Seasonal changes in microzooplankton feeding behavior under varying eutrophication level in the Bahía Blanca estuary (SW Atlantic Ocean)

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ARTICLE INFO

ABSTRACT

Dilution experiments were conducted during a two-year survey in the Bahía Blanca Estuary (SW Atlantic Ocean) to evaluate seasonal variations of microzooplankton grazing (m) and phytoplankton growth (μ). The trophic interactions between these groups were investigated in relation to anthropogenic eutrophication. For this purpose, two sites exposed differently to sewage input in the estuary were selected. The percentage of primary production grazed by microzooplankton averaged 79%, although this percentage showed high seasonal variability. The lowest consumer control was found in winter and summer, and was associated with high density of chain-forming diatoms. Conversely, the microzooplankton grazing exerted heavy control on phytoplankton biomass during spring and fall, in concurrence with the highest density of nanoflagellates. Results showed average differences between μ and m close to zero during most of the annual cycle, suggesting microzooplankton control of autotrophic prey in the two sites. Under high exposure to sewage, however, experimental results evidenced the prevalence of non-linear feeding response, poor trophic phasing at low phytoplankton growth rate and lower reactivity of microzooplankton grazing. Regardless their exposure to sewage input, microzooplankton was an important loss term of phytoplankton in the estuarine system and appear as a primary factor defining phytoplankton biomass accumulation.

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1. Introduction

Microzooplankton grazing is an important pathway of phytoplankton loss in most aquatic systems, from the oligotrophic open-ocean to nutrient rich coastal waters (Calbet and Landry, 2004). They can rapidly synchronize productivity patterns and stabilize phytoplankton populations due to their short generation time (Strom, 2002). This rapid trophic response can minimize algal export to bottom layers, and thereby reduce the impact of disruptive algal blooms (Stoecker et al., 2005).

Several methods have been used to quantify in situ grazing rate of microzooplankton. The size-fractionation method, based on the incubation of different size fractions of plankton (Capriulo and Carpenter, 1980; Sato et al., 2007; Burian et al., 2013), assumes that consumers are larger than their prey, so micro- and nano-sized consumers can be separated from prey by filtering seawater through different mesh sizes. The grazing effect is then estimated by comparing prey growth rate in the presence and absence of consumers. Microzooplankton grazing can be also assessed by measuring the uptake of fluorescently-labeled tracers (Sherr et al., 1987). This approach has been successfully applied for the determination of grazing rate on homogeneous prey assemblages (Albright et al., 1987; Callieri et al., 2002; Sanders and Gast, 2011). Different prey taxa, however, may stain op-

timally with different dyes and staining protocols (Martínez et al., 2014), which makes the measurement of grazing on natural mix prey assemblages difficult. To date the most widely used method for the estimation of microzooplankton grazing is the dilution technique (Landry and Hassett, 1982). Its widespread use has generated a great amount of information that allowed comparing the impact of microzooplankton grazing on the global scale (Calbet and Landry, 2004; Schmoker et al., 2013). The technique is based on the reduction of encounter rate between consumers and their prey by the serial dilution of natural seawater with particle-free seawater. It has the advantage of simultaneously measuring microzooplankton grazing and phytoplankton growth assuming that the impact of grazing is linear with respect to the dilution factor (Landry et al., 1995). Several factors can modify the linear functional response of microzooplankton in dilution experiments. For instance, consumers may exhibit different growth rates along the dilution gradient (Dolan et al., 2000; First et al., 2007), and prey concentration may saturate microzooplankton feeding, thereby changing the clearance rate of consumers (Gallegos, 1989; Moigis, 2006).

The Bahía Blanca Estuary is a highly productive and eutrophic system located in the south western Atlantic Ocean coast, Argentina. The area is characterized by pronounced seasonality that shapes the annual cycle of plankton. Phytoplankton show two recurring productivity events occurring during summer and winter (Guinder et al., 2010, 2013). During the warmer season, zooplankton are characterized by the presence of omnivorous copepods and high ciliate biomass while during the colder season, mesozooplankton are dominated

by herbivorous copepods and detritivore cirriped larvae and microzoo-plankton abundance remains low (Berasategui et al., 2009; Barría de Cao et al., 2011; Dutto et al., 2012). In the last three decades, the estuary has experienced cumulative organic pollution due to the dumpling of untreated sewage (Lara et al., 1985; Marcovecchio et al., 2008). Previous studies conducted on the sewage plume showed high phytoplankton biomass and dominance of small-sized, non-siliceous algae (López Abbate et al., 2015). The potential pathways of phytoplankton loss, however, has received less attention (Diodato and Hoffmeyer, 2008; Dutto et al., 2014).

