between 5 and 20 m depth (Fig. 2d), whereas in winter fluorescence was homogeneous in the water column with a slight increment in the top 10 m.

Methodological approach and direct fungal detection in seawater

Typical hyphal structures and spores were clearly identified in fungal culture tissue added to filtered seawater and stained with Calcofluor White demonstrating the effectiveness of our method in identifying filamentous fungi in the seawater matrix. Fungal structures were identified by epifluorescence microscopy (Fig. 3a–e) and scanning electron microscopy in seawater samples (Fig. 3g, h). Fungal filaments were easily distinguished from detritus and other filaments from non-fungal sources (Fig. 3f). Septate hyphae were found as single or grouped filaments with spores on the particulate material retained on filters. The size of fungal structures identified ranged between ~10 and >400 μm long and ~1–4 μm wide; however, during productive periods most of the fungal filaments (>80%) were identified in the size fraction ≥90 μm.

Fungal, chlorophyll-*a* and prokaryote variability

Fungal carbon ranged from 0.01 to ca. 40 μg C L^{-1} throughout the observational period of almost 3 years and showed a marked seasonality in waters from the depth of maximum fluorescence (Fig. 4a). Significant differences were observed between fungal biomass during upwelling (summer and spring) and non-upwelling (winter and autumn) periods (Mann–Whitney test, $P < 0.05$). The highest values of fungal carbon (≥4 μg C L^{-1}) were observed during the austral spring and summer (Fig. 4a). Maximum fungal biomass (~40 μg C L^{-1}) was recorded in March 2009 at the depth of maximum fluorescence and was higher than prokaryote carbon (~29 μg C L^{-1}, Fig. 4a). During the austral winter (April to August), fungal biomass fell to values in the range of 0.01–2 μg C L^{-1} at the depth of the maximum fluorescence, 2–4 orders of magnitude lower than prokaryote biomass that ranged between 10 and 23 μg C L^{-1} at this depth during the same period (Fig. 4a).

Phytoplankton biomass ranged between 16 and 1,780 μg carbon L^{-1} (0.3–31 μg chlorophyll-*a* L^{-1}) during the study period in waters from the depth of maximum fluorescence and showed a drastic increment at the beginning of the productive season (around September) up to maximum values during spring and summer (October 2007, October 2008–January 2009 and late September 2009) (Fig. 4a).

Prokaryote abundance ranged between ~10 and 44 μg C L^{-1} (10^8–10^9 cells L^{-1}) in waters from the depth of maximum fluorescence during the study period with significant increments during May 2007, January and March 2008, and March and November 2009. High values of prokaryote carbon were recorded in January and March 2008 and March and November 2009 (~31, 44, 29 and 39 μg C L^{-1}, respectively), coincided with the decline of chlorophyll-*a* and the increment in fungal carbon (Fig. 4a).

The vertical distribution of fungal carbon in the water column during spring 2008 (Fig. 4b) was characterized by the highest values (0.1–11 μg C L^{-1}) in the top 20 m and in the range of 0.04–0.08 μg C L^{-1} below 20 m (Fig. 4b). Fungal, prokaryotes and phytoplankton biomasses were maxima at the surface and decrease with depth in the water column (Fig. 4b–d).

In waters from the depth of maximum fluorescence, the fungal biomarker 18:2ω6 showed a similar pattern to that observed for fungal biomass estimated by microscopy counts (Fig. 5), with a high abundance during the austral spring and summer (September 2007 to March 2008). Moreover, a significant correlation was observed between both the variables (Pearson correlation, $r = 0.7$, $P < 0.05$).

Extracellular enzymatic hydrolysis in the water column

Water column hydrolysis of the proteinaceous substrate (MCA-Leu) at depths less than 20 m ranged from 4 to 229 nM h^{-1} from March 2007 to October 2008. Below 20 m, hydrolytic activity was in the range of 1 and 20 nM h^{-1} (Fig. 6a). Extracellular hydrolysis of substrate MUF-cel was at least one order of magnitude lower than activity on proteinaceous substrates. The highest potential rates of hydrolysis of glucosidic substrate (MUF-cel) were observed in surface and bottom waters (~10 and 20 nM h^{-1}, respectively), whereas at 30 and 40 m they

