Effect of ammonium input over the distribution of iron in the seawater and the phytoplankton in a mesocosm experiment in a north Patagonian fjord

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ABSTRACT

The distribution and concentration of iron in seawater and plankton were studied under different ammonium concentrations in a 22 day mesocosm experiment. The objective was to assess the possible effects of aquaculture on the phytoplankton and the biogeochemistry in fjords of Chile. Brackish and marine water were used in two different setups, each one with 1 control and 4 different NH_4^+ concentrations. Total Chelex labile (TFe_{Ch}), dissolved Chelex labile (DFe_{Ch}) and DGT labile (Fe_{DGT}) iron measurements were performed in seawater, while the particulate iron content was determined as total (PFe) and fractionated (PFe_{SF}) for the plankton community. The average concentration per treatment revealed higher values for both TFe_{Ch} and DFe_{Ch} in the marine system compared to the brackish system. TFe_{Ch} showed an overall increasing trend in time and with increasing ammonium concentration, positively correlated to the chlorophyll and particulate organic carbon content. DFe_{Ch} on the other hand presented an inverse pattern as expected. Fe_{DGT} had on average lower concentrations compared to DFe_{Ch} with final concentrations significantly lower in treatments with artificial ammonium addition. PFe had an increasing trend in time and with increasing ammonium in both systems. However, when normalized to chlorophyll-a or particulate organic carbon the trend was inverted, showing that at higher ammonium loading the iron per chlorophyll-a or

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particulate organic carbon decreases. PFe_{SF} major changes occurred in the marine system in the ratio between the 20-140 µm and the 2-20 µm fractions, suggesting a possible community structure shift. Overall, ammonium input indicated an effect over iron in the seawater and the particulate matter, depending on the iron form and the microbial assemblage. The further changes in the microbial composition due to ammonium addition may affect the cycling of iron, having possible positive or negative feedbacks in the major biogeochemical cycles.

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1. INTRODUCTION

The fjord ecosystem in Chile constitutes a nearly pristine environment. However, it is experiencing a growing anthropogenic influence and aquaculture is one of the main influences. This industry has seen and increased expansion in the last two decades, causing growing concern about the environmental impact. Salmon farming releases nutrients as dissolved inorganic species through excretion (Ammonium (NH_4^+) and phosphate (PO_4^{3-})), particulate organic nitrogen (PON) and phosphorus (POP) through defecation, and its dissolved components (DON and DOP) through resuspension from the particulate fractions (Olsen and Olsen, 2008). Oxygen depletion and decreased biodiversity among others are well documented effects for the marine sediments and benthic fauna. However, current knowledge of how waste release affects the structure and function of the pelagic ecosystems is still scarce (Cloern, 2001;Olsen et al., 2006). It has been proposed that this waste release may alter nutrient stoichiometry in the seawater determining to some extent how the marine environment responds to increasing anthropogenic inputs of nutrients (Arrigo, 2005).

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Major biogeochemical cycles (carbon, nitrogen and phosphorus) in the marine environment, are strongly dependent on marine microbes as this group is directly responsible for approximately half of the earth's primary production (Field et al., 1998). In coastal areas such as the Chilean fjord regions, the macronutrient nitrogen is mainly contributed as nitrate NO_3^- with the incoming deep water (nutrient-rich) and/or the surface layers through the run-off of inland fertilizers. After phytoplankton uptake, nitrate follows a series of metabolic processes that occur in order to be finally assimilated as NH_4^+. Within these steps in the nitrogen cycle, as well as for that of major elements, are involved metabolic processes that are dependent on the availability of certain "micro-nutrients". Trace metals such as Mn, Fe, Co, Ni, Cu, Zn and Cd are involved in several biological processes capable of influencing the biochemical cycling in aquatic systems such as of carbon (e.g. carbon-concentrating mechanism involves the Zn metalloenzyme carbonic anhydrase) or nitrogen (e.g. Fe requirements for

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metalloenzymes in nitrogen cycle) (Morel and Price, 2003). As most of these elements are continuously exported out the photic zone into deeper water as settling organic biomass, biological processes (uptake, trophic transfer, regeneration, excretion and decomposition) are critical in controlling the fate of these bioactive metals in the ocean (Wang et al., 2001). In the case of iron, the key role it plays on major biochemical cycles in the marine environment is well acknowledged (Martin et al., 1991;Martin, 1991;Morel et al., 1991). Specifically related to nitrogen, Fe is involved in the nutrient uptake by diatoms as NO_3^- undergoes sequential reduction to nitrite and ammonium, each step involving the assimilatory nitrate reductase and assimilatory nitrite reductase enzymes respectively (Zehr and Ward, 2002).

Through this feedback control mechanism between the so-called "macro-" and "micro-" nutrients, it can be expected that a species shift in the macronutrient load (e.g. NO_3^- to NH_4^+) in a given environment, may affect the long-term cycling of trace elements. The biologically "new" versus "regenerated" production, based on the NO_3^- : NH_4^+ uptake ratio in the water column, is a determinant factor favoring growth rates of certain groups of primary producers (Thompson et al., 1989). Therefore, increased input of dissolved inorganic nutrients (NH_4^+ and PO_4^{3-}) by aquaculture activities may have a direct effect on the phytoplankton community structure (Olsen and Olsen 2008). The general response from marine pelagic ecosystems to nutrient enrichment is reflected in increased nutrient uptake by phytoplankton and bacteria and hence growth rate, with the consequent increased autotrophic biomass transfer to higher trophic levels (Olsen and Olsen 2008). However, knowledge on the capacity for phytoplankton to biologically uptake and metabolize these surplus of nutrients, strongly link to the bioavailability of certain trace metals, is still scarce.