This study aims to determine the effect of organic pollution on the trophic relationship between microzooplankton and their prey. Growth and grazing rates were measured by the dilution technique during a two-year survey in two sites with different exposures to sewage inputs in the Bahía Blanca Estuary. The working hypotheses are that strong temperature gradient due to high seasonality controls the impact of microzooplankton grazing on phytoplankton, and that anthropogenic nutrient loading further interferes on microzooplankton grazing by affecting phytoplankton yield and composition.

2. Materials and methods

2.1. Study area

The Bahía Blanca Estuary (38°45′–39°40′S, 61°45′–62°30′W) is located in the south western Atlantic Ocean coast, Argentina (Fig. 1). This area shows a temperate climate with marked seasonality (Montecinos et al., 2000). The estuary is a shallow and vertically homogeneous system that shows inverted salinity gradient as it experiences low influence from continental drainage (annual mean $2.7 \text{ m}^3 \text{ s}^{-1}$), high evaporation rate and restricted water circulation in the inner reach (Perillo et al., 2001).

Two sampling sites were carefully selected by considering similar geomorphological features, but contrasting conditions with regard to their exposure to sewage input (Fig. 1). Both sites are located within tidal channels ranging 50–300 m width and 4–7 m depth. The first site, Bahía del Medio (BM), is not directly impacted by human stress (Baldini et al., 1999; Hoffmeyer and Barría de Cao, 2007), while the second site, Canal Vieja (CV), is directly affected by anthropogenic pollution since it is located within the channel that receives the untreated domestic effluent from the adjacent Bahía Blanca city (300,000 inhab.). The volume of sewage effluent represents 23% of total freshwater received by the estuary, significantly contributing to decreasing local salinity. The environmental condition of both sites were described by Dutto et al. (2012, 2014) and López Abbate et al. (2015). The authors noted that the concentration of all inorganic nutrients and particulate organic carbon were consistently higher in the sewage-affected site. Severe eutrophication in the area promoted hypoxia and water acidification. Micro-sized plankton biomass showed a saturation relationship with nutrients (i.e. the increase of nutrient concentration beyond a threshold value leaded to a decrease in the phytoplankton and microzooplankton biomass) and was strongly driven by the influence of temperature. In addition, mesozooplankton in
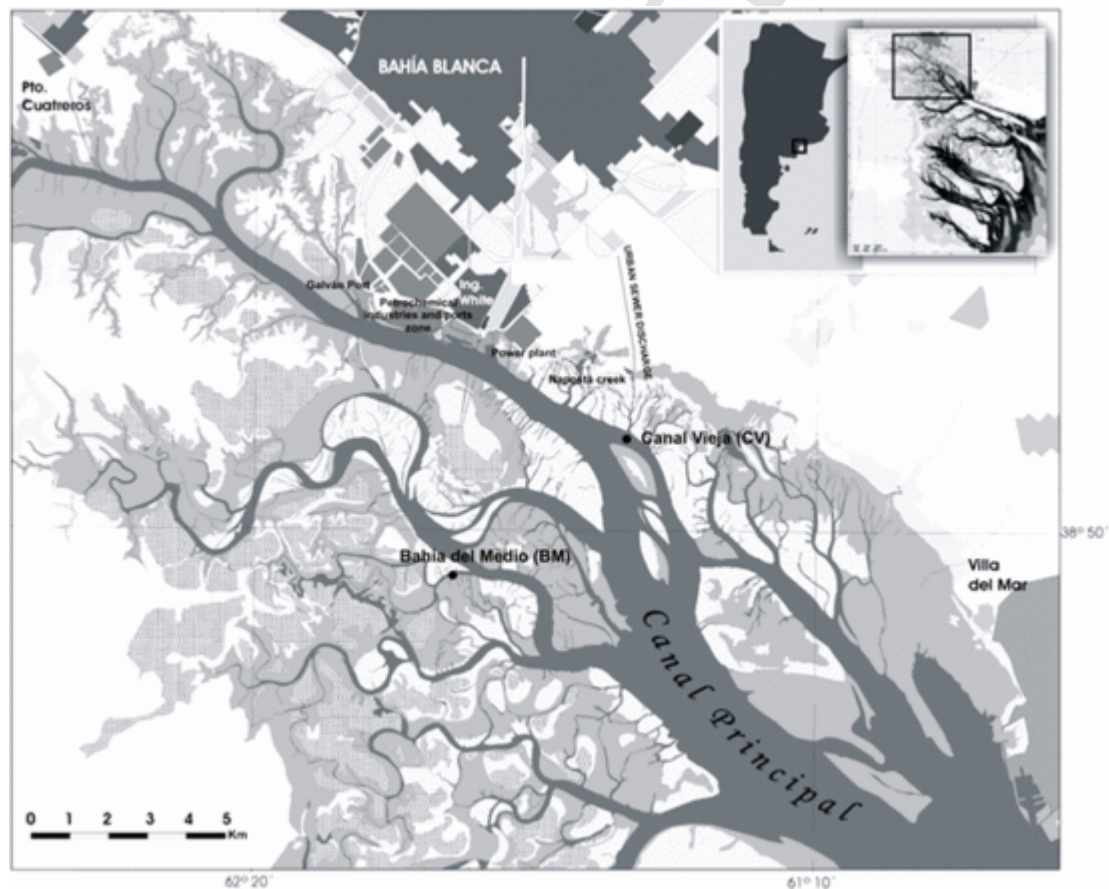