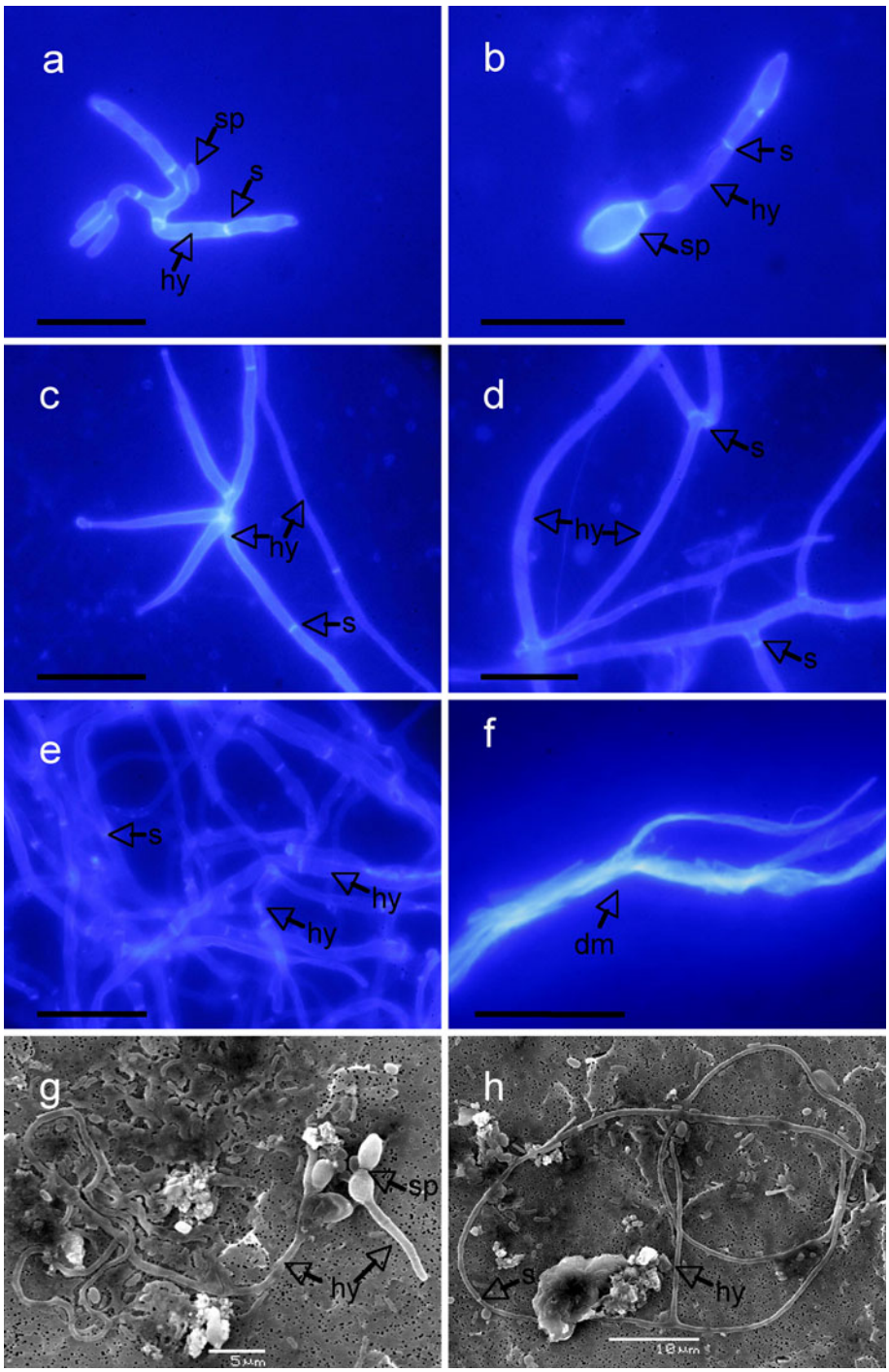

Fig. 3 Fungal structures identified by epifluorescence (**a**, **b**, **c**, **d**, **e**) and scanning electron microscopy (**g**, **h**) in seawater during austral spring 2008 (October) and summer 2009 (early March). Fungal structures were clearly distinguished from detrital material (**f**). *Bars* are 10 µm, *sp* spores, *hy* hyphae, *s* hyphal septa, *dm* detrital material

were minimal or not detected during the study period (Fig. 6b). The highest activities for proteinaceous and glucosidic substrates were observed at the depth of the maximum fluorescence.

Extracellular enzymatic activity on proteinaceous and glucosidic substrates showed a seasonal pattern in waters from the depth of maximum fluorescence, with maximum activities of $\sim$229, 100 and 133 nM h^{-1} for the MCA-Leu substrate during the austral spring and summer (October 2007, January 2009 and November 2009; Fig. 7), respectively. Significant differences were observed between extracellular enzymatic activity on proteinaceous substrate during upwelling and non-upwelling periods (Mann–Whitney test, $P < 0.05$). Secondary weak increments in proteolytic activity (30–40 nM h^{-1}) were observed between April and June of 2007 and April and May 2009 (Fig. 7).