Considering this, it can be expected that within a certain environment following nutrient enrichment through NH_4^+, the nitrogen cycling and the stoichiometry of trace elements linked to it may be modified. Therefore, it is likely that a NO_3^- to NH_4^+ shift achieved through progressive nutrient addition would imply long-term changes in the Fe requirements by phytoplankton with the consequently effect on the trophic transfer to higher trophic levels. In order to assess the probable feedbacks processes between nitrogen and iron marine cycles and the implications in the base of the pelagic marine food web, a mesocosm experiment simulating the nutrient enrichment occurring in fjords ecosystems due to salmon farming was carried out. In it, the concentration and variation in time of different fractions of Fe in the seawater and plankton community under different NH_4^+ concentrations were measured.

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2. MATERIALS AND METHODS

2.1 Study Area

Experiments were carried out during the austral summer season between January and February 2011 at the facilities of the Huinay Scientific Field Station (42°22'46"S – 72°25'12"W) in the Comau fjord, Northern Patagonia (Fig. 1). The fjord has north-south location, with a maximum depth of nearly 500 m and a width of 2-8.5 km. Its hydrography features a two layer system with the presence of a permanent low salinity layer (LSL) between the surface and ~5 m, product of the mixing of freshwater (precipitation and river runoff) with oceanic water. Salinity in the first 10 m ranges from 18 to 28 PSU during the year, remaining constant below 10 m. Dissolved oxygen ranges from 7.5 $ml.L^{-1}$ in surface waters to 4 $ml.L^{-1}$ at 20 m (Jantzen et al., 2013;Pickard, 1971;Sánchez et al., 2011; Häussermann and Försterra, 2009;Iriarte et al., 2013). The sharp salinity gradient was the criteria to use two types of water to perform the experiments.

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2.2 Mesocosms set up and sampling

A total of 10 ($1m^3$) containers were filled with water collected at depths of 2 m (brackish) and 10 m (marine) respectively, each one representing one treatment (Fig. 1). Water pumped into the containers was collected with a peristaltic pump (Multifix type M80), placed in a peer and using plastic hose (35 mm diameter) projected 30 m offshore with a bottom depth at the sampling point ca 30 - 45 m. Seawater was pumped to a 30 L mixing bottle and then distributed simultaneously to all mesocosms containers by gravity-fed pipe flow. The water was not prescreened, in order to contain the different taxonomic groups at the various trophic levels of the natural plankton assemblage. The length of the experiments was 22 days and during this period the containers remained closed. Sampling was performed every third day after proper mixing of the containers and before any other kind of samples. A hose connected to the peristaltic pump was introduced in each tank pumping 2-3 Lt into 5 L plastic bottles, which were taken to the laboratory for processing (Table 1). All material used, except from containers was acid washed in ultra-pure HNO_3 (Double quartz distilled from reagent grade). The containers were washed with the ambient seawater thoroughly and preconditioned by the same seawater for one day. Initial concentrations for all treatments corresponded to water collected by the peristaltic pump at the respective depths for brackish and marine systems before starting the experiments.

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2.3 Nutrient additions

In order to simulate the nutrient enrichment occurring in the water containers were supplied with four different concentration (treatments) of macronutrients (nitrogen, phosphorus and silicon) as ammonium chloride (NH_4Cl), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and sodium metasilicate enneahydrate ($Na_2SiO_3 9 \cdot H_2O$) every third day at a fixed ratio. Although salmon aquaculture does not add silicon into the marine environment, it was supplied as the fjords ecosystem in southern Chile has continuous and in excess natural input of it, thus preventing potential nutrient limiting specifically for diatoms. The five units used per system, were denominated as Control, Natural, Conc 1, Conc 2 and Conc 3, where "Control" corresponded to the unit with no addition of nutrients, whereas "Natural", received a nutrient input at the average ratio for N:P:Si, occurring in the natural environment (González et al., 2010;González et al., 2011;Iriarte et al., 2013). The three other units received the experimental nutrient concentrations from the lowest (Conc1) to the highest (Conc3) (Table 2). All macronutrient solutions were prepared from reagent grade and treated by adding Chelex-100 resin (Bio-Rad Laboratories) to remove the iron (see Section 2.5). The solutions were collected in acid washed PE bottles.

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2.4 Laboratory work

Processing of the samples collected in the field as performed under Class-100 laminar flow hood (AirClean-600 PCR Workstation) minimized possible contamination, whereas laboratory worked was carried out in a class 1000 clean laboratory at the Department of Chemistry at NTNU. After processing, all samples were analyzed by High Resolution Inductive Coupled Plasma Mass Spectrometry (HR-ICP-MS) Element 2 (Thermo-Finnigan) with PFA-Schott type spray chamber and nebulizer.

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2.5 Chelex samples

Samples for total Chelex labile (TFe_{Ch}) and dissolved Chelex labile (DFe_{Ch}) iron were collected in acid washed PE bottles, where a volume (~150 ml) of water was collected and added ~0.8 mL of Chelex-100 slurry (Bio-Rad Laboratories). The chelating ion-exchange resin was cleaned by putting in 2-3 M ultra-pure HNO_3 for 2 hours and washed with deionized water (18.2 MΩ) (two times), then adding 13.5 M Ammonia solution (Suprapur Merck) to maintain in the ammonium form. For the DFe_{Ch}, 0.2 µm acid washed filters (0.45 + 0.2 µm Sartorious Sartobran 300) and all-plastic syringes (PE) were used to filter the water.

All samples were double bagged and placed in a shaker (65 - 80rpm) for 48 – 72 hours. After this period, each sample was transferred to an acid-washed Poly-prep Chromatography column with a built-in polyethylene frit (pore size 100 – 300 µm size) (Bio-Rad Laboratories), where the water was washed out through and the Chelex-100 resin containing the material was retained in the frit. Each column was then first rinsed with deionized water (18.2 MΩ) and secondly with ~10mL of 0.1M Ammonium Acetate buffer (pH: 9,6 prepared from 13.5M ammonia (suprapur Merck) and 17.4M acetic acid (traceSELECT ultra Fluka) to remove the residue of seawater matrix, then afterwards packed and stored at 4˚C. In the clean laboratory, extraction of trace metals was done in a two-step acidifying process, obtaining a final 5 mL 0.6 M HNO_3 sample (Ardelan et al., 2010;Öztürk et al., 2002).