Fig. 1. Sampling area showing the location of two sampling sites exposed to low (Bahía del Medio, BM) and high anthropogenic eutrophication (Canal Vieja, CV).

the sewage-affected site are characterized by a higher contribution of detritivore species.

2.2. Experimental set-up

Feeding experiments were done during 14 surveys from July 2008 to November 2010. Dilution series were prepared by gently mixing different amounts of whole water and < 0.7 μm water in acid-cleaned polycarbonate bottles (1 l). Four dilution treatments (D) were prepared: 25%, 50%, 75% and 100% (whole water). An additional highly diluted treatment (5%) was prepared in July 2008, in order to detect saturated feeding (Gallegos, 1989). Seawater from each site was obtained from the sub-superficial layer (0.5 m depth), and pre-screened by reverse filtration with a 135 μm mesh pore net to eliminate larger grazers. Experimental bottles (3–5 replicates) were daily (24 h) deployed at each sampling site to maintain in situ temperature and photoperiod. Bottles were placed on net bags near the surface (0.5 m depth) and fixed to the bottom by an anchor.

Incubation bottles were not amended with inorganic nutrients as in highly eutrophic systems there are usually no significant differences between nutrient amended and not amended treatments (Li et al., 2011; York et al., 2011). In the Bahía Blanca Estuary, nutrient levels are usually above limiting concentrations (Popovich et al., 2008; Spetter et al., 2015), although exceptions to this may occur in post-bloom conditions in late winter–early spring (August–September), mainly with dissolved phosphate (Popovich et al., 2008). Nutrient limitation during the incubations was tested by computing the ratio between the initial concentration of inorganic nutrients and phytoplankton carbon and confronting the results with the Redfield ratio (Redfield et al., 1963).

2.3. Phytoplankton growth and microzooplankton grazing

Rate estimates of phytoplankton growth (μ) and mortality (m) were obtained by chlorophyll a measurements at the beginning and end of an incubation period (24 h) in all dilution series following the method described by Landry and Hassett (1982). This method assumes that the relationship between microzooplankton grazing rate, m, and chlorophyll a concentration can be described by a linear model. Thus, model fit was tested in every experiment; when dilution experiments yielded non-linear slopes as a result of saturated feeding (concave curves), the three-point method for the estimation of μ and m was used (Gallegos, 1989). Non-linear models were constructed by subdividing the dilution interval into sections of equal m (Moigis, 2006). The optimal model (linear vs. non-linear) was then obtained by computing the index of error, which estimates the fitting of each model (Moigis, 2006). The lowest index value is used to determine the optimal model fitting. The squared correlation coefficient between x (D) and y (μ_0) of both models was used to additionally contrast the model fitting (Moigis, 2006). Non-linear curves were further analyzed in order to compute the concentration of food at which grazing becomes saturated (Ps), following the methodology in Redden et al. (2002).

The proportion of primary production consumed by microzooplankton was estimated as the quotient between m and μ. The ratio was then transformed to their arctangent in order to reduce the effect of large values. Range and average percentage were converted back to percent production consumed using the tangent function (Calbet and Landry, 2004). The reasonable cause of non-linear feeding responses was obtained after evaluating the composition of plankton at the beginning of experiments. The calculation of feeding rates from experiments conducted in October 2010, could not be obtained due to

problems in the handling of chlorophyll a samples from that particular experiment.

2.4. Sample processing and data analysis

Chlorophyll a concentration was determined as described in Lorenzen and Jeffrey (1980). Water samples (500 ml) were filtered through 0.7 μm GF/F filters and then read in a spectrophotometer. The concentration of chlorophyll a at the beginning of the incubation (t_0) in the undiluted treatment ($D_1 = 100\%$), was used to calculate the concentration in the diluted bottles by the following equation:

Chlorophyll $a_{(t0, Dn)}$ = Chlorophyll $a_{(t0, D1)}D_n$.

Final chlorophyll a concentration was determined in all experimental bottles. Two to four replicate seawater samples were analyzed for the concentration of dissolved nitrate, nitrite, ammonium, phosphate and silicate as described in APHA-AWWA-WEF (1998) using a Technicon AA-II Autoanalyzer expanded to five channels.