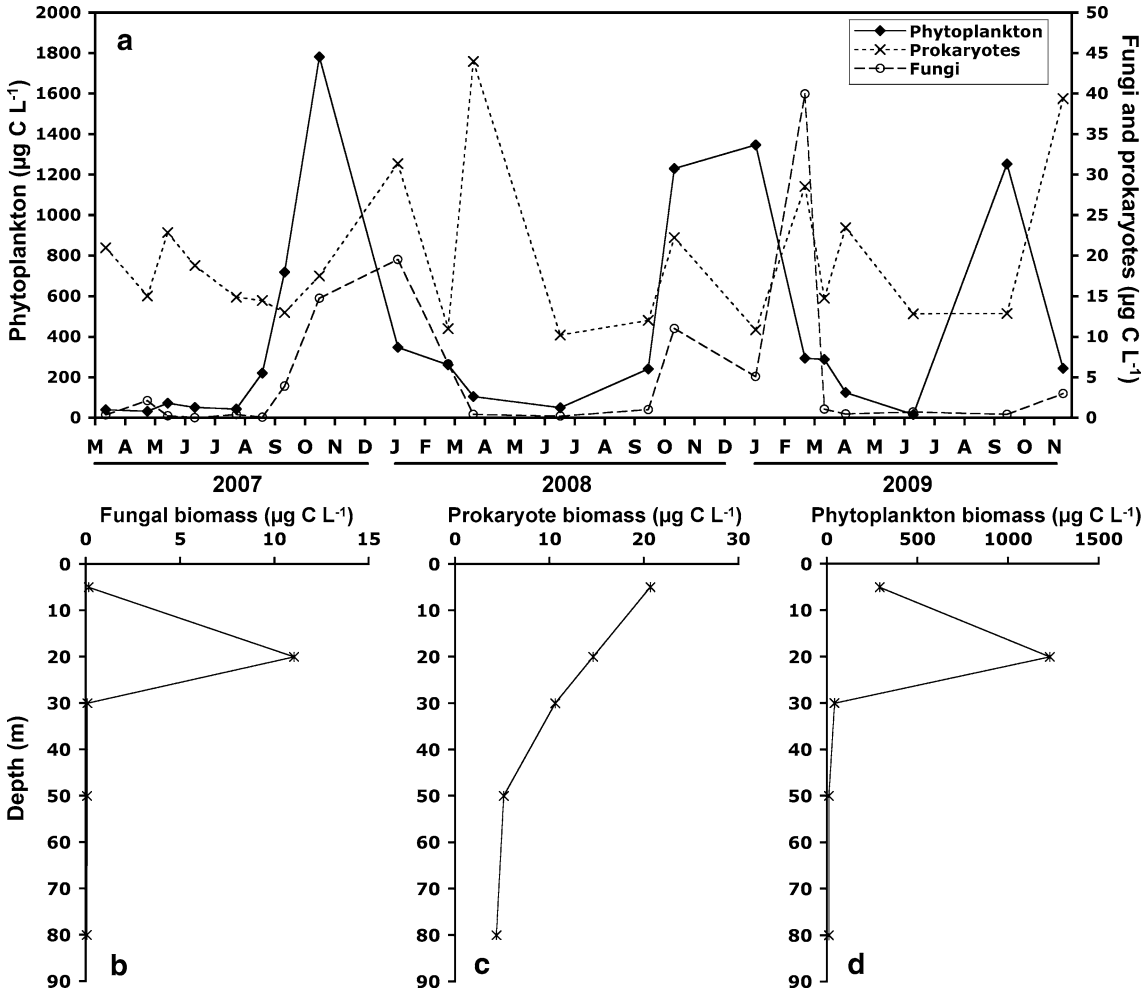

Fig. 4 Temporal variability in phytoplankton, fungal and bacterial carbon in surface waters (**a**), and vertical distribution of fungal (**b**), prokaryotic (**c**) and phytoplankton biomass (**d**) during austral spring 2008 (October 2008) at Station 18. The coefficient of variation of duplicate samples was less than 20% for fungal biomass

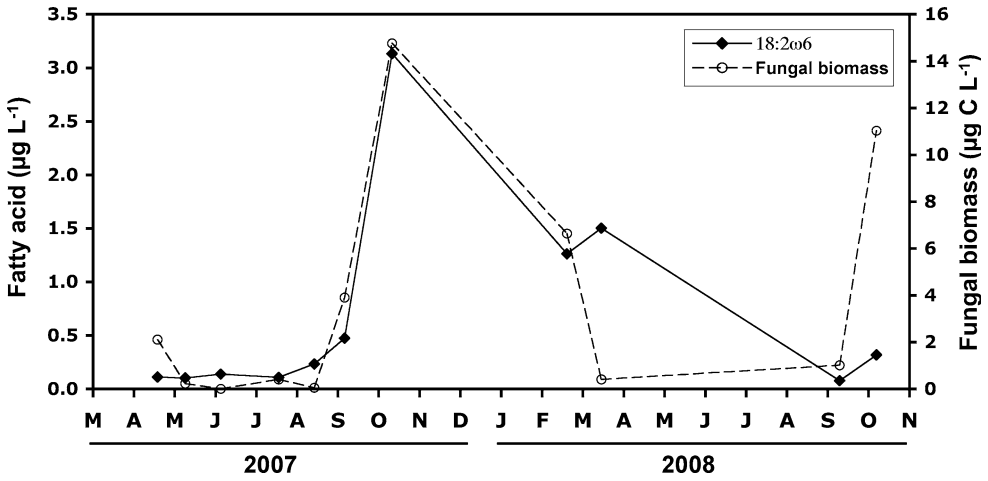

Fig. 5 Concentration of the fatty acid biomarker of fungi (18:2ω6) and fungal biomass in waters from the depth of maximum fluorescence between March 2007 and October 2008. The coefficient of variation of the biomarker method was 14%

Although a similar pattern was observed for both the substrates, hydrolysis of glucosidic substrates (between 0 and 10 nM h^{-1}) were in the lower limit of proteolytic activity, and during austral autumn and winter (March–August 2007 and March–September 2008) were minimal or not detected in seawater (Fig. 7). Maximum potential

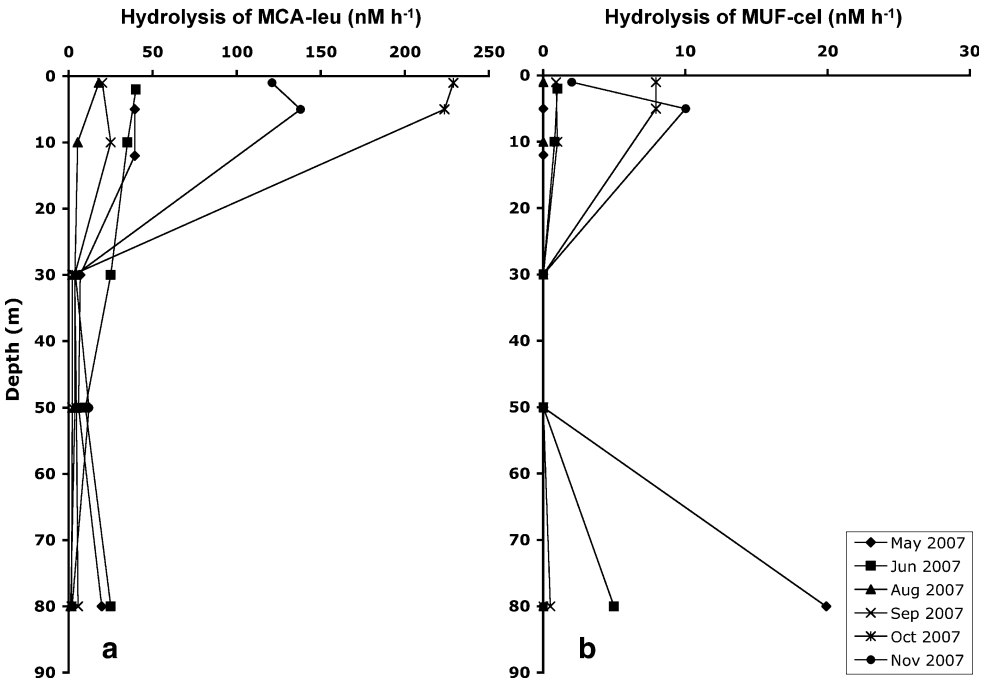

Fig. 6 Vertical profiles of extracellular enzyme activity on proteinaceous (**a**) and glucosidic substrates (**b**) from June to November 2007 at Station 18. The coefficient of variation of duplicate experiments of the same water sample was less than 17%

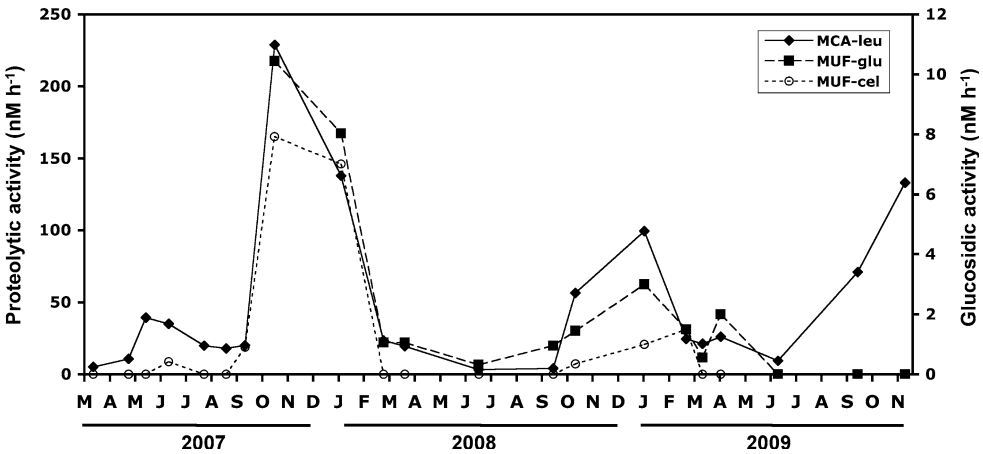

Fig. 7 Temporal variability of extracellular enzyme hydrolysis of proteinaceous (MCA-Leu) and glucosidic substrates (MUF-glu and MUF-cel) in waters from the depth of maximum fluorescence during 2007, 2008, 2009 at Station 18. The coefficient of variation of duplicate experiments of the same water sample was less than 17%

activity of extracellular hydrolysis in waters from the depth of maximum fluorescence during austral spring and summer (September–December 2007, October 2008–January 2009, and November 2009) coincided with both the highest phytoplankton biomass and the onset of the increment in fungal biomass (Fig. 4a).

Size-fractioned extracellular enzyme activity

Four size-fractionated hydrolysis experiments (Fig. 8a) were carried out in 2009 (March, April, June, and November). Hydrolysis of proteinaceous substrate ranged from 10 nM h^{-1} in June to 133 nM h^{-1} in November (Fig. 7), while hydrolysis of glucosidic substrates (1.5–2 nM h^{-1}; Fig. 7) was only detected during March and April and in consequence was not considered in the analysis of size-fractionated hydrolysis.

Most of hydrolysis of proteinaceous substrate was verified in sizes higher than 25 μm during periods of higher phytoplankton and fungal biomasses (over 90 and ca 70% in March and November 2009, respectively) (Fig. 8a, b). In contrast, during autumn and winter, when phytoplankton and fungal biomasses decline (Fig. 8b), extracellular enzymatic hydrolysis was mainly distributed in size fraction 0.2–3 μm (∼64 and 40% in April and June 2009, respectively) (Fig. 8a).

Fungi were distributed in all but the 0.2- to 3-μm size class, although they were mainly distributed in the size classes >25 μm, and during March and November 2009 over 90% of fungal biomass was found in size classes 25–90, and >90 μm (Fig. 8c). The increment in fungal biomass in the size classes >25 μm during the end of summer and spring (March and November 2009) coincided with the increment of

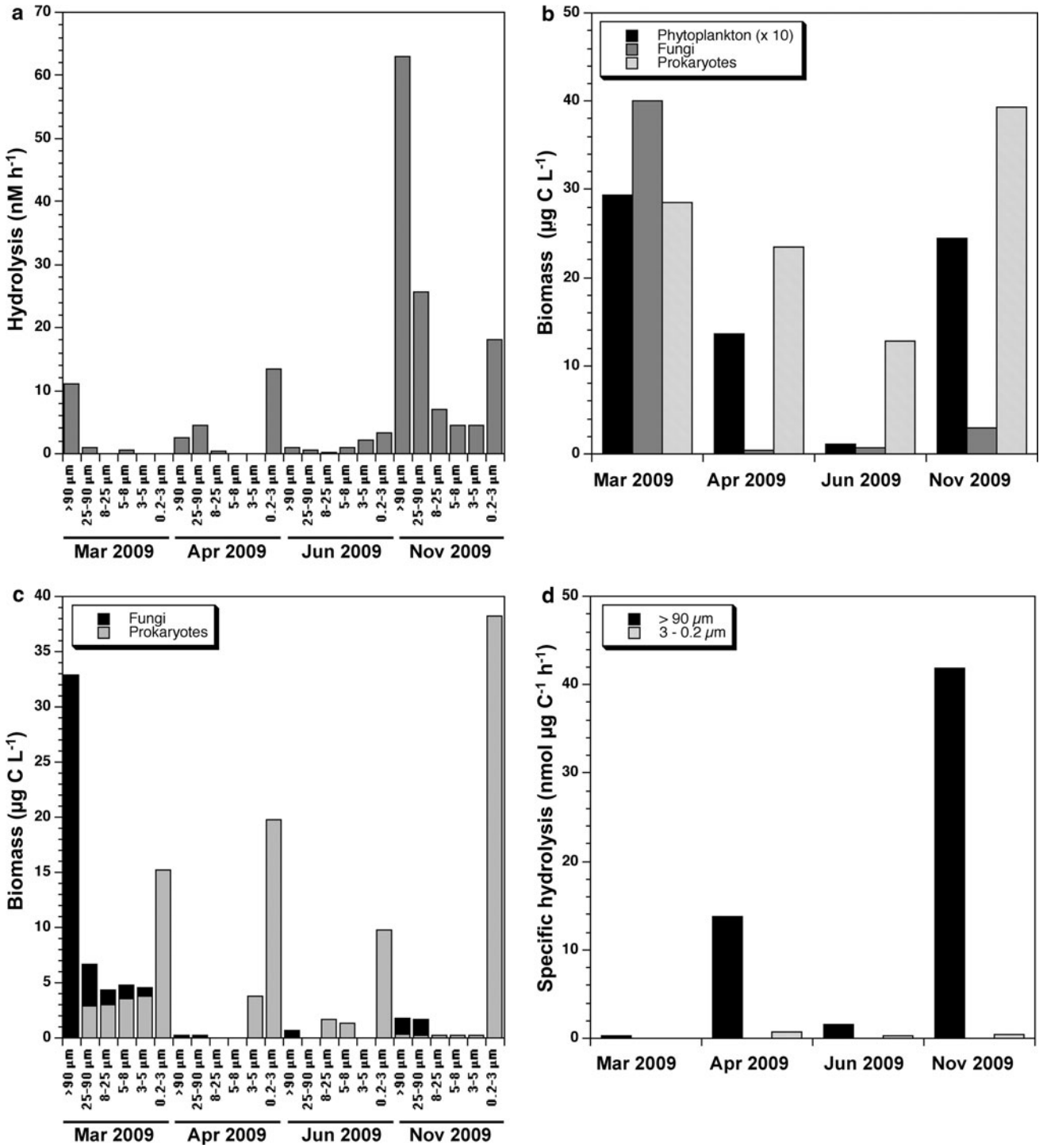

Fig. 8 Water column size-fractioned extracellular enzymatic hydrolysis rates of the proteinaceous substrate MCA-Leu (**a**), total biomass of phytoplankton, fungi and prokaryotes (**b**), size-fractioned biomass of prokaryote and fungi (**c**) and specific activity of fungi and prokaryotes (**d**). Experiments were carried out during low (April, June) and high (March, September) phytoplankton production conditions in waters from the depth of maximum fluorescence at Station 18

extracellular enzymatic hydrolysis in these size fractions (Fig. 8a, c). Prokaryote carbon biomass showed low variability during the experimental period (13–39 µg C L^{-1}, Fig. 8b) and was mainly distributed in the size class 0.2–3 µm (53–97% of prokaryote carbon). During April and June 2009, when fungal carbon was minimal, extracellular hydrolysis was mainly distributed in the prokaryote size class (60 and 40% of hydrolysis, respectively, Fig. 8a, c).

Specific rates of hydrolysis of fungi and prokaryotes were calculated using biomasses in size classes >90 μm (fungi) and 0.2–3 μm (prokaryotes). Specific hydrolysis by fungi (>90 μm) ranged between 0.3 and 42 nmol MCA-Leu (μg fungal carbon)$^{-1}$ h^{-1}, and it was higher during the study period than the specific activity in the prokaryote size fraction (0.2–3 μm), which ranged from 0.3 to 0.7 nmol MCA-Leu (μg prokaryote carbon)$^{-1}$ h^{-1} (Fig. 8d). During November 2009, maximum specific hydrolysis 42 nmol MCA-Leu (μg fungal carbon)$^{-1}$ h^{-1} was estimated to be in the fungi size fraction (Fig. 8d).

Discussion

Extracellular enzymatic activity of the microbial community during an annual cycle

The vertical pattern of leucine aminopeptidase activity (MCA-Leu substrate) shows surface water values up to 100 times higher (>100 nM h^{-1}) than those found below the photic zone (about 10 nM h^{-1}) during the austral spring and summer (October 2007 and January 2008; Fig. 6a). These values are within the range of the only previous report of extracellular hydrolysis for the Humboldt Current System (Pantoja et al. 2009). The pattern of extracellular enzymatic activity observed is a reflection of the enhanced plankton activity in the photic zone (Lalli and Parsons 1996).

We also observed a seasonal pattern in extracellular enzymatic activity in waters from the depth of maximum fluorescence in the upwelling ecosystem off central-south Chile. This pattern was characterized by an enhancement in potential rates of hydrolysis on proteinaceous and glucosidic substrates associated with the increment in phytoplankton biomass under upwelling conditions during spring and summer (September 2007 to February 2008, October 2008 to March 2009 and September to November 2009; Figs. 4a, 7). Thus, the maximum rate of 229 nM h^{-1} for leucine aminopeptidase activity was detected in October 2007, when water column temperature, salinity, oxygen, nitrate and fluorescence indicate the occurrence of upwelling events (Fig. 2). The estimated activity of leucine aminopeptidase was in the range of proteolytic activity reported for other coastal regions (Rosso and Azam 1987; Rath et al. 1993; Pantoja et al. 2009), and the hydrolysis by β-glucosidase and 1,4-β-glucanase was in agreement with previous studies in seawater (Hoppe 1983; Rath et al. 1993; Misic et al. 2006).

The marked seasonality in the coastal upwelling ecosystem off central-south Chile (Strub et al. 1998; Daneri et al. 2000; Figueroa and Moffat 2000; Sobarzo et al. 2007) results in the enhancement of photosynthetic production of organic matter (Daneri et al. 2000; Montero et al. 2007), and presumably an associated release of dissolved organic matter from plankton activity and mortality (Biddanda and Benner 1997; Strom et al. 1997; Biersmith and Benner 1998; Carlson 2002). Thus, our results showing the increment in extracellular enzymatic hydrolysis in surface waters (Fig. 7) during periods of high phytoplankton biomass (Fig. 4a) might be explained by the availability of new organic matter synthesized during active upwelling in the Humboldt Current System. This hypothesis is supported by previous studies of leucine aminopeptidase and β-glucosidase activity in coastal waters of the Baltic Sea that evidenced a seasonal pattern in enzyme activity related to the increment of organic matter during the productive period (Naush et al. 1998). Other evidence is derived from mesocosm studies that have shown an enhancement of extracellular activity during diatom bloom development (Smith et al. 1995; Riemann et al. 2000), as well as studies in trophic gradients where hydrolytic activity increased from oligotrophic to eutrophic conditions in aquatic ecosystems (Karner et al. 1992; Rath et al. 1993; Jorgensen et al. 1999; Kiersztyn et al. 2002).

Fungi in the Chilean upwelling ecosystem

We used an adaptation of the Calcofluor White stain assay (Damare and Raghukumar 2008; Jebaraj and Raghukumar 2009; Rasconi et al. 2009) for the direct detection of fungal hyphae in seawater by epifluorescense microscopy. The microscopic observations permitted us to clearly distinguish septate hyphae and spores from detrital material (Fig. 3). Considering that our estimates of fungal biomass considered only septate hyphae, it is very likely that our values underestimate total fungal biomass since non-septate hyphae and non-filamentous fungi, such as yeasts, are unaccounted for. The abundance of fungal filaments coincided with the temporal distribution of the phospholipid fatty acid 18:2ω6 in seawater (Fig. 5), which has been used as fungal marker in terrestrial environments (Vestal and White 1989; Frostegard and Baath 1996; Olsson 1999; Boschker and Middelburg 2002; Kaur et al. 2005) and has been found as a major constituent (11–37%) of fatty acids of marine fungi (Cooney et al. 1993; Devi et al. 2006; Das et al. 2007). This association is not surprising since phospholipid fatty acids are appropriate biomarkers for viable cells since they occur as membrane lipids and are rapidly hydrolyzed after cell death (White et al. 1979; Balkwill et al. 1988). Although the fatty acid 18:2ω6 is also a constituent in the membrane of some microalgae, it represents a minor fraction in diatoms (Nichols et al. 1993; Zhukova and Aizdaicher 1995), which are the dominant phytoplankters in the Humboldt Current System off Chile (Anabalón et al. 2007; Montero et al. 2007; Vargas et al. 2007).

Our results show a vertical distribution of fungal biomass closely related to that of phytoplankton biomass in

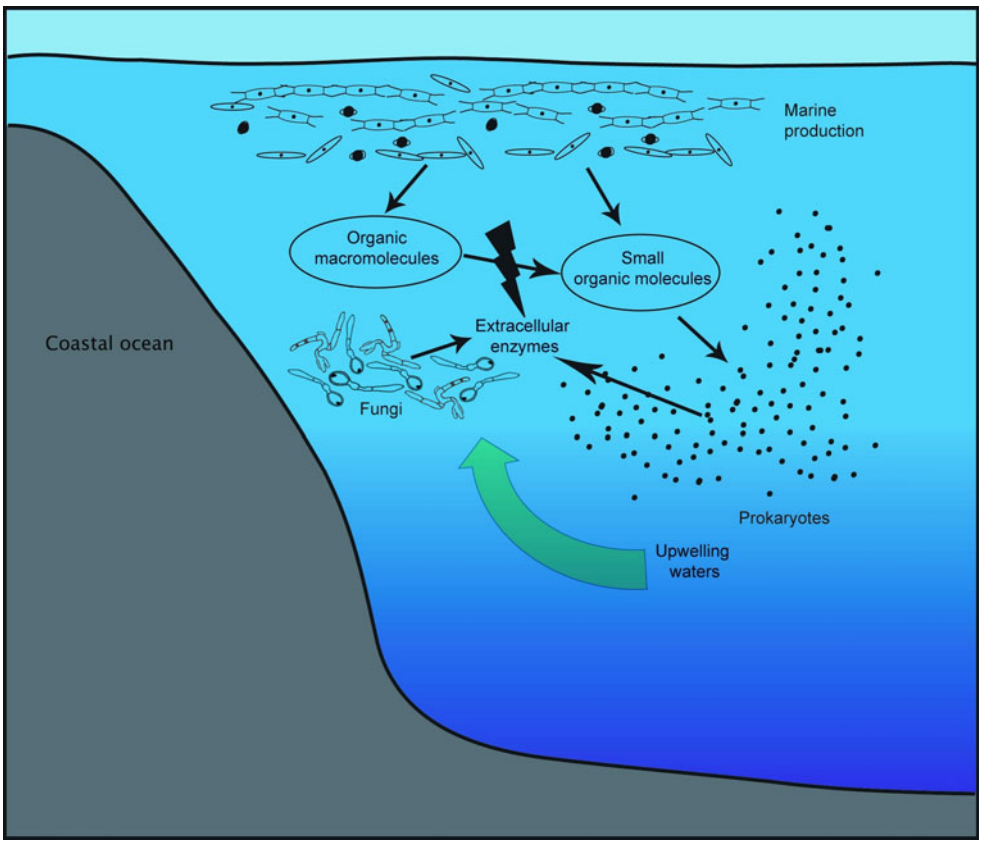

Fig. 9 Proposed conceptual model for degradation of organic matter including fungi-degrading organic polymers in the coastal upwelling ecosystem off central-south Chile

the water column (Fig. 4b, d) and suggest a seasonal pattern in the distribution of fungal biomass associated with the increment in organic matter available during active upwelling and phytoplankton growth in the photic zone (Fig. 4a). Thus, high fungal carbon, up to values that rival those of prokaryotes, were observed during periods of high photoautotrophic biomass and extracellular hydrolytic activity in waters from the depth of maximum fluorescence (Fig. 4a), reaching values ca. two times higher than those of prokaryote biomass at the end of the active upwelling period (March 2009) in the Humboldt Current System. That pattern is also consistent with observations in freshwater ecosystems (Gulis et al. 2006; Gessner et al. 2007), where environmental factors and mainly the availability of detritus could determine temporal variability in the composition and abundance of fungal communities.

Extracellular hydrolytic activity in the marine environment: the role of fungi

In the marine ecosystem, extracellular hydrolysis has been mainly attributed to bacterial enzymes (Rosso and Azam 1987; Chróst 1991; Hoppe et al. 2002), i.e. in the size class 0.2–3 μm (Hoppe 1983; Rosso and Azam 1987). However, correlations between extracellular enzymatic activity and microbial parameters such as bacterial secondary production and prokaryote cell abundance are frequently variable or weak (Arnosti 2003). Moreover, there are reports of significant extracellular enzymatic hydrolysis in the size fraction where bacterial abundance and activity are low (>3 μm, Hoppe 1983; Unanue et al. 1993; Crumb et al. 1998, and >10 μm Chróst and Riemann 1994), suggesting that prokaryotes are not the only microorganisms able to process organic polymers in aquatic ecosystems. Since most of the filamentous fungi were distributed in the largest size classes (i.e. >25 μm), size-fractionated hydrolysis experiments are able to separate the enzymatic activity associated with fungi from that of prokaryotes, which are distributed mainly within the size fraction 3–0.2 μm (Azam and Hodson 1977). Contrary to the results of Hoppe (1983) and Rosso and Azam (1987), who found minimal extracellular enzymatic activity in the fraction >3 μm, our results showed the highest potential rates of hydrolysis on proteinaceous substrates at the end of summer and spring (early March and November 2009) occurring in the sizes fractions >25 μm, which were dominated by fungal structures, while prokaryote cells were at the detection limit or not detected in those size classes (Fig. 8). After summer, when productivity in this ecosystem becomes low (Daneri et al. 2000; Montero et al. 2007),

hydrolysis in the size fraction dominated by prokaryotes (3–0.2 μm) increased over 60% in mid-April and ~40% in June 2009, in contrast to hydrolysis in the size fractions associated with fungi (>25 μm) that were responsible for 40% (April) and 19% (June) of the activity (Fig. 8). Our results agree with those found by Riemann et al. (2000), who observed an increment in extracellular enzymatic activity in the size fraction >1 μm during diatom bloom in a mesoscosm, although they attributed the activity to attached bacteria and their supposed higher specific activity, since fungi are not stained with DAPI.

Here, we provide an alternative explanation by demonstrating that fungi play a significant role in enzymatic hydrolysis of proteinaceous substrates during productive periods (Fig. 8). An upper limit of fungal macromolecular breakdown can be estimated and compared with photosynthetic production of carbon. Depth-integrated (using the trapezoidal method) hydrolysis potential rates of MCA-Leu for the water column profiles of October 2007 and January 2008 (Fig. 6a) during the productive season were converted to carbon units using the factor 72 μg C (μmol MCA-Leu)-1 (Hoppe et al. 1988), resulting in integrated rates of hydrolysis of 5 and 7 g C m^{-2} d^{-1}, respectively. Since fungi were responsible for up to 90% of extracellular hydrolysis during the high productive season (Fig. 8a), and considering the historical highest primary production rates reported for the area (18 and 25 g C m^{-2} d^{-1}, January 2005 and December 2003, Montero et al. 2007), then about 30% of photosynthetic carbon could be processed by fungi in this coastal upwelling ecosystem.

These results support the hypothesis that fungi are an important component of the marine microbial community and co-participate with bacteria and archaea in the breakdown of organic matter during productive periods in surface waters of the coastal upwelling ecosystem off central-south Chile. Our results highlighted a further hypothesis to be tested in the future: prokaryotes are heterotrophs that process organic matter throughout the year in this coastal ecosystem, whereas fungi, as in terrestrial environments (Carlile et al. 2001; Lindahl et al. 2010), use a more opportunistic nutritional strategy by growing during productive periods when substrates are highly available.

Based on our results and considering the increasing number of reports on the occurrence of fungi in different marine environments, we suggest that the current model of the microbial loop in waters of upwelling ecosystems underestimates the role of fungi during the processing of organic matter. Consequently, we propose a more complete model for the degradation of organic matter in this coastal upwelling ecosystem (Fig. 9), in which fungi participate in the processing of biopolymers in the Humboldt Current System, particularly in the productive periods.

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