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2.6 DGT samples

Diffusive gradient in thin films (DGT) (Zhang and Davison, 1995) samples were collected for labile iron (Fe_{DGT}), placing three DGT samplers in acid washed plastic sealed containers with a known volume (~2000 mL) of water. Containers were placed in a shaker (60 - 80rpm) for 48 – 72 hours and then the samplers were collected and stored at 4ºC. In the clean laboratory, all DGT samplers were processed over a Teflon sheet, where the first two layers (filter and gel) were removed using plastic tweezers. The third layer, containing the Chelex-gel was transferred to an acid washed PE tube and 1 mL 3M ultra-pure HNO_3 was added. The PE tubes containing the Chelex-gel were put on a shaker at (60 - 80rpm) for a 12 hour period. Afterwards, content in the PE tubes was transferred to new acid-washed PE tubes, keeping the Chelex-gel in the previous tube. To assured total transfer of material, 4 mL of deionized water (18.2 MΩ) were used to rinse the previous tubes and then poured into the new ones, obtaining a final 5 mL 0.6 M HNO_3 sample (Ardelan et al., 2009).

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2.7 Size fraction filtration

Simple and sequential fractionation filtration was performed respectively to determine the concentration and distribution of the particulate iron within the plankton community as total (PFe) and in different size fractions (PFe_{SF}) present in the mesocosms. The former, implied filtration only through 0.2 µm, while the later, encompassed a range of five size classes: 0.8 – 2 µm (picoplankton), 2 - 10 µm (nanoplankton), 10 – 20 µm (larger nanoplankton), 20 – 140 µm (microplankton) and >140 µm (mesozooplankton). Filtration up to the 10 µm was performed with a simple filtration system fitted to a peristaltic pump and using acid washed

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filters (isopore membrane, polycarbonate, hydrophilic, 0.2, 2, 10 µm, 47 mm), whereas filtration from 20, 140 and 220 µm fractions were performed with acid washed meshes (Nitex) with different pore size, with the retained material then afterwards washed into 0.2 µm filters. Filtration volumes ranged from ≥ 2000 mL for larger fractions to a 100 mL for the smaller ones. Samples were kept frozen until further processing. In the clean laboratory, samples underwent High Performance Microwave Reactor (Ultra Clave UC Milestone) digestion, by placing the filters into Teflon tubes, adding 5 mL of 7M ultra-pure HNO_3 and then inside the UC for two hours. After the digestion process, samples were set to final dilution of 61 ± 0.3 mg. Original volume was calculated using the density of deionized water (18.2 MΩ) (0.998 gr.cm^{-3} at 20 °C) and initial volume filtered to obtain final concentration.

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2.8 Blanks and detection limits

The detection limit used here is three times the standard deviation calculated from the measured method blank values. All values reported here lie above the blank value determined, first subtracted from the blanks obtained from HR-ICP-MS values and then calculated to the appropriate concentration. Method blanks and detection limits of the analysis performed in HR-ICP-MS for each technique are presented (Table 3). Method efficiency was determined by addition of Chelex-100 resin for certified reference materials CASS-4 and NASS-6 from the National Research Council of Canada (Ardelan et al., 2010).

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3. RESULTS

3.1 Iron variability in the mesocosms seawater

Measurements for TFe_{Ch}, DFe_{Ch} and Fe_{DGT} during the experiment presented high variability between treatments and among the two systems (Fig 2). Overall, TFe_{Ch} and DFe_{Ch} concentrations presented higher values on the marine compared to the brackish system. On the other hand, average Fe_{DGT} concentration for all treatments showed no difference between the brackish (4.0 ± 2.4 nmol.L^{-1}) and marine (3.9 ± 2.3 nmol.L^{-1}) system (Table 4). TFe_{Ch} distribution for every treatment in both systems, tended to follow the same distribution pattern in time, with an initial increase followed by a maximum and a posterior decrease (Fig. 2a and Fig. 2b). Mean TFe_{Ch} concentrations for the Control and Natural treatments in both systems presented the lowest values (brackish: 7.7 ± 2.9 nmol.L^{-1} and 11.9 ± 2.8 nmol.L^{-1} and marine: 12.7 ± 5.4 nmol.L^{-1} and 13.5 ± 2.8 nmol.L^{-1} respectively) among all five treatments (Table. 4). DFe_{Ch} distribution in time, exhibited a lower range of variability compared to

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TFe_{Ch}, with a more consistent decreasing trend in time for both systems (Fig. 2c and Fig. 2d). Except for the Natural treatment in the marine system, final concentrations for all treatments in both systems presented lower values than initial ones (Table 4).

Conversely to TFe_{Ch} and DFe_{Ch}, Fe_{DGT} distribution in time revealed no clear trend in any system (Fig. 2e and Fig. 2f). However, average in time per treatment revealed lower Fe_{DGT} compared to DFe_{Ch} in the brackish and marine systems (Table 4). Values in the brackish system ranged from very low 0.3 ± 0.1 nmol.L^{-1} up to 6.6 ± 2.2 nmol.L^{-1}, while those in the marine system presented a broader range (up to 9.5 ± 2.0 nmol.L^{-1}). In both systems the final concentrations in the Control and Natural treatments presented the highest values, being above initial ones, whereas the final concentrations for the other treatments were below initial values. A comparison between the final concentrations in the revealed significant differences between the Natural and the three artificial nutrient addition treatments (Conc 1, Conc 2 and Conc 3) (1-way ANOVA; DF: 14, Holm-Sidak), for both brackish and marine systems.

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3.2 TFe_{Ch}, DFe_{Ch}, Fe_{DGT} and NH_4^+ loading

No significant relationship was found between TFe_{Ch} and DFe_{Ch} and between DFe_{Ch} and Fe_{DGT} for all 5 treatments neither for the brackish nor the marine system. However, comparing TFe_{Ch} and DFe_{Ch} only for treatments with artificial nutrient addition (Conc 1, Conc 2 and Conc 3), a negative correlation (R^2 = 0.535) appears in the brackish system (Fig. 3a). Relative to the NH_4^+ loading, concentrations for TFe_{Ch} presented positive correlation for both the brackish (R^2 = 0.606) and marine (R^2 = 0.839) systems, reflecting the increasing trend in time observed with increasing NH_4^+ concentration. Contrary to TFe_{Ch}, the DFe_{Ch} exhibited a negative trend in both systems, but this was not significant (Fig. 3b and Fig. 3c).

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3.3 Iron variability in the mesocosm plankton community

PFe in all treatments presented a higher mean value (116.3 ± 75.1 nmol.L^{-1}) in the marine compared to the brackish system (80.6 ± 44.6 nmol.L^{-1}). The average in time for each treatment with artificial NH_4^+ addition in both systems, showed higher values compared to those in the Control and the Natural treatments. The trend exhibited by PFe content in the plankton biomass followed an increase in time and with increasing NH_4^+ loading (in Conc 3 in both systems, there was a noticeable decrease at the end of the experiment) (Fig. 4a). However, when standardized by the total Chlorophyll (Chl-a) content, PFe:Chl-a reflected an inverse trend with a decreasing concentration with increasing NH_4^+ loading in time in both

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systems (Fig. 4b). The same change in trend occurred when PFe was standardized by the particulate organic carbon (PFe:POC) (Fig. 4c). Moreover, standardized values plotted against Chl-a or POC for the three artificial addition treatments, the PFe showed a negative exponential correlation (R^2: 0.507 to 0.960), supporting the trend observed (Fig. 5).

PFe_{SF} values followed the same trend as PFe in both the brackish and marine systems as expected. A decreasing trend in time in the controls, and in the same way, higher values per fraction in treatments with artificial nutrient addition exhibited the accumulation trend of Fe with increasing NH_4^+ loading (Fig. 6). The proportion of PFe_{SF} in the 0.8 - 2µm fraction, remained constant with no significant changes among treatments in the brackish system, whereas in the marine system, treatments with artificial nutrient addition presented a decrease (Conc 1: 10.9 % Conc 2: 17.5 % and Conc 3: 13.7 %) compared to Control (34.8 %) and Natural (35.3 %). PFe_{SF} in the fraction >140 µm, exhibited higher proportion in all treatments with artificial nutrient addition compared to the Control and Natural for both the brackish and marine system, yet it was not significant in either case.

3.4 PFe_{SF} in the 20-140 and 2-20 µm fraction

To compare PFe_{SF} contained in the plankton community in the size spectrum from 2 to140 µm, the microplankton - nanoplankton Fe ratio (Fe µ:n ratio) was estimated between the 20 – 140 µm fraction (containing the microplankton) and the 2-10 and 10–20 µm fractions added (containing the nanoplankton) (Fig. 8a and Fig. 8c). In the brackish system, the Fe µ:n ratio presented no significant differences between treatments. On the other hand, in the marine system the mean Fe µ:n ratio for Control (0.8 ±0.2) and Natural (0.8 ±0.4) treatments compared to Conc 1 (2.4 ± 0.4) and Conc 2 (1.9 ± 0.3) presented significant differences (1-way ANOVA, DF: 14 P: < 0.002; Holm-Sidak). These higher ratios imply an increase of the proportion in the PFe_{SF} of the 20 – 140 µm fraction relative to the 2-20 µm fraction

3.5 Phytoplankton assemblages

The total abundance of phytoplankton presented average higher values in the brackish systems compared to the marine system, being proportional to the NH_4^+ concentration in both cases. Thus, Control and Natural presented the lowest values, while Conc 2 and Conc 3 presented the highest values (Fig. 8b and Fig. 8d). Abundance by main groups revealed initial high values (> $2x10^6$ cell.ml^{-1}) and dominance of nanoplankton in the brackish system, while diatoms and nanoplankton were both present in low numbers (3 - $7x10^5$ cell.ml^{-1}) in the

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marine system. For the brackish system a rapid decline of the nanoplankton abundance was observed, maintaining low values in all treatments (except some peaks), while in the marine system the nanoplankton remained low throughout the experiment in all treatments. On the contrary, diatoms in the treatments with NH_4^+ addition grew steadily until the abrupt decline in both systems despite of the low initial values and the higher nanoplankton abundance in the brackish system (Fig 8b and Fig 8d). Concerning the diatom composition, no significant trend was found related to the water type or for NH_4^+ concentration. Both systems showed the dominance of chain-forming centric diatoms as *Chaetoceros spp*. (medium and small sizes) and *Guinardia delicatula*. By the middle of the experiment, medium size *Chaetoceros spp*. represented > 70 % in all treatments, both in the brackish and marine systems.

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4. DISCUSSION

4.1 Iron variability in the mesocosms

The Fe distribution pattern appeared to be correlated to the POC and Chl-a trends observed along the experiment. This implies a direct biological role in the changes in distribution of the different fractions of the element in the seawater (Fig. 7). TFe_{Ch} and DFe_{Ch} followed this pattern with opposite trends, particularly that of TFe_{Ch} exhibiting a remarkably similar trend to that followed by the POC. Given the observed decreasing concentration in DFe_{Ch}, the increase of TFe_{Ch} is presumed as a product of an increase in the particulate iron, via physical-chemical changes and/or uptake by the biota. A possible explanation could be that enhanced biological activity (e.g. release of organic ligands and exudates) might have induced chemical changes in the speciation in a fraction of iron previously not available (e.g. complexed) to the chelex-100 resin, forming newly dissolved iron (including colloids) that afterwards progressively transformed in time into TFe_{Ch}.

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A relevant factor related to the observed results, was the high biomass production attained in the containers. Compared to the productivity of the region (Iriarte et al., 2007;González et al., 2010;González et al., 2011;Iriarte et al., 2012), the concentrations of Chl-a and POC observed in the treatments, were much higher due to the artificial nutrient addition. This thus contributed to the formation of aggregate material at high rate that could be noticed at simple observation. Possible results of this type of particle aggregation could have been enhanced adsorption of the dissolved form onto the aggregates and coagulation of colloids, therefore removing portion of the dissolved fraction and transforming it into particulate. In a mesoscale

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experiment Wong et al. (2006) reported a quick transformation of the dissolved iron to particulate forms, with as much as 70% of the added iron transformed in the non-dissolved form after less than 24 h. The trend described lower colloidal iron percentages as the experiment progressed while the particulate fractions increased. The mechanism alleged to be involved, could be a combination of biological uptake (Chen et al., 2003;Nodwell and Price, 2001) or simply adsorption of dissolved (including colloidal) iron to the plankton cell surfaces as well as aggregation of oxyhydroxides (Wong et al., 2006). Accordingly, the decrease observed in TFe_{Ch} towards the end of the experiment, coupled to that of Chl-a and POC, seems to point to the settlement of the dying phytoplankton, and therefore suggesting that the PFe attached was eventually exported to the bottom of the mesocosm containers.

Among other factors that could account for a poor correlation between different fractions of iron, mechanical artifact could be an important one. It is known that colloids encompass a size range from 1 to 1000 nm in diameter (Wells, 1998), lying within the boundaries of the dissolved and particulate matter, consequently subjected to possible bias via artificial manipulation. Moreover, colloid production rates could be enhanced by biological action, presumably through a combination of cell exudation and lysis, microbial degradation of particulate organic matter, sloppy feeding and excretion by zooplankton (Wells and Goldberg, 1994), all factors which were gradually increased in the experiment by the NH_4^+ input. The combined factors above, could have modulated the increase of TFe_{Ch}, and therefore the positive linear correlation with NH_4^+ input and TFe_{Ch}. The rather poor negative correlation for DFe_{Ch} and NH_4^+ could be related to the mismatch between newly DFe_{Ch} becoming available and the uptake by phytoplankton, making an expected decreasing DFe_{Ch} trend less evident.

In the case of the Fe_{DGT}, the fact that the average concentration for all treatments was lower than that of DFe_{Ch}, would suggest that the Chelex labile dissolved fraction, was probably not all readily bioavailable. Nevertheless, when looking at the trends in time between Fe_{DGT} and DFe_{Ch}, it was evident the high variation, exhibited at some points opposite trends (Fig. 7a and Fig. 7c). The average ratio between DFe_{Ch} and Fe_{DGT}, higher for the marine (1.44) compared to the brackish (1.27), suggest differences in the proportion of the bioavailable fraction between the systems. However, given such variability the latter could not prove significant, rather it establishes a gross pattern. Such variability can be argued as the response to a dynamic system in which biological (release of organic ligands) and chemical (kinetics

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and equilibrium) forcing, modulated changes in the iron speciation in short periods of time. Despite all the variation, at the end of the experiment the significant lower Fe_{DGT} concentrations for all the treatments with NH_4^+ compared to the Natural one, reflected the decrease of the bioavailable fraction of iron as a consequence of the increased uptake by the growing phytoplankton biomass.

Further sources of variability affecting the results could be invoked. Although it was carefully performed, the fieldwork carried out was not under 100% ultraclean conditions. Indeed, the high peaks obtained for both TFe_{Ch} and DFe_{Ch} samples appeared to be clear sample artifact. Moreover, provided methodological errors were ruled out, high variation could have been the result of an enclosed system and the experimental setup. Such case could be the NH_4^+ rate supply (4.6 $\mu mol.L^{-1}.d^{-1}$) applied in treatment Conc 3. A significantly high concentration (Olsen et al., 2006) was applied due to expected possible toxic effects, in that way the plankton community was exposed to drastic changes in short periods of time that would not have occurred in a natural environment.

4.2 Iron content in the planktonic community

Determination of the PFe and PFe_{SF}, aimed to provide an estimation of the particulate iron relative to the particulate carbon pool in the system and hence within the planktonic community revealed that changes occurred in time of the concentration and distribution. The results presumably reflect the effects of the NH_4^+ input over the planktonic composition in both systems. PFe standardized both by POC and Chl-a, showed a negative exponential relation with NH_4^+, for both the brackish and marine systems, while PFe_{SF} (not standardized by fractionated POC and Chl-a data) revealed a significant change in the ratio in the iron content between two fractions (micro and nano) of the plankton community in the marine system. The observed differences for the brackish and marine systems, can be attributed to a great extent to the biological features (different microbial assemblages) under constant physical forcing (i.e. presence of permanent LSL) and other hydrographic parameters proper of fjord ecosystems (Pickard, 1971). The LSL particularly affects the water column features, reducing light penetration, nutrient exchange and limiting wind-induced mixing, during periods of strong water column stratification (Gibbs, 2001). The result is an environmental partitioning, with an often less productive (primary productivity) brackish layer based on nutrient recycling, dominated regularly by the nano-plankton size class, whereas below the halocline there is a marine layer with peak productivity (10 - 15m) based on constant nutrient

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input (deep nutrient-rich waters) and with micro-phytoplankton as the dominant component (Sánchez et al., 2011).

Based on the higher Fe µ:n ratio observed in the marine system, could be argued that micro-phytoplankton (diatoms in particular), takes advantage of the NH_4^+ input at less energy expense. Therefore, a plankton community dominated by big diatoms could be expected to be resembled in the marine system. However, it was evident that diatoms also outweighed the nanoplankton abundance and biomass in the brackish system. The evidence of successful growth of diatoms based on NH_4^+ is substantial. The diatom *Thalassiosira pseudonana* exhibited an 8% increase in growth based on NH_4^+ compared to NO_3^-, under saturating light and Fe-replete conditions (Thompson et al., 1989), while another study found higher growth rates for ammonium-grown cells than for nitrate-grown cells of several species under the same conditions (Levasseur et al., 1993). On the other hand, an oceanic diatom isolated from the subarctic Pacific was found to have no difference in the growth rates of nitrate- and ammonium-grown cells under Fe-replete conditions (Muggli et al., 1996). It is worth noting that NO_3^- (not added as nutrient), concentrations in the marine system showed a decreasing trend in the Control and Natural, while in the treatments with artificial addition NO_3^- concentration remained constant during the experiment (data not shown), thus supporting the idea of preferential ammonium uptake. Nevertheless, Price et al. (2005) found contrasting results when growing diatoms with NO_3^- and NH_4^+ under high and low Fe-mediums. While the iron quota (Q) was higher for NO_3^- under low Fe, at high Fe, was higher for NH_4^+ grown cells. The main reasons pointed out that the growth conditions and species differences may be responsible for the contrasting results.

As the Fe µ:n ratio here discussed above, the Fe:C ratio is determined mainly by the differences in the community species composition and its physiological state. In contrast to the relatively constant Redfield ratios of C: N: P, the cellular Fe:C ratios vary markedly (by a factor of 30) as a function of the iron available (Bruland et al. 2001). Hence, most of the data available on estimation of trace element quotas come from laboratory cultures (e.g. Sunda and Huntsman 1995), with still few data from natural environments (Cullen and Sherrell, 1999). In such a way, in the scenario with the natural community studied here, There was a high variability but with a tendency to reducing iron uptake, due to the conditions of NH_4^+ excess supply and diatoms dominance (Sunda and Huntsman, 1995). Accordingly, the POC based Fe:C ratio obtained per treatment on both systems, followed a rather decreasing trend

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on time with increasing NH_4^+ concentration (Fig. 8b and Fig. 8d). However, a more pronounced reduction for the brackish mesocosms was observed despite that initial Control and Natural values being higher compared to marine ones. Given the dominance of diatoms in both systems, it could be argued these would reflect species- specific differences (e.g. diatom species with different Fe requirements). Nevertheless, the diatom composition found (at the genus level) in both systems did not differ significantly to account for the different slopes observed in Fe:C in time.

On the other hand, looking on the composition within the nanoplankton size fraction it is observed that nanoflagellates constituted on average double (5% versus 10%) of the total abundance in the brackish system compare to the marine system. Pico- and nano-plankton with higher surface to volume ratios and a more efficient uptake at low nutrient concentrations (Chisholm, 1992;Price et al., 1994), thrive in environments were the main source of nitrogen is recycled like NH_4^+, hence having lower Fe requirements. Conversely, diatoms in general have a higher requirement for iron to satisfy certain metabolic demands (Bruland et al., 2001). This is even more so for coastal assemblages since they have been shown to have an order-of-magnitude higher iron requirement (on a Fe:C basis) than oceanic species (Sunda and Huntsman 1995). Thus it could be argued that nanoflagellates having a higher contribution to the carbon pool to the brackish system, would have a consequently decrease in the iron to carbon proportion (Schmidt and Hutchins, 1999;Sunda and Huntsman, 1997). On the contrary, diatoms (higher contribution in the marine system), needing to meet minimum Fe requirements could account for the rather smoothly decrease in the Fe:C ratio observed in the marine system.

Despite the reduction in the Fe:C ratio, the estimated ratios as a proxy for the Fe content of plankton community in the Comau fjord during the experiment covered a broad range (265 up to 4224 µmol:mol^{-1}). Medians values (more representative here) were between 419 to 1430 and 753 to 1765 µmol:mol^{-1} for the brackish and marine mesocosms respectively, situating these values above to what is reported on the literature for phytoplankton, (2.3 to 370 µmol:mol^{-1}) (Sunda and Huntsman, 1995;Ho et al., 2003;Sarthou et al., 2005). The values reported here account for the potential total cellular iron, but this did not distinguish between external and intracellular iron content. Reported values range from 63 to 90% of intracellular iron of total cellular iron under Fe limiting conditions for different cell diameters and phytoplankton class species (Sunda and Huntsman, 1995). Moreover, the study reports

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intracellular max concentrations of 1700 µmol:mol^{-1} for diatom species at high Fe conditions, 30 times higher than needed to support maximum growth rate. Therefore, the values reported here could be reduced by at least 20%.For further comparisons, a range of carbon to chlorophyll ratios from the literature and the Fe:Chl found here, where used to estimate the Fe:C by other means (Table 5). Values obtained were higher than the POC based Fe:C but with a consistent trend, thus seeing a decrease in the Fe:C at higher NH_4^+.

When addressing the Fe:C ratio, it was considered that Chl-a only accounts for the autotrophic component of the plankton community (i.e. diatoms, autotrophic dinoflagellates and autotrophic nanoflagellates), whereas POC renders the complete amount of organic carbon in the whole community (i.e. including bacteria and protists). Likewise, POC measurements include all organic carbon whether of autochthonous or allochthonous origins, which might be of particular relevance in fjords ecosystem subjected to constant input of terrigenous origin (Syvitski et al., 1987;Vargas et al., 2011). In northern Patagonia fjords, where the Comau fjord is located, are not influenced by glaciers and thus have significantly lower loads of fine inorganic sediments than most fjords further south (Pickard, 1971), where the input of marine-derived organic carbon on surface sediments varies widely and can account between for 13 to 96% (average 61%) (Sepúlveda et al., 2011). Yet, a study of the Comau fjord reports allochthonous contributions to the sediments of 23.6 to 89.9 % at depths of 35 m and 475 m respectively (Silva et al., 2011). In this sense, it is important to highlight that this high variability could have a significant effect on the composition of the water collected for the experiments, in particular the brackish system. The pycnocline formed by the fresh water input act as barrier, therefore retarding the water mixing, with the probable consequence of temporary accumulation of organic carbon of allochthonous origin in the first meters of the water column. As observed in the initial POC content, the brackish water showed a three-fold difference (263.9 ± 23.3 mg.m^{-3}) compared to the marine (70.1 ± 11.8 mg.m^{-3}) mesocosm, hence increasing the carbon -to- iron ratio and simulating a lower Fe:C in the plankton community for the brackish mesocosms. Regardless of the above, and given that both Chl and POC related to the iron showing the same trend, the POC based Fe:C ratio estimation can be still considered a reliable approach.

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SUMMARY

The addition of NH_4^+ in our experimental set up revealed that there was an effect on the distribution of the different forms of iron measured in the seawater as well as in the particulate matter representing the content of iron in the plankton community.

The response for both type of water was different, the effect depended on the iron form measured and was correlated either positively or negatively with increased NH_4^+ concentration, as seen from the TFe_{Ch} and DFe_{Ch} correlation in the brackish system. The physical-chemical changes observed in Fe measured in seawater, suggest that besides from dissolvable Fe, TFe (non-filtered) is related to the biological uptake, and that under certain conditions should not be neglected as potential bioavailable source of Fe.

In the presence of an excess of nutrients a dominance of microphytoplankton and a decrease in Fe:C was observed in both water systems. However, the fact that Fe µ:n and Fe:C ratios, species composition, Chl-a and POC were considerably different, highlights the key role of the microbial assemblage within each type of water and therefore in the natural environment. Determining Q in key species is important to infer over the physiological state, environmental adaptation or possible growth limitation in phytoplankton and therefore is useful to understand possible ecosystem changes (e.g. a phytoplankton community with a low Fe:C but rather high Chl-a yield, as seen here, could result in higher efficiency in carbon export).

Likewise microcosm essays, these results are product of enclosed manipulated systems and therefore interpretations must take this into account. Natural systems are complex and this could be seen in the differences observed in the brackish and marine systems. Consequently, to better understand the impact of the anthropogenic influence (e.g. increase input of NH_4^+ via salmon aquaculture) on the biogeochemical cycling of major elements and presumably that of micronutrients such as iron in the pelagic ecosystem in the fjords of Chile, more emphasis needs to be allocated to study the linkages in between and the role of the marine biota within.

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Table 1. Date, sampling day (sday), system and parameters measured (Chelex labile (Total: TFeCh and dissolved: DFeCh) and DGT labile (FeDGT) iron) for the different treatments in the mesocosm experiment.

Date	Sday	System	Parameters
23 / 24.01.2011	1	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - Fe_{DGT}
26 / 27.01.2011	2	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - Fe_{DGT} - PFe
29 / 30.01.2011	3	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - Fe_{DGT} - PFe_{SF}
01 / 02.02.2011	4	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - PFe
04 / 05.02.2011	5	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - PFe_{SF}
07 / 09.02.2011	6	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - Fe_{DGT} - PFe
10 / 11.02.2011	7	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - PFe
13 / 14.02.2011	8	Marine / Brackish	TFe_{Ch} - DFe_{Ch} - Fe_{DGT} - PFe_{SF}

Table 2. Concentration (µM) and ratio for the different macronutrients added as NH_4Cl for Nitrogen (N), $NaH_2PO4{\cdot}H_2O$ for Phosphorus (P) and $Na_2SiO_3.9H_2O$ for Silicon (Si) in the different treatments for the brackish (6 to10) and marine (1 to 5) systems, in the mesocosm experiment. Control units had no nutrient addition.

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Treatment	Mesocosm	N	P	Si	N:P	N:Si
Control	1 and 6					
Natural	2 and 7	6,5	0,4	3,2	15,3	2
Conc 1	3 and 8	26,4	1,1	13,1	24,2	2
Conc 2	4 and 9	65,8	2,7	32,6	24,2	2
Conc 3	5 and 10	102,8	4,3	51,0	24,2	2

Table 3. Concentration (nM), Standard deviation (Std) and relative standard deviation (RSD %), for the blanks analyzed in HR-ICP-MS for the chelex, DGT and fractionation samples in the mesocosm experiment. Concentration (nM) and percentage of recovery (% Rec) for the certified reference materials (NRC). *Method blanks: 20 ml deionized water 18.2 MΩ water +10 ml ammonium acetate solution treated exactly as sample with 0.8 ml clean Chelex-100 and eluted similarly.** With pre-concentration factor 30.

Blank	n	nM	Std	% RSD	Ref. nM	% Rec.
Chelex-100	5	0,02	0,01	39,07		
DGT	8	0,03	0,01	23,79		
Fractionation (0,8 µm filter)	3	0,02	0,00	24,28		
Fractionation (2 µm filter)	3	0,01	0,00	11,06		
Fractionation (10 µm filter)	3	0,01	0,01	50,51		
Method blank*	8	0.08 nmol	0,02	28.6		
Procedural detection limits**		0.1				
Standard						
CASS-4	4	10,5	1,4	12,9	12,7	82,2
NASS-6	4	6,2	0,5	7,4	8,8	70,5

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Table 4. Concentration (nM) for total Chelex labile (TFe_{Ch}), dissolved Chelex labile (DFe_{Ch}) and DGT labile (Fe_{DGT}) iron for all treatments in the brackish and marine systems in the mesocosm experiment.

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		TFe_{Ch}					DFe_{Ch}					Fe_{DGT}				
	Sday	Con	Nat	Conc1	Conc2	Conc3	Con	Nat	Conc1	Conc2	Conc3	Con	Nat	Conc1	Conc2	Conc3
Brackish	1	8.3	8.3	8.3	8.3	8.3	8.2	8.2	8.2	8.2	8.2	4.7±1.8	4.7±1.8	4.7±1.8	4.7± 1.8	4.7±1.8
	2	9.4	13.3	9.3	7.1	15.1	8.0	3.5	4.9	6.9	5.4	2.6±1.5	0.3±0.1	3.4±2.6	0.3± 0.0	2.1±2.9
	3	10.3	12.1	14.9	14.5	23.6	5.2	4.6	4.7	6.4	4.3				3.7± 2.6	2.3 ±1.0
	5	7.6	15.6	24.2	25.6	20.2	1.4	6.9	2.0	3.7	2.8	6.3±2.4	6.3±1.7	6.6±2.2	6.2± 0.1	5.7±1.7
	8	2.9	10.0	15.9	24.6	9.6	1.0	5.4	4.6	2.8	2.7	4.3±1.9	6.3±0.5	2.4± 0.4	2.4± 0.8	0.9 ±0.8
Marine	1	13.1	13.1	13.1	13.1	13.1	6.1	6.1	6.1	6.1	6.1	4.1±1.4	4.1±1.4	4.1± 1.4	4.1±1.4	4.1 ±1.4
	2		11.7				6.2	6.3	12.1					1.5± 0.5	2.5±3.2	
	3	12.0	15.1	16.2	21.0	19.9	7.5	7.7	6.8	6.4	4.4				2.6±0.5	4.3±3.5
	5	9.4	17.4	31.7	30.4	23.3	6.2	6.8	4.2	4.8	7.4	2.4±0.9	2.1±1.1	4.3± 1.3	8.2±1.7	1.8 ±1.1
	8	7.5	10.4	24.3	14.1	9.2	6.6	3.5	2.9	2.8	3.6	9.5±2.1	6.3±2.6	2.9± 0.5	2.8±0.9	2.1±0.4

Table 5. Chlorophyll to carbon ratio (Chl:C) (mol:mmol^{-1}) and iron to carbon ratio (Fe:C) (µmol:mol^{-1}) for a range of values for *T. pseudomona* (Sunda and Huntsmann 1995). Fe:C (µmol:mol^{-1}) based on Fe:Chl (µmol:mmol^{-1}) and Chl:C from the literature, for all the treatments in the brackish and marine systems in the mesocosm experiment.

Reference			Control		Natural		Conc 1		Conc 2		Conc 3	
Chl:C	Fe:C		Fe:Chl	Fe:C	Fe:Chl	Fe:C	Fe:Chl	Fe:C	Fe:Chl	Fe:C	Fe:Chl	Fe:C
0,13 0,26	13 1770	**Brackish**	59969	7616 15472	121318	15407 31300	24971	3171 6443	28578	3629 7373	9553	1213 2465
		Marine	53321	6772 13757	43888	5574 11323	62126	7890 16029	47562	6040 12271	13315	1691 3435

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Figure 1. Study area, sampling site (Huinay Stn) and deployment of the brackish and marine systems for the mesocosm experiment in the Comau Fjord, Chile during January-February 2011.

Figure 2. Total Chelex labile iron (TFe_{Ch}) (a.-b.), dissolved Chelex labile iron (DFe_{Ch}) (c.-d.) and DGT labile iron (Fe_{DGT}) (e.-f.) ($nmol.L^{-1}$), in the brackish (left) and marine (right) systems for all treatments in the mesocosm experiment. Gap between points: missing data. Error bars: standard deviation (n=3).

Figure 3. Relation between a.) TFe_{Ch} ($nmol.L^{-1}$) versus DFe_{Ch} ($nmol.L^{-1}$) for both systems (Conc 1, Conc 2, Conc3), TFe_{Ch} and DFe_{Ch} versus NH_4^+ concentration in the b.) brackish and c.) marine systems for all treatments (average for all days) in the mesocosm experiment. Error bars: standard deviation (n=5).

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Figure 4. Distribution of a.) PFe ($nmol.L^{-1}$), standardized by b.) chlorophyll-a (Chl-a) ($nmol.ugr^{-1}$) (PFe:Chl-a) and c.) particulate organic carbon (POC) ($nmol.ugr^{-1}$) (PFe:POC), for all treatments along time in the brackish and marine systems in the mesocosm experiment.

Figure 5. PFe standardized by chlorophyll-a (Chl-a) ($nmol.\mu g^{-1}$) and by particulate organic carbon (POC) versus chlorophyll-a ($\mu g.L^{-1}$) and particulate organic carbon ($\mu g.L^{-1}$) in the a.-b.) brackish and c. – d.) marine systems, for treatments with artificial nutrient addition (Conc 1, Conc 2 and Conc 3) in the mesocosms experiment.

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Figure 6. Distribution of the PFe_{SF} ($nmol.L^{-1}$), in the plankton community, contained within the different size classes (µm) in the a.) brackish and b.) marine systems for all treatments along time in the mesocosm experiment.

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Figure 7. Total Chelex labile iron (TFe_{Ch}), dissolved Chelex labile iron (DFe_{Ch}), DGT labile iron (Fe_{DGT}) ($nmol.L^{-1}$), Chlorophyll (Chl-a) ($\mu g.L^{-1}$) (Right axis) and particulate organic carbon (POC) ($\mu g.L^{-1}$) (Left axis) concentrations in the a.- b.) brackish and c.- d.) marine systems for all treatments in the mesocosm experiment. Isolated points: contamination outliers. Dash line: missing data. Error bars: standard deviation (n=3).

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Figure 8. Distribution of PFe_{SF} ($nmol.L^{-1}$) in three fractions (2 – 10µm, 10 – 20µm and 20 - 140µm) of the plankton community (Left axis), ratio between PFe_{SF} in the 20 – 140 µm and 2 – 10 + 10 – 20 µm (encompassing the microplankton and nanoplankton fractions respectively) (Right axis). Abundance ($Cell.L^{-1}$) of total phytoplankton, diatoms (microplankton) and nanoplankton and iron to carbon ratio (Fe:C) ($\mu mol:mol^{-1}$) (Right axis) in the a.-b.) brackish and c.-d.) marine systems for all treatments along time in the mesocosms experiment. Error bars: standard deviation (n=3).

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