Sub-samples from the initial undiluted treatment ($D_1 = 100\%$) were taken for plankton (nanoflagellates, phytoplankton and microzooplankton) quantification by light microscopy. This was done by settling a variable volume (10–50 ml, depending on sediment and plankton concentration) of preserved seawater sample (Lugol's iodine) in Utermöhl chambers during 24 h. The entire chamber was analyzed under a Wild M20 inverted microscope (Hasle, 1978). Autotrophic nanoflagellates were included into the phytoplankton category, although some heterotrophic nanoflagellates could have been miscounted as autotrophic due to the difficulty to distinct between these two groups in Lugol's fixed samples. To facilitate the interpretation of feeding experiments, the composition of microzooplankton at the beginning of experiments was organized in five different categories: i) *Tintinnidium balechi*, ii) ciliates (other tintinnid species and aloricated ciliates), iii) dinoflagellates, iv) mixotrophes (*Cyrtostrombidium longisomum*, *Lohmaniella oviformis* and *Strombidium capitatum*), and v) micrometazoans (rotifers and crustacean nauplii). The tintinnid species *T. balechi*, was presented as a separate category because there is no available information about its feeding behavior. Differences on the mean concentration of plankton, chlorophyll a and environmental conditions between sites were tested by the non-parametric Mann–Whitney U-test.

3. Results

3.1. Environmental conditions

The mean values of environmental condition are summarized in Table 1, and the raw data were presented in López Abbate et al.

Table 1

Mean values (± 1 SD) of environmental variables in both sites. Values in bold, indicate significant differences between sites (Mann–Whitney U-Test, $p < 0.05$).

	CV	BM
Temperature (°C)	15.51 ± 4.57	14.24 ± 5.51
Salinity	35.49 ± 7.09	**37.99 ± 2.19**
pH	8.39 ± 0.41	8.42 ± 0.36
Dissolved oxygen (mg l^{-1})	5.83 ± 1.17	**6.38 ± 0.89**
Turbidity (NTU)	88.23 ± 69.03	109.14 ± 67.61
Ammonium (μM)	**83 ± 93.08**	15.02 ± 13.32
Nitrite (μM)	**1.38 ± 1.34**	0.5 ± 0.8
Nitrate (μM)	**4.36 ± 3.74**	1.93 ± 3.36
Phosphate (μM)	**7.07 ± 9.53**	1.76 ± 0.87
Silicate (μM)	**71.45 ± 31.48**	57.45 ± 20.71
Particulate organic matter (μg l^{-1})	**2740.04 ± 1153.48**	2201.53 ± 1085.87

(2015). The sewage-affected site showed lower values of salinity and dissolved oxygen and higher values of all inorganic nutrients and particulate organic matter. Also, dissolved inorganic nitrogen (i.e. ammonium, nitrate and nitrite) and phosphate were in excess with respect to silicate.

3.2. Phytoplankton and microzooplankton abundance and composition

Nanoflagellates and nano-sized diatoms constituted the most abundant prey items, while the contribution of autotrophic dinoflagellates was negligible (Fig. 2). The temporal pattern of phytoplankton was characterized by higher cell densities in summer and fall and lower in winter and spring. Diatoms dominated phytoplankton in summer and winter, while nanoflagellates were the most abundant group in fall and spring. Phytoplankton density was higher under sever eutrophication, due to a higher density of nano-sized phytoplankton (Mann–Whitney U-test, $p < 0.05$), while the concentration of phytoplankton in the size range from 5 to 20 µm remained unchanged (Fig. 2). Main differences on phytoplankton composition between sites were due to a higher contribution of nanoflagellates in CV (Mann–Whitney U-test, $p < 0.05$). This planktonic group constituted 75% of total phytoplankton abundance in CV, while its relative contribution in BM was 60%. In addition, the relative percentage of

diatoms in BM (39%) was higher than in CV (24%). The dominant diatom species in both sites were *Thalassiosira* spp., *Paralia sulcata* and *Cyclotella* sp. In spite of different phytoplankton density, the initial concentration of chlorophyll *a* was higher in BM (Mann–Whitney U-test, $p < 0.05$).

Microzooplankton were characterized by the dominance of tintinnids and aloricated ciliates (Fig. 3). Tintinnids were mainly represented by *T. balechi*, *Tintinnopsis parva* and *Tintinnopsis gracilis*, while aloricated ciliates were dominated by *Strombidium* spp. and *Strombidinopsis* spp. In general, microzooplankton abundance and biomass were higher during the warmer months and lower during colder months (Fig. 3). Under sever eutrophication, microzooplankton abundance was significantly lower (Mann–Whitney U-test, $p < 0.05$). This was largely due to a lower density of *T. balechi*, which represented 90 and 65% of total microzooplankton abundance in BM and CV respectively. In fact, the concentration of ciliates, heterotