

# Assessing the micro-phytoplankton response to nitrate in Comau Fjord (42°S) in Patagonia (Chile), using a microcosms approach

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Received: 6 February 2012 / Accepted: 27 September 2012 / Published online: 11 October 2012  
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**Abstract** Anthropogenic (aquaculture) changes in environment nutrient concentrations may affect phytoplankton (biomass and taxa composition) in marine coastal waters off the Chilean Patagonia. The effects of adding nitrate ( $\text{NO}_3^-$ ) to natural phytoplankton assemblages were evaluated considering biomass, cell abundance, and taxonomic composition. Microcosm experiments were performed in the spring, summer, and winter in the Comau Fjord located in Subantarctic Patagonia. At the end of the experiments,  $\text{NO}_3^-$  decreased rapidly and was undetectable in treatments, indicating a strong  $\text{NO}_3^-$  deficiency associated with an exponential increase in Chl-*a* concentrations, particulate organic nitrogen, and carbon in these

treatments. Moreover, given the depleted nitrate concentrations of the spring and summer experiments, the micro-phytoplankton taxa structure shifted from mixed diatom and dinoflagellate assemblages (*Ceratium* spp., *Dinophysis* spp., *Coscinodiscus* sp., *Rhizosolenia pungens*) to assemblages dominated by blooms of the classic chain-forming diatoms found in temperate and cold waters such as *Chaetoceros* spp., *Skeletonema* spp., and *Thalassiosira* spp. Thus, nitrogen sources (i.e., nitrate, ammonia) may influence phytoplankton abundance and biomass accumulation dynamics in the northern section of Patagonia. It also emphasizes the importance of diatom taxa in regards to the short-term response of phytoplankton to changing

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environmental nutrient conditions due to natural (decreasing freshwater stream flow) and anthropogenic (aquaculture) events. This situation may be one of the future scenarios in the Patagonian fjords, thus stressing the needs for active environmental monitoring and impact assessment.

**Keywords** Diatoms · Nitrate · Chlorophyll-a · Phytoplankton structure · Patagonian fjords

## Introduction

There are many processes that drive the succession of phytoplankton assemblages, mainly phytoplankton blooms, and their interactions are complex. One of such processes is the competition between phytoplankton species for “limiting” nutrients (Tilman 1982; Tilman et al. 1982). In freshwater, estuarine, and coastal marine environments, recognized growth-limiting nutrients are nitrogen (N), phosphorus (P), and silicic acid (Si). The concentrations and ratios (N/P, Si/N, Si/P) of nutrient supplies and the resulting environment concentrations have often been used to determine which nutrient is “limiting” primary production or diatom biomass and growth. Results from competition and nutrient uptake laboratory experiments have revealed taxonomic trends regarding diatoms, dinoflagellates, and green algae. In general, diatoms are superior competitors for P and Si; green algae are intermediate for P, and dinoflagellates are better at using more reduced nitrogen sources (ammonia and organic nitrogen) (Glibert and Terlizzi 1999; Lomas and Glibert 1999; Iriarte et al. 2005). As a consequence, diatoms are favored when Si/P and Si/N are high, whereas dinoflagellates and green algae are favored when Si and N/P are low (Margalef 1978; Sommer 1994). Although these experimental results have been extrapolated to explain natural trends, there is still debate on this subject given the interactions of multiple nutrient requirements (i.e., Fe+Si, Fe+N) (Leynaert et al. 2001; Sedwick et al. 2002; Shipe et al. 2007).

The increase or decrease in nutrient loading due to anthropogenic causes has been associated with changes in environmental nutrient concentration, and these changes, in turn, have been used to explain shifts in the phytoplankton assemblage composition (Piehler et al. 2004). In some coastal waters, a gradual decrease in molar Si/N and Si/P ratios has been accompanied by an increase in the ratio of flagellates to diatoms (Turner and Rabalais 1994).

Continuous anthropogenic enrichment of rivers by organic and inorganic N or P compounds could also lead to low Si/N ratios (Bronk et al. 1994; Turner et al. 2003), thereby modulating primary productivity and phytoplankton biomass accumulation (Piehler et al. 2004). Furthermore, nitrate accumulation displays a negative non-linear correlation with particulate organic carbon in aquatic ecosystems (Taylor and Townsend 2010).

Unlike results for marine systems in the Northern Hemisphere, seasonal N and P limitations may occur in Patagonian fjords when surface Si(OH)<sub>4</sub>-rich freshwater, which is generally low in N and P, does not mix with oceanic deep water (with relatively greater N and P concentrations) (Iriarte et al. 2007; González et al. 2010; Torres et al. 2011). The Inner Sea of Chiloé (41–43°S) is characterized by the occurrence of harmful algal blooms (HABs) mainly from micro-phytoplankton taxa such as dinoflagellates (*Dinophysis acuminata*, *Alexandrium catenella*) and diatoms (*Pseudo-nitzschia* spp., *Leptocylindrus* spp., *Chaetoceros convolutus*, *Rhizosolenia setigera*) (Clement 1994; Seguel et al. 2005; Iriarte and González 2008). These HABs cover a wide region of the Patagonian marine ecosystem and may have ecological implications, influencing the biomass/nutrient ratio in the water column. Frequent blooms of some micro-phytoplankton species have negatively affected salmon fish farms, most of which are located in embayments and fjords along in the Inner Sea of Chiloé. Specifically, since 1993, routine records of micro-phytoplankton samples (Monitoring Programme from Instituto Tecnológico del Salmon) have revealed the frequent blooms of harmful diatoms such as *Leptocylindrus danicus*, *Leptocylindrus minimus*, and *C. convolutus* to be associated with low-oxygen waters and fish mortalities in salmon cages.

Increased loads of nitrogen from anthropogenic activities in southern Chile (e.g., salmon cage farming; Soto and Norambuena 2004) may potentially increase the N/Si ratio off areas near the coast (fjords, embayments), favoring the growth of non-silicified phytoplankton taxa at the expense of diatoms. Furthermore, inorganic nitrogen is thought to limit primary production rates in southern Chile (1–2 gCm<sup>-2</sup>day<sup>-1</sup>) (Iriarte et al. 2007) as a consequence of a lower inorganic N/P ratio (9:1) compared with the theoretical Redfield ratio of 16:1 as well as the high content of orthophosphate (>0.8 μM) estimated in the subsurface layer of the northern section of the Patagonian marine ecosystem (Silva 2006). The phytoplankton bloom dynamics, including those of HABs, despite their large impact on

aquaculture health and environmental issues, remain an unanswered question and a major research challenge in coastal waters of the Patagonian marine ecosystem. In a near-future scenario, we would expect to observe a higher concentration of nitrogen sources (i.e., nitrate, ammonia, urea) due to human activities in marine (aquaculture) and terrestrial (land change use, agriculture) systems, resulting in the dominance of potentially harmful phytoplankton species in the coastal waters of southern Chile. Here, to understand the dynamics of micro-phytoplankton blooms and their relationship with environmental nutrients in the Patagonian fjords area, classical microcosm experimental-oriented studies are fundamental. The main objective of this study was to determine the short-term (weekly) response of phytoplankton under different nitrate treatments. In order to accomplish this goal, we carried out a set of manipulative seasonal experiments to examine what relationship might exist between nutrient availability as a controlling factor and the observed taxa composition and total biomass of phytoplankton assemblages during the austral spring, summer, and winter. This study focused on two main questions: (1) Does phytoplankton biomass increase after the addition of  $\text{NO}_3^-$ ? (2) Is there any change in the structure of the phytoplankton assemblage thriving particular HAB species after the addition of nutrients? We used in situ incubations to test artificial nutrient enrichment in phytoplankton to mimic natural changes in phytoplankton nutrient uptake dynamics. This approach would give insight to extrapolate natural change induced by global warming and to contribute with information to national and international HAB and aquaculture monitoring programs.

## Materials and methods

### Study region

The southern fjord region of Chile, one of the major fjord regions of the world, is located on the southeastern border of the Pacific Ocean and extends over more than 1,000 km between  $41^\circ$  and  $55^\circ\text{S}$ . The fjords of southern Chile are an area of interaction between the high-nutrient Subantarctic Waters (SAAW) (Silva and Neshyba 1979; Palma and Silva 2004) and freshwater from the fjords (coastal runoff), resulting in a strong vertical and horizontal salinity gradient (Dávila et al. 2002; Acha et al. 2004; Valle-Levinson et al. 2007).

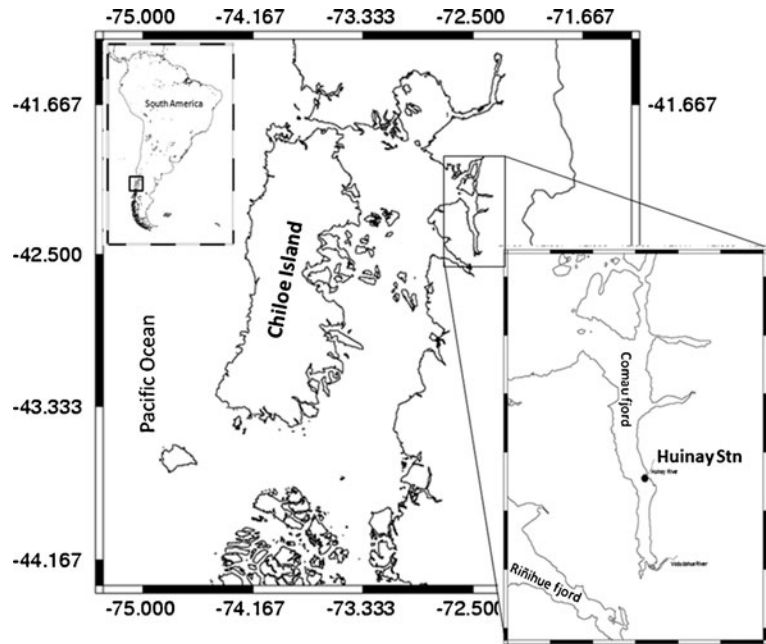
Freshwater input into rivers in this region is modulated by both pluvial and nival seasonal regimes. Oceanographically, the region can be considered a transitional marine system, influenced by deep oceanic waters with high salinity and nutrients and by surface freshwater with low salinity and nutrients (except for silicic acid). The nearly pristine Comau Fjord ( $42.2^\circ\text{S}$ ) is located on the eastern border of the Pacific Ocean, in the northern section of the Chilean Patagonia (Fig. 1). This is one of three north-western-oriented Patagonian fjords leading into the Gulf of Ancud (along with the fjords Reloncaví and Reñihue). These three fjords are not influenced by glaciers and, thus, have significantly lower loads of fine inorganic sediments than most fjords further south (Pickard 1970). The Comau Fjord is more than 30 km long, running mostly south to north, but turning westward at its connection with the Gulf of Ancud through the Comau Channel (Fig. 1). Precipitation originates from passages of the Southern Westerlies and increases strongly towards the south. Annual precipitation averaged 5,800 mm ( $2,000\text{--}6,000\text{ mm year}^{-1}$ ) between 2005 and 2008 and was highly seasonal, with a wet austral winter (June–July) and a dry summer (December to March) (Dávila et al. 2002).

### Experimental design

We carried out three experiments to assess the relationship between  $\text{NO}_3^-$  and  $\text{Si}(\text{OH})_4$  concentrations and short-term shifts in the structure of the phytoplankton assemblage. Seawater was collected from a depth of 5 m (40 % PAR irradiance) in Bahía Ilque, an oceanic-influenced embayment 200 km from Comau Fjord, in 50-L Nalgene polyethylene carboys using a Teflon diaphragm pump. We selected water from a depth of 5 m given the generally low nutrient concentrations in the austral spring, summer, and winter (Table 1). Water was filtered (0.7 m MFS glass-fiber filter) to remove native phytoplankton, and carboys were maintained in the dark at  $4^\circ\text{C}$  until performing the experiments (within 48 h).

The experiments were conducted with native phytoplankton assemblages collected from one selected station (Huinay Biological Station) at the Comau Fjord in the austral spring (October–November 2008), austral summer (January–February 2009), and austral winter (August 2009). A 50-mL micro-phytoplankton concentrated inoculum (20  $\mu\text{m}$  mesh size net and pre-screened by 200  $\mu\text{m}$  mesh size to avoid large zooplankton), using a Teflon diaphragm pump and collected at a depth of

**Fig. 1** Map of the sampling location for phytoplankton experiments in the Comau Fjord, Huinay Station (42.39°S, 72.44°W) performed in the austral spring (October–November, 2008), austral summer (January–February, 2009), and austral winter (August, 2009) seasons. Freshwater sampling sites from Huinay and Vodudahue rivers for reference are indicated by filled circles



**Table 1** Dissolved inorganic nutrient concentration in the subsurface water of Ilque Bay (oceanic water type) used for seawater microcosm incubations throughout the spring, summer, and winter

Season	Treatment	Initial day			Final day		
		NaNO <sub>3</sub>	Nutrients NaH <sub>2</sub> PO <sub>4</sub>	(μM) Na <sub>2</sub> O <sub>3</sub> Si	NaNO <sub>3</sub>	Nutrients NaH <sub>2</sub> PO <sub>4</sub>	(μM) Na <sub>2</sub> O <sub>3</sub> Si
Spring 2008	ALL	37	2	37	2.5	0.4	26.5
	ALL–Si	39	2	18	12.0	0.5	5.4
	CONTROL (ALL–NO <sub>3</sub> )	12	2	32	3.5	1.2	18.4
	ONLY 3×Si	18	2	77	2.8	0.2	53.3
	“Oceanic seawater”	14	2	12			
Summer 2009	ALL	16.4	1.6	34.8	0	0.6	1.9
	ALL –Si	17.9	2.1	8.3	0	1.1	2.7
	CONTROL (ALL–NO <sub>3</sub> )	2.2	1.8	22.4	0	1.6	7.1
	ONLY 3xSi	4.6	1.2	58.6	0	0.6	32.9
	“Oceanic seawater”	5.9	1.6	13.9			
Winter 2009	ALL	22	2.4	40.3	0.7	1.9	13.3
	ALL–Si	22.1	2.7	20.7	0	1.4	15.5
	CONTROL (ALL–NO <sub>3</sub> )	3.1	1.0	30.2	4.3	0	21.3
	ONLY 3×Si	2.2	2.2	32.2	4.3	1.3	20.1
	“Oceanic seawater”	6.0	0.4	24.7			
	Huinay River	0.3–2.1	0–0.5	13.9–17.2			
Vodudahue River	0.7–2.5	0–0.2	12.2–17.2				

Dissolved inorganic nutrient concentrations in enriched seawater on the initial day (day 0) and after 10 days of “on deck” incubation in the Comau Fjord ( $n=2$ , for each treatment and sampling day). Freshwater inorganic dissolved nutrients at Huinay and Vodudahue rivers at the Comau Fjord

10 m (PAR, 90–180  $\mu\text{molm}^{-2}\text{s}^{-1}$ ), was added to each 4-L transparent polycarbonate bottle filled with filtered seawater, to initial cell concentrations of 100–250 cells  $\text{mL}^{-1}$ . Although we note that the microcosms approach (4-L bottle) has its limitations, we used it as a model system for asking our questions on phytoplankton populations and community properties during a short time scale. In addition, microcosm experiments were useful for high replication throughout time (seasons) and gave us statistical power over the observed results. Finally, microcosms gave us the control to manipulate features of the system (i.e., nitrate) that are difficult to control and manipulate in highly heterogeneous natural environments such as fjords ecosystems. Four treatments were prepared for the bioassays (Table 1): ALL, which included the addition of  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{Si}(\text{OH})_4$ ; ALL–Si, which included the addition of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ , but not Si; CONTROL, consisting of untreated seawater which included only the addition of  $\text{PO}_4^{3-}$  (but not  $\text{NO}_3^-$  or  $\text{Si}(\text{OH})_4$ ; hereafter referred to as ALL– $\text{NO}_3^-$  treatment); and ONLY 3 $\times$ Si, which consisted of the addition of 60  $\mu\text{M}$  Si ( $\text{OH})_4$  (the addition of 60  $\mu\text{M}$  Si was thrice the concentration observed in near-surface waters throughout all seasons). Orthophosphate concentration was maintained constant to natural concentrations fluctuating between 1 and 2  $\mu\text{M}$  (Table 1) in order to compare the effects of nitrate and silicic acid on phytoplankton taxa, abundance, and biomass. Three replicates of each treatment were incubated in 4-L transparent polycarbonate bottles and randomly placed in a running-water container under natural light conditions near the pier. Carboys were incubated for 10 to 13 days. During the spring and summer experiments, on days with irradiance above 200  $\mu\text{molm}^{-2}\text{s}^{-1}$ , neutral density screening was used to avoid adding another factor to the outcome (e.g., temperature). The experimental temperatures were 10–11  $^\circ\text{C}$  for spring, 14–16  $^\circ\text{C}$  for summer, and 9–10  $^\circ\text{C}$  for winter seasons, and these values varied (+2  $^\circ\text{C}$ ) compared with environmental temperature conditions. Bottles were inverted eight times a day to avoid sedimentation of phytoplankton cells. In our experimental approach, total autotrophic biomass (Chl-*a*) and taxa abundances (cell counts) were defined as main phytoplankton assemblage descriptors. These

two properties were selected given that the phytoplankton response to exogenous environmental factors such as nutrient supply is expressed in chlorophyll-*a* biomass and the relative contribution (cell numbers) to the total assemblage made by the component taxa in a sample (Tilman et al. 1982; Oviatt et al. 1989).

#### Phytoplankton biomass and micro-phytoplankton abundance

Seawater samples (50 mL each, two replicates) for chlorophyll-*a* (Chl-*a*) were filtered through a 0.7- $\mu\text{m}$  MFS glass-fiber filter and analyzed using a digital Turner P700 fluorometer (Parsons et al. 1984). For phytoplankton cell counts, 50-mL subsamples were stored in clear plastic bottles and then preserved in a 1 % Lugol iodine solution. Another set of 50-mL subsamples was placed in a sedimentation chamber and left to settle for 24 h, and the bottom of the chamber was observed using an inverted microscope (Utermöhl 1958). Phytoplankton taxa were occasionally monitored using a 20- $\mu\text{m}$  mesh-size net (micro-phytoplankton) at three fixed stations from 2007 to 2010 (November 2007–December 2009;  $N=49$  samples). An irregular time series of phytoplankton taxa emphasized the trends observed in this fjord. Samples for phytoplankton were stored in clear plastics bottles and fixed and preserved in a 1 % Lugol iodine solution. Micro-phytoplankton was identified to genus or species level, when possible, and divided into diatoms (Bacillariophyceae: Centric and Pennates) and thecate dinoflagellates (Dinophyceae) (Tomas 1997).

#### Heterotrophic and autotrophic nanoflagellate abundances

Water samples (50 mL) were preserved in plastic tubes with 2.5 % glutaraldehyde (final concentration). A subsample (25–50 mL) was filtered (<50 mm Hg) through blackened Millipore filters (diameter, 13 mm; pore size, 0.2  $\mu\text{m}$ ). Filters were mounted between two drops of immersion oil (Nikon) and viewed under blue light illumination using a Nikon microscope equipped for epifluorescence (100 W Mercury light, reflector 505 nm, excitation 465–495 nm, barrier filter 515–555 nm). A stratified counting design provided counts for different-sized cells (Booth 1988). In order to observe the response of the



bacterial component facing different inorganic nutrient regimes during phytoplankton growth, samples (15 mL) for bacterial abundance were preserved in cold glutaraldehyde (2.5 % final concentration) and kept in the dark until the bacteria were counted using an epifluorescence microscope (Nikon) according to Porter and Feig (1980).

#### Particulate organic carbon and nitrogen

Samples were collected to determine particulate organic carbon (POC) and particulate organic nitrogen (PON) during the austral spring (October–November 2008), austral summer (January–February 2009), and austral winter (August 2009) experiments. These samples (0.2 L) were filtered through pre-combusted MFS (glass fiber filters with 0.7- $\mu\text{m}$  nominal pore size) filters and stored frozen until later analysis. Measurements were conducted in an Europa Hydra 20–20 continuous-flow isotope ratio mass spectrometer following combustion at 1,000 °C at the UC Davis Stable Isotope Facility Laboratory (USA), using acetanilide as a standard (Bodungen et al. 1991).

#### Inorganic nutrients

Analyses of  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{Si}(\text{OH})_4$  were carried out in situ by collecting 50 mL of seawater from depths of 0, 2.5, 5, 10, 15, and 20 m during spring (October–November 2008) and summer (January–February 2009) at Comau Fjord. In addition, 50 mL water samples for all nutrients were collected during the time course incubations of all seasons: austral spring (October–November 2008), austral summer (January–February 2009), and austral winter (August 2009). All samples were stored frozen until analysis (Parsons et al. 1984). Additional freshwater samples were collected in the adjacent Huinay and Vodudahue rivers (Fig. 1) for comparative analyses.

#### Seasonal primary productivity and total phytoplankton chlorophyll *a*

Primary production (PP) estimates at the Comau Fjord in earlier studies were measured using the method described by Steemann-Nielsen (1952). Water samples for PP estimates were collected at depths of 0, 5, 10, 15, and 30 m using 5-L PVC Go-Flo oceanographic bottles. Sampling was conducted in November, 2005

(spring), January, 2006 (summer), September, 2006 (winter), and December, 2006 (spring). Samples were incubated in 125-mL polycarbonate bottles (two clear + one darkened) and placed in an on-deck natural light incubator for ca. 4 h (between 10:00 AM and 14:00 PM). The temperature was regulated (+1 °C) by running surface seawater over the incubation bottles. For the subsurface samples (5, 10, 15, 30 m), light intensity was attenuated using a screen to approximate light to the level found at the depth where water was collected. Light irradiance (photosynthetically active radiation (PAR)) was determined using a submersible Ramses-ACC2-UV-VIS hyperspectral radiometer sensor (Trios Optical Sensors) in the spring and summer experiments. To each bottle, 20 to 40  $\mu\text{Ci}$  sodium bicarbonate ( $\text{NaH}^{14}\text{CO}_3$ ) was added. Samples were manipulated under subdued light conditions during pre- and post-incubation periods. Filters (MFS glass fiber, 0.7  $\mu\text{m}$ ) were placed in 20-mL plastic scintillation vials and kept at  $-15$  °C until reading (15 days later). To remove excess inorganic carbon, filters were treated with HCl fumes for 24 h. Scintillation cocktail (8 mL, Ecolite) was added to the vials, and radioactivity was determined in a Beckmann scintillation counter. Seawater samples (50 mL each, two replicates) for chlorophyll-*a* (Chl-*a*) were filtered through a 0.7- $\mu\text{m}$  MFS glass-fiber filter and analyzed using a digital Turner P700 fluorometer (Parsons et al. 1984).

#### Statistical analyses

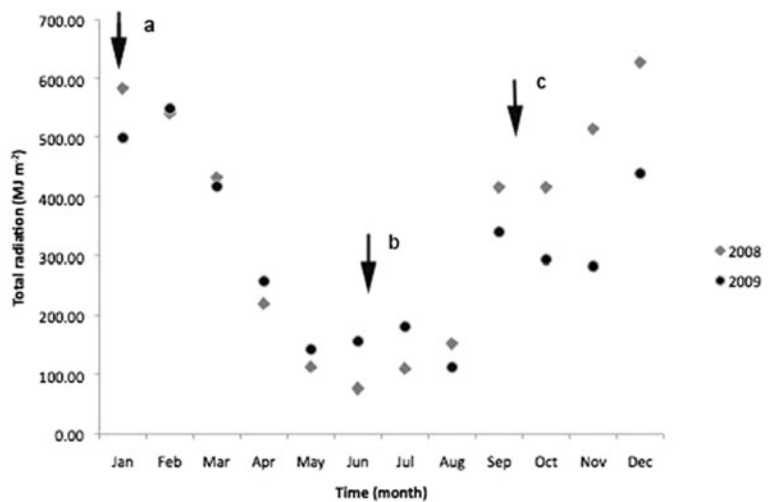
Significant differences among treatments were evaluated using a parametric, balanced, one-way ANOVA (Zar 1984) with Chl-*a* as a dependent variable for each seasonal experiment. An a posteriori Tukey test was used to make multiple comparisons of means of significant factors ( $p < 0.05$ ). The homogeneity of variance for Chl-*a* was verified with a Bartlett test.

## Results

#### Seasonality in nutrients, biomass, and primary productivity

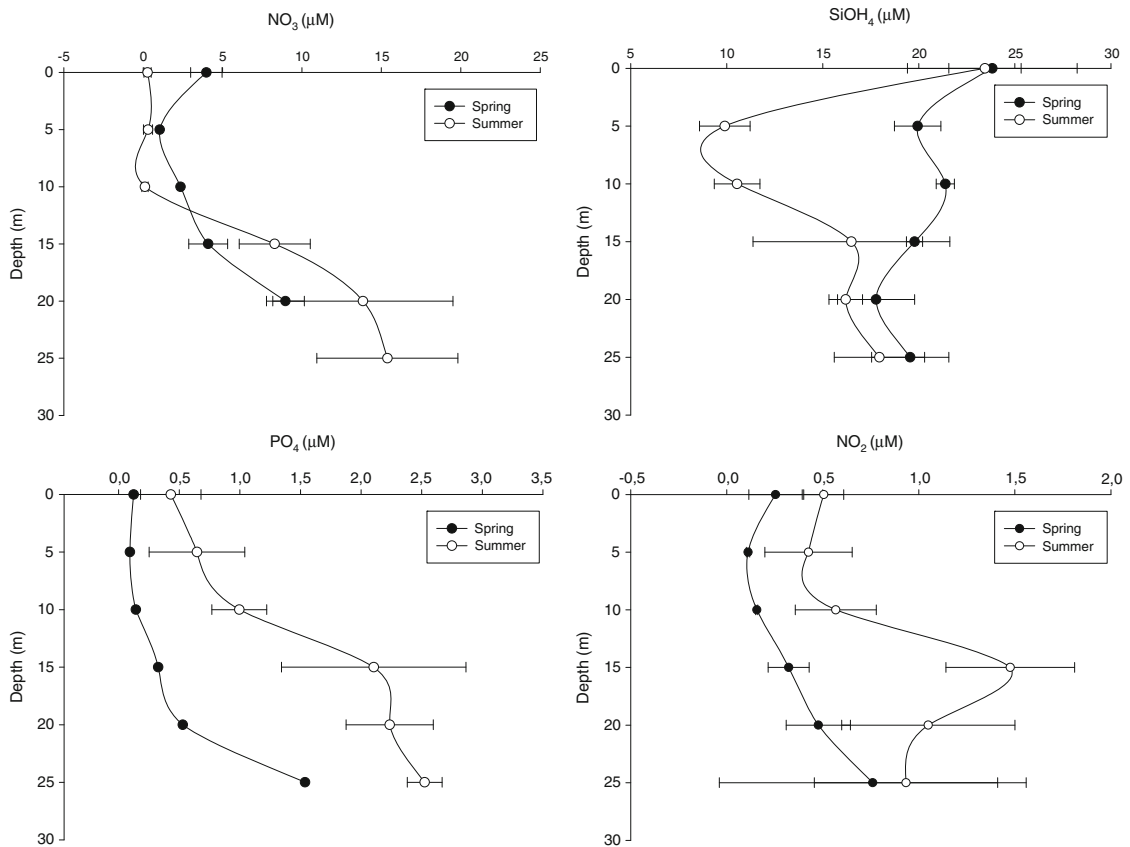
Solar irradiance recorded at the Huinay Station during the study period (2008–2009) showed a marked seasonal variability during summer/spring and winter experiments (Fig. 2). In the Comau Fjord, the top

**Fig. 2** Monthly mean irradiance variation from January, 2008 to December, 2009 at the Huinay meteorological station in the Comau Fjord. Arrows indicated sampling periods of summer (a), winter (b), and spring (c)



10 m of the water column were remarkably poor in  $\text{NO}_3^-$  ( $<5 \mu\text{M}$ ) and  $\text{PO}_4^{3-}$  ( $<1 \mu\text{M}$ ) in the austral spring and summer (October–November, 2008 and January–February, 2009) (Fig. 3). The vertical distribution of silicic acid showed maximum values of

14.5–20  $\mu\text{M}$  in the surface layer (0–5 m) and a sub-surface minimum below the halocline (10–15 m). Near the mouths of the Huinay and Vodudahue rivers, silicic acid concentrations were high, fluctuating between 16 and 55  $\mu\text{M}$ , whereas  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  were low



**Fig. 3** Vertical profiles of inorganic nutrients of the Comau Fjord during the austral spring. Open circles show the vertical distribution of nutrients during the austral summer; closed circles show the vertical distribution of nutrients in the austral spring

(<2.0 and <0.5  $\mu\text{M}$ , respectively). Seasonal Chl-*a* measurements at the Huinay station showed high autotrophic biomass (up to 5.3  $\text{mg Chl-}a\text{m}^{-3}$ ) in the austral summer (January, 2009) and low values (<2.5  $\text{mg Chl-}a\text{m}^{-3}$ ) in the spring (October–November, 2008). Both PP and Chl-*a* have been measured seasonally (spring–summer) in the Comau Fjord since 2005, revealing a subsurface maximum of photosynthetic rates and Chl-*a* biomass estimates below the halocline (Fig. 4).

#### Phytoplankton biomass and nutrients in experiments

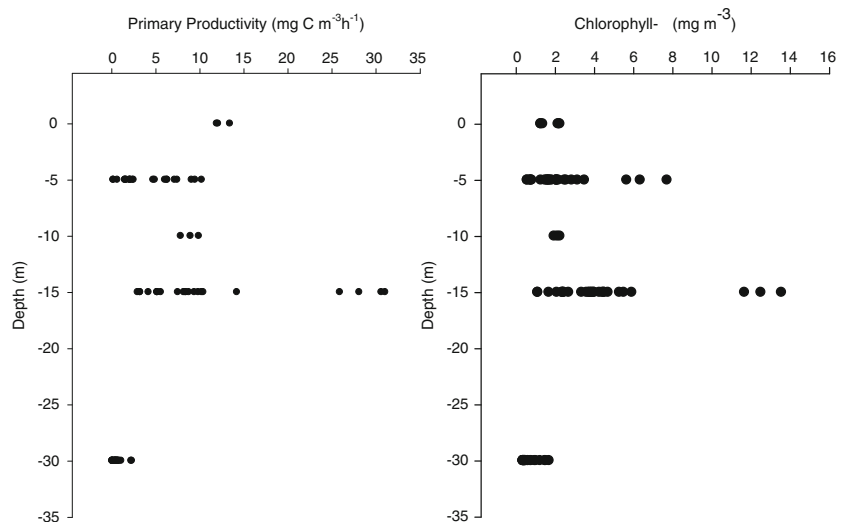
During the three experiments performed in the spring, summer, and winter, the initial phytoplankton Chl-*a* was low (<0.5  $\text{mg Chl-}a\text{m}^{-3}$ ) and remained low until the middle of the experiments (day 5 or 6) in all treatments (Fig. 5, upper panel). A significant effect of the nutrient addition was only observed on day 9 of the incubation in two treatments (ALL and ALL–Si). Phytoplankton Chl-*a* reached higher levels later, during the last 4 days of incubation. The Chl-*a* concentration increased in treatments ALL ( $\text{NO}_3^- + \text{PO}_4^{3-} + \text{Si(OH)}_4$ ) and ALL–Si compared with the CONTROL (ANOVA<sub>spring</sub>,  $F=3.5$ ,  $df=4$ ; Tukey's test,  $p=0.00007$ ; ANOVA<sub>summer</sub>,  $F=9.3$ ,  $df=7$ ; Tukey's test,  $p=0.000003$ ; ANOVA<sub>winter</sub>,  $F=7.0$ ,  $df=7$ ; Tukey's test,  $p=0.01$ ). During days 10 to 13 of the experiments, the highest Chl-*a* in ALL and ALL–Si was best related to nitrate depletion, with the highest mean phytoplankton biomass occurring in the spring (34  $\text{mg}$

$\text{Chl-}a\text{m}^{-3}$ ) and summer (20  $\text{mg Chl-}a\text{m}^{-3}$ ). Average Chl-*a* in the ALL and ALL–Si treatments of the winter experiment were four to five times higher than the mean values of the spring and summer experiments (100  $\text{mg Chl-}a\text{m}^{-3}$ ). At the end of the experiments,  $\text{NO}_3^-$  decreased rapidly and was undetectable in ALL and ALL–Si, indicating a strong  $\text{NO}_3^-$  deficiency associated with an exponential increase in Chl-*a* concentrations in these treatments. In the spring experiment, final  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$ , and  $\text{Si(OH)}_4$  concentrations were <4, 0.2–1.9, and 1.9–53.3  $\mu\text{M}$  in almost all treatments. In all four treatments of the summer experiments,  $\text{NO}_3^-$  was depleted on day 10, and in the treatments, ALL and ALL–Si,  $\text{SiO}_4$  levels fell below 3  $\mu\text{M}$ .

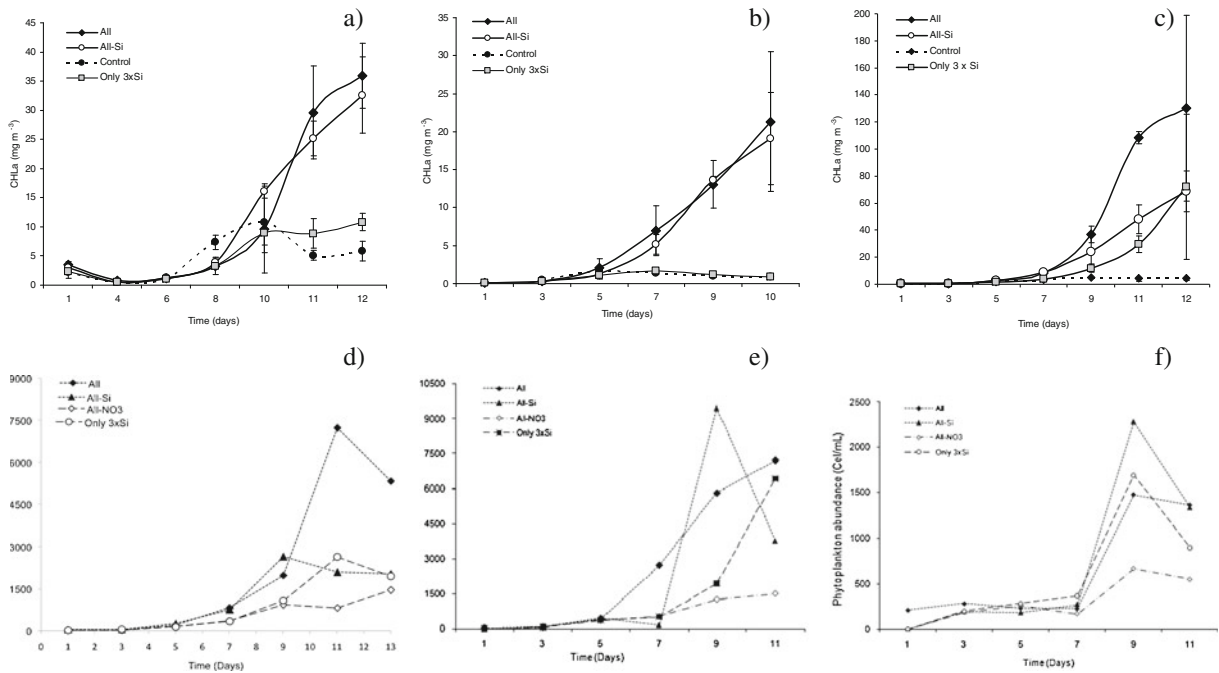
#### Micro-phytoplankton and abundances in experiments

At the end of all experiments, micro-phytoplankton abundance reflected the observed differences in phytoplankton Chl-*a* biomass for the treatments (Fig. 5, lower panel). The initial micro-phytoplankton abundance was low (inoculum, 250  $\text{cells mL}^{-1}$ ) and consisted mainly of mixed diatom and dinoflagellate assemblages in both spring and summer, with *Rhizosolenia pungens*, *Ceratium lineatum*, *Ceratium fusus* and *Coscinodiscus* sp., *Thalassionema nitzschioides*, *Dinophysis* spp., *Chaetoceros* spp. as the dominant taxa, respectively (Fig. 6). On the contrary, throughout the winter experiment, the assemblage was dominated by diatom taxa such as *Detonula pumila* and *Skeletonema* spp. (Fig. 6). In all

**Fig. 4** Vertical distribution of primary production ( $\text{mg C m}^{-3} \text{h}^{-1}$ ) and total chlorophyll-*a* ( $\text{mg m}^{-3}$ ) through the photic zone at the Huinay station in November, 2005 and 2006, January 2006, and September 2006







**Fig. 5** Short-time course of chlorophyll-a concentration ( $\text{mg m}^{-3}$ , upper panel) and micro-phytoplankton abundance (cells per liter, lower panel) in experiments performed in the spring (a, d), summer (b, e), and winter (c, f) seasons in the Comau fjord. Seawater “microcosms” (4-L bottles), including all microbial community (bacteria, nanoflagellates, micro-phytoplankton), were incubated under four different treatments: seawater

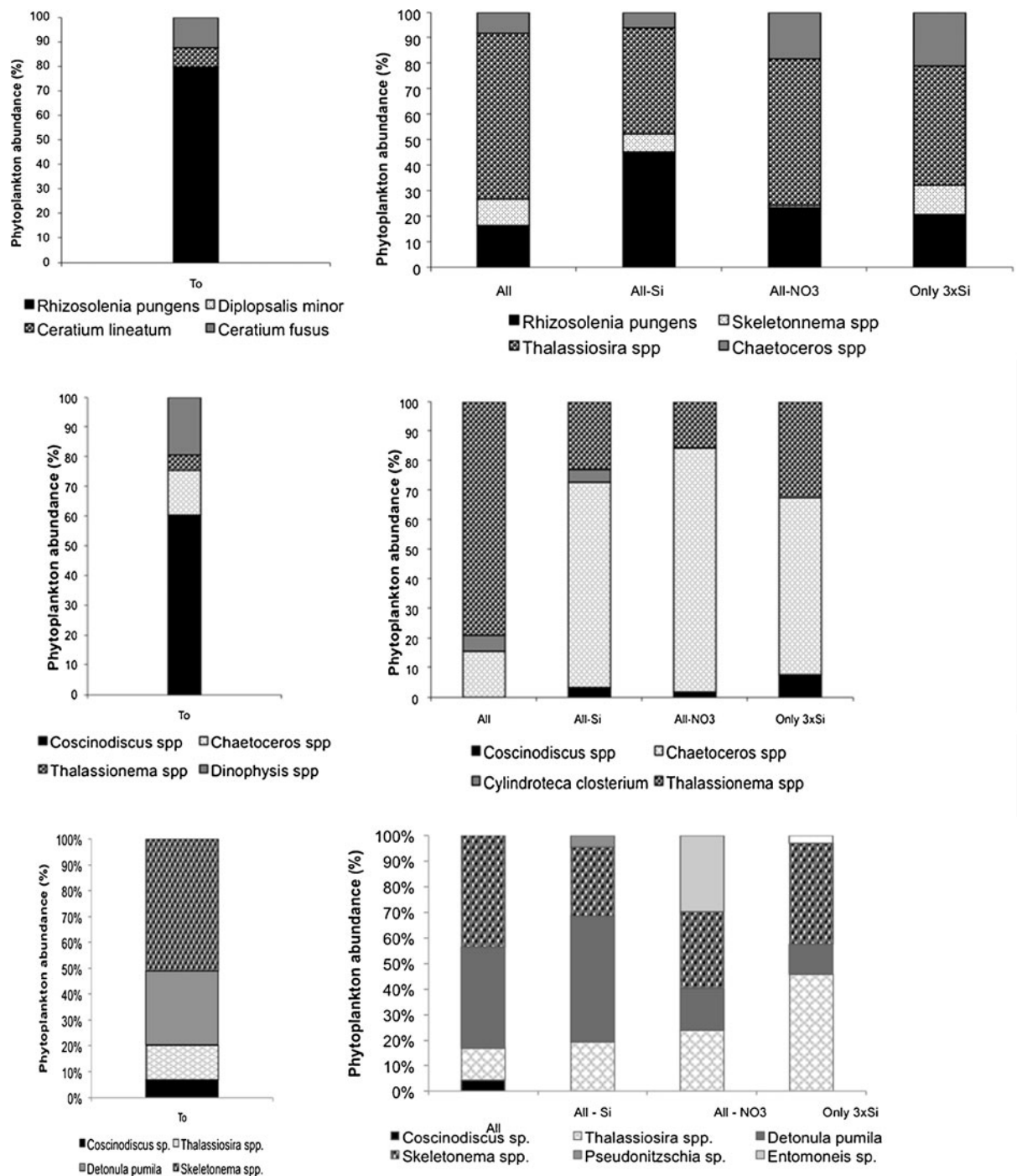
enriched with nitrate, orthophosphate and silicic acid (ALL), seawater enriched only with nitrate and orthophosphate (ALL–Si), seawater without nitrate addition (CONTROL=ALL–NO<sub>3</sub>), and seawater enriched with three times silicic acid (ONLY 3×Si). Values are the means±1SD for samples from duplicate bottles maintained until day 10

treatments of the three experiments, diatom cell abundance responded slowly to the addition of nutrients and became the dominant phytoplankton group. After 10 days of the experiment, centric and pennate diatoms were the most abundant group, making up more than 90 % of the micro-phytoplankton abundance, mainly in the ALL and ALL–Si treatments of the spring (up to 1,500–2,200 cells mL<sup>-1</sup>), summer (up to 6,000–9,500 cells mL<sup>-1</sup>), and winter (up to 7,000 cells mL<sup>-1</sup>) experiments. Specifically, at the end of the spring experiment, the phytoplankton composition changed to *Thalassiosira* spp. and *R. pungens* making up more than 70 % of the assemblage, particularly in the ALL and ALL–Si treatments. The contribution of *Chaetoceros* spp. increased in the ONLY 3×Si treatment. During the summer experiment, the phytoplankton assemblage in the ALL treatment was dominated by *Thalassionema* sp. (80 %), but *Chaetoceros* spp. dominated (60–80 %) the other treatments. Dinoflagellates showed high absolute and relative abundances at the beginning of the

spring (20 cells mL<sup>-1</sup>) and summer (40 cells mL<sup>-1</sup>) experiments, but with all treatments, these abundances decreased during the experiments. During the winter experiment, there was no evidence of change in the main taxa, and the fast-growing *Skeletonema* spp. and *D. pumila* made up more than 70 % of the total cell abundance in the ALL and ALL–Si treatments at the end of the experiment. During this experiment, *Entomoneis* sp. and *Thalassiosira* spp. were the main taxa in the CONTROL (ALL–NO<sub>3</sub><sup>-</sup>) and ONLY 3×Si treatments, respectively.

Nanoflagellate abundances

The abundances of autotrophic (ANF) and heterotrophic (HNF) nanoflagellates were counted in order to study the response of these components to different inorganic nutrient regimes during phytoplankton growth. Minor, but highly variable, differences were observed in the cell abundances of both groups for all

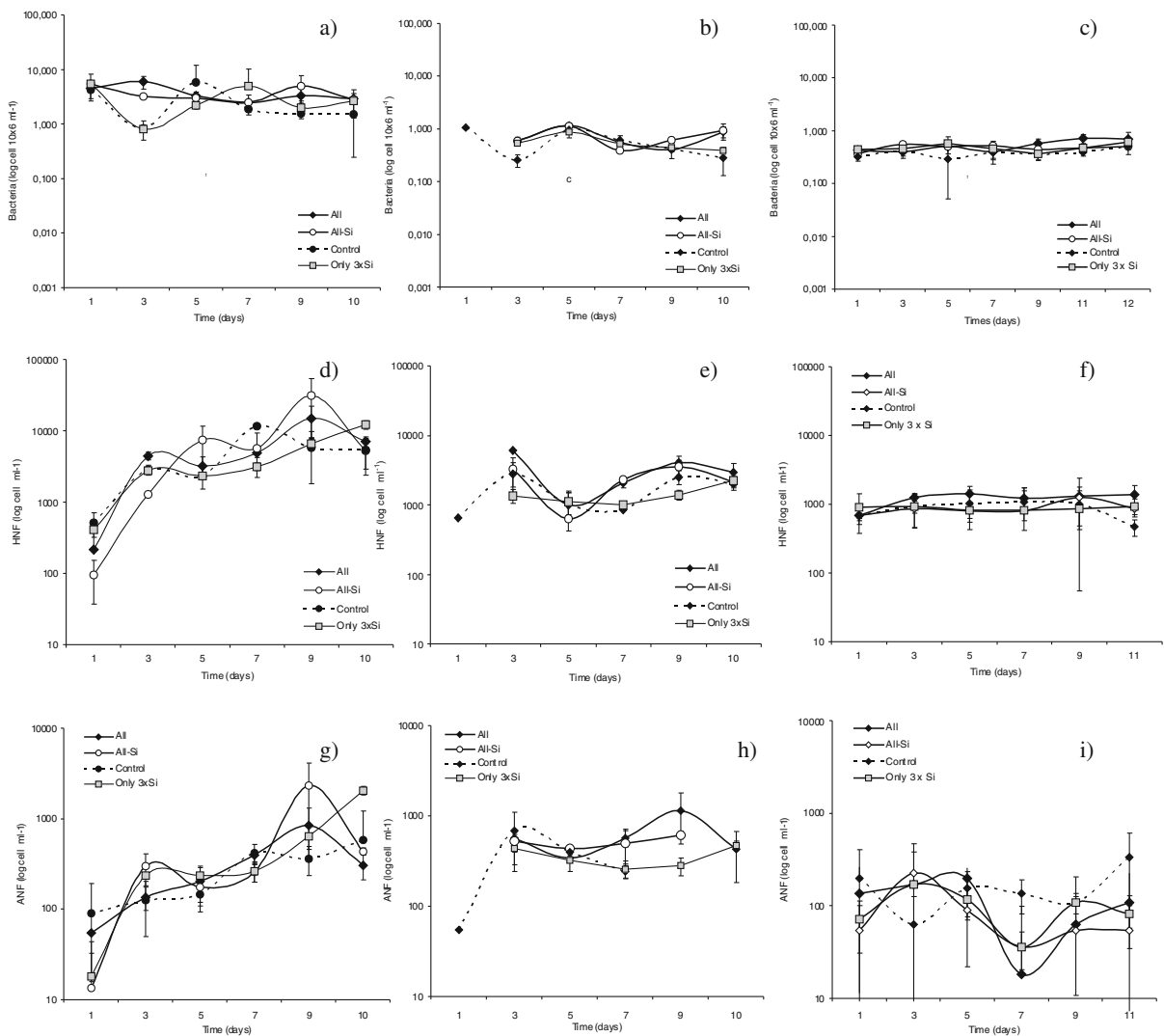


**Fig. 6** Contribution of predominant micro-phytoplankton taxa to total abundance (as cells per milliliter) on the initial day and day 10 of the austral spring (a), austral summer (b), and austral

winter (c) experiments performed in the Comau Fjord. Each value represents one sample

treatments and seasons. The initial average HNF abundances for the spring, summer, and winter experiments were 307, 3,366, and 742 cells $\text{mL}^{-1}$ . After 10 days,

these had increased one order of magnitude in all treatments (7,477 cells $\text{mL}^{-1}$ ), with higher abundances for the treatment ONLY 3 $\times$ Si in the spring (Fig. 7, middle



**Fig. 7** Dynamics in microbial growth during phytoplankton experiments in the spring (a, d, g), summer (b, e, h), and winter (c, f, i) seasons. *Upper panel*: bacterial abundance (log cells per milliliter). *Middle panel*: heterotrophic nanoflagellate abundance

(log cells per milliliter). *Lower panel*: autotrophic flagellate abundance (log cells per milliliter). Values are the means±1SD for samples from duplicate bottles maintained until day 10

panel). However, HNF abundances did not substantially change in the summer (mean, 2,325 cells $\text{mL}^{-1}$ ) or winter (mean, 914 cells $\text{mL}^{-1}$ ) experiments. Initial mean abundances for ANF were 43, 555, and 114 cells $\text{mL}^{-1}$  in the spring, summer, and winter (Fig. 7, lower panel). After 10 days, these abundances had increased one order of magnitude in all treatments (mean, 844 cells $\text{mL}^{-1}$ ) in the spring, with higher abundances for the treatment ONLY 3×Si. As with HNF, ANF abundances did not substantially change in the summer (mean=502 cells

$\text{mL}^{-1}$ ) or winter (mean=144 cells $\text{mL}^{-1}$ ) experiments. Minor differences were observed in the cell abundances of bacteria abundances as a response to autotrophic biomass or nutrient enrichment for all treatments and seasons. The initial average bacterial abundance in the treatments was 4.8 in the spring and 0.4 cells $\times 10^6 \text{mL}^{-1}$  in the summer and winter incubations, respectively (Fig. 7, upper panel). In the spring, after 10 days, the bacterial abundance had decreased nearly twofold (mean, 2.4 cells $\times 10^6 \text{mL}^{-1}$ ) in all treatments. However,

during the summer and winter experiments, bacterial abundance showed a constant pattern during the experimental period (mean =  $0.5 \text{ cells} \times 10^6 \text{ mL}^{-1}$ ).

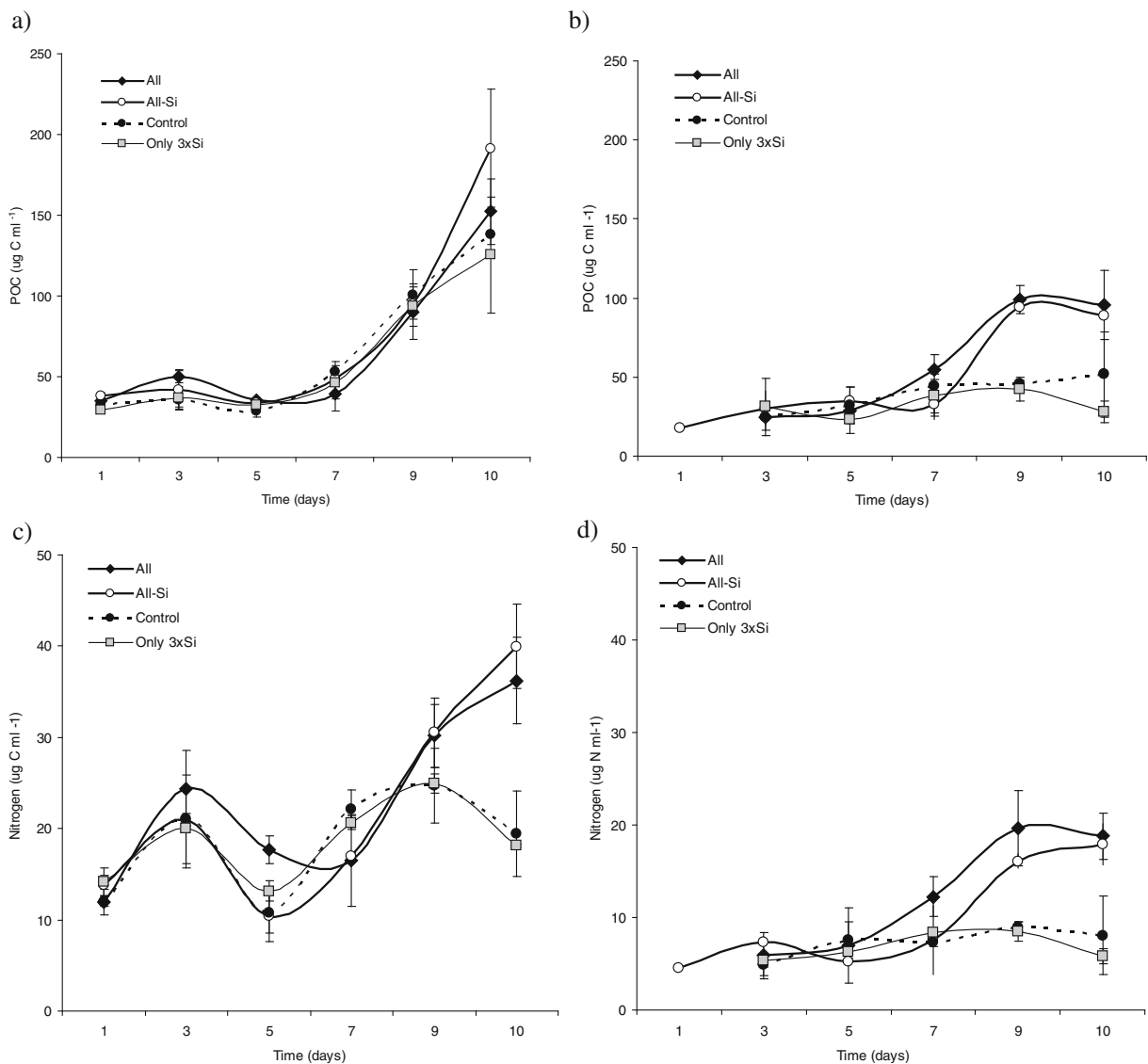
#### Particulate C/N ratio

The ratio of particulate carbon to nitrogen (C/N) of the microbial community at the end of the spring and summer experiments ranged from 4 to 7. The maximum POC and PON were reached for two treatments, ALL (POC, 96–152; PON, 19–36  $\mu\text{gC mL}^{-1}$ ) and ALL-Si (POC, 89–191; PON, 18–40  $\mu\text{gC mL}^{-1}$ ) and coincided with those of

Chl-*a*, micro-phytoplankton cell abundances (Fig. 8), and assemblages dominated heavily by diatoms, together with a severe decrease in nitrate and silicic acid concentrations, especially during the summer experiment.

#### Discussion

In this study, we showed that phytoplankton responded to the addition of nitrate in terms of Chl-*a*, cell abundance, and taxonomic composition of phytoplankton. Our observations supported the assumption that, in



**Fig. 8** Short-term course of particulate organic carbon (POC) and particulate organic nitrogen (PON) during the austral spring (a, c) and austral summer (b, d) experiments in the Comau Fjord

southern fjord systems, diatoms represent a significant component of the overall biomass (chlorophyll-*a*, POC, PON) and might be one of the main contributors to the structure of the pelagic food web in this highly stratified environment. The micro-phytoplankton assemblages of the Comau Fjord showed a 5-day lag (slow phytoplankton growth) followed by relatively rapid changes in the Chl-*a* biomass, cell abundances, and taxa/assemblage in response to enhanced NO<sub>3</sub><sup>-</sup> concentrations in the spring, summer, and winter seasons. The fact that NO<sub>3</sub><sup>-</sup> pools in ALL-Si (but not PO<sub>4</sub><sup>3-</sup>) became exhausted (ranging between 0 to 12 μM in all experiments) after 10 days suggests a NO<sub>3</sub><sup>-</sup> limitation of diatoms in the fjords in the spring and summer. A nitrate limitation has been suggested for phytoplankton primary productivity in the Chilean fjord system (Saggiomo et al. 1994; Iriarte et al. 2007) and other coastal marine systems (Pinckney et al. 1999; Lagus et al. 2004; Pehler et al. 2004). Furthermore, both this experimental result and the in situ NO<sub>3</sub><sup>-</sup>/PO<sub>4</sub><sup>3-</sup> (9.0) and Si(OH)<sub>4</sub>/NO<sub>3</sub><sup>-</sup> (>1.0) ratios observed in the cold waters of northern Patagonia (Inner Sea of Chiloé) are lower than the expected Redfield ratio (Iriarte et al. 2007; González et al. 2010; Aracena et al. 2011), indicating a probable inorganic nitrogen limitation (e.g., nitrate).

Altered nutrient ratio and increased loading of NO<sub>3</sub><sup>-</sup> or reduced nitrogen (NH<sub>4</sub>) may affect the species composition of the phytoplankton, with increased growth of harmful diatoms as one possible outcome. Micro-phytoplankton (>20 μm, centric and pennate diatoms) was responsible for approximately 60 % of the autotrophic biomass in the treatments ALL and ALL-Si in the spring, summer, and winter. When compared with nanoplankton (<20 μm, nanoflagellates), the biomass of the micro-phytoplankton in these treatments was closely related to the taxa composition of the phytoplankton assemblages. Therefore, centric and pennate diatom populations in the Comau Fjord are able to respond quickly to nutrient pulses, facilitating high diatom abundances in the spring and early summer. It is well known that short-term nitrate addition influences phytoplankton dynamics and biomass accumulation (as carbon and nitrogen) and that diatoms tend to dominate initial phases of such microcosm experiments when enough nutrients are available (Dugdale 1967; Margalef 1978). On the other hand, the lower representativity of dinoflagellate taxa during the experiments agreed with the view that dinoflagellates are inferior competitors than diatoms in nutrient-deficient waters because they are capable of using organic forms or less oxidized forms (i.e., ammonia and urea)

(Fan et al. 2003; Lomas 2004; Iriarte et al. 2005). The diatom taxa observed during the experiments (e.g., *Thalassiosira* spp. and *Chaetoceros* spp., *Rhizosolenia* sp.) have been described as typical for the spring bloom conditions of southern fjords (Iriarte et al. 2007; Alves-de-Souza et al. 2008; Iriarte and González 2008; González et al. 2010). Two related phytoplankton taxa, *Chaetoceros* spp. and *Rhizosolenia* sp. observed in our study have negatively affected salmon fish farms, most of which are located in the Inner Sea of Chiloé. The Phytoplankton Monitoring Programme from Instituto Tecnológico del Salmon identified several harmful algae species including diatoms such as *C. convolutus*, *L. danicus*, and *L. minimus* and *R. setigera* which are associated with salmon mortalities due to asphyxia and with low-oxygen water column.

In our experiments, the observed pattern in the changing phytoplankton biomass follows the general idea that large biomass pulses are due to micro-phytoplanktonic taxa, mainly chain-forming populations. The 10–12 day response of diatom abundances and Chl-*a* to the NO<sub>3</sub><sup>-</sup> treatments in the experiments implies that brief nitrate pulses to the surface could explain the diatom blooms of relatively high phytoplankton biomass (>2 mgm<sup>-3</sup>) and primary productivity (1–3 gCm<sup>-2</sup>day<sup>-1</sup>) in the fjord systems of southern Chile in the spring and summer seasons. Although the single limiting nutrient approach could be simplistic (compared with Tilman's resource ratio hypothesis; Tilman 1982), we give evidence to support the hypothesis that interactions of nutrient and column stratification could explain observed phytoplankton assemblage structure (Reynolds 1980) especially for southern fjords (Alves-de-Souza et al. 2008). Furthermore, from sedimentary information, it is evident that large to medium diatoms (e.g., *R. pungens*, *Skeletonema* spp., *Thalassiosira* spp, and *Chaetoceros* spp.) may become more abundant in the stratified waters of the Patagonian fjords (Rebolledo et al. 2011). The relatively high contribution of marine (versus freshwater) diatom species to the present time sediments in the fjords suggests relatively high rates of production under conditions of moderate to high nutrient availability. In these stratified marine systems, high and fast diatom growth and, thus, high PP and biomass could be thought to offer a competitive advantage during nitrogen limitation. Specifically, the notorious increment of the diatoms *R. pungens* over the past 20 years in the sediments have been suggested by the decrease in silicic acid flux associated with the recently lower rainfall and river stream flow rates around the Inner



Sea of Chiloé (Rebolledo et al. 2011). Finally, we hypothesize that this weakly silicified diatom is adapted to lower silicic acid and higher nitrogen environmental conditions and should be considered a functional key phytoplankton species that could cause harmful algal blooms in southern Chile. Here, the phytoplankton bloom dynamics, including HABs, despite their tremendous impact on productive activities (aquaculture), health, tourism, and environmental issues, still remain an unanswered question and a major research challenge.

Throughout most of the year, a permanent low-salinity layer in the upper 5 to 10 m of the water column imposes a strong barrier to (1) the entrance of inorganic nutrients from a deeper, saltier layer of oceanic water and (2) the export of phytoplankton carbon out of the euphotic layer. Furthermore, under stratified conditions, nutrients (mainly N and P) added from below the pycnocline would not be directly available for phytoplankton growth in the upper  $\text{Si}(\text{OH})_4$ -rich freshwater layer. In the Comau Fjord, maximum estimates of subsurface primary productivity ( $2\text{--}4\text{ gCm}^{-2}\text{d}^{-1}$ ) and Chl-*a* are located below the pycnocline during most of the annual cycle. Specifically, in this highly stratified system, the micro-phytoplankton dominance may result from the infrequent vertical advection of deep, nutrient-rich waters by internal and tidal waves (Valle-Levinson et al. 2007). The interplay of the availability of “seed” stocks could play a crucial role in obtaining higher subsurface phytoplanktonic biomass, but rapid and periodic changes in horizontal and vertical advection may also provide opportunities for the accumulation or enhancement of growth rates of large-sized phytoplankton through the interaction of mixing, augmented by changes in the environmental conditions of light and nutrients. During summer, the high in situ PAR irradiance ( $K_d$   $0.24\text{--}0.31\text{ m}^{-1}/90\text{--}180\text{ }\mu\text{molm}^{-2}\text{s}^{-1}$ ) at a depth of 10 m was similar to the light conditions provided in experiments, ensuring the phytoplankton were not light-deficient. However, the marked seasonality in light conditions, closely related to the seasonal pattern of primary production observed in the fjord, and the distinct trends in the decrease of PAR irradiance just below the surface layer in estuarine and continental waters, suggest that PAR attenuation may be also a key factor in limiting primary production (Pizarro et al. 2000). The amount of nitrate added in the ALL and ALL-Si treatments to support the observed high values of autotrophic biomass ( $20\text{--}30\text{ mg Chl-}am^{-3}$ ) was almost ten times greater than the concentrations in the top 5 m of the

surface waters of the Comau Fjord ( $2\text{--}5\text{ }\mu\text{M}$ ), suggesting a greater importance or critical state of the nitrate supply from below the pycnocline. However, the low surface Chl-*a* suggests that the semi-permanent stratification of the water column, due to the presence of haline stratification, acts as a strong barrier restricting the upward distribution of inorganic nutrients and, hence, phytoplankton growth. The development of nutritional strategies by phytoplankton in variable environments subjected to pulsing nutrients could be advantageous given nutrient-deficient conditions. It has been hypothesized that moderate uptake rates, large internal storage in nutrient pools, and low cell division—the so-called “storage response” (Collos 1986)—could be one main strategy in vertically stratified environments. In our study, a delayed phytoplankton growth response at the early stage of the experiments (until the fifth day) in terms of biomass and cell abundance was observed in three microcosm bioassays, offering some support for this prediction. On the other hand, the strong latitudinal gradients in sea surface temperature recorded over  $10^\circ$  latitude (decreasing southwards) may also limit primary production. However, the low correlation between discrete temperatures and primary production rates along the study area ( $41\text{--}50^\circ\text{S}$ ), suggest that temperature is not related to the spatial pattern of primary production along Patagonia marine system (González et al. 2011).

## Conclusions

We concluded that, from small-volume and short-term nutrient addition experiments, nitrate concentration influences phytoplankton population dynamics, accumulation of biomass, and diatoms tend to dominate initial phases of such microcosm experiments. While it is complex to make predictions to extrapolate experimental results to system-level scenarios, our approach provided insights into one of the main factors responsible for the taxa shifts and biomass accumulation of phytoplankton assemblages. Patagonian fjord ecosystems have experienced new scenarios mainly due to anthropogenic activities (intensive aquaculture) along these shorelines (Iriarte et al. 2010). This activity, like routine fertilizer and feed additions on salmon-cage coastal sites, may modulate the seasonal phytoplankton blooms, as well as promoting the growth of harmful algal species (diatoms and/or dinoflagellate species). Herein, we suggest that changes in the nutrient ratios



(i.e., low Si/N and high N/P ratios) of the coastal waters of the Patagonian fjords presently exposed to high levels of human activities (e.g., aquaculture, land change use, agriculture) could affect the phytoplankton species composition and total biomass (i.e., from low to high), constituting a major concern in terms of potential future scenarios.

**Acknowledgments** This research was funded by FONDECYT 1080187 (J.L. Iriarte). We thank the Huinay Foundation for providing marine laboratories and vessel facilities, as well as phytoplankton data from the Comau Fjord, and our colleagues G. Försterra and V. Häussermann for their help at the Huinay Scientific Station. We appreciate the valuable help of Patricio Ampuero and Caroll García during the experimental work and microscopic analysis, and Pirjo Huovinen for allowing us to use her submersible Ramses-ACC2-UV-VIS hyperspectral radiometer sensor (Trios Optical Sensors). This research was also partially funded by the Centro de Investigación de Ecosistemas de la Patagonia (CIEP), Programa de Financiamiento Basal COPAS–Sur Austral, and WAFOW/NTNU–Norwegian Research Council Latin American–Norway Programme. The winter experiment was part of the MSc. Thesis of Ms. Pamela Labbe at Universidad de Concepción, who was funded by a 1-year scholarship from COPAS–Sur Austral. This is publication 55 of Huinay Scientific Field Station.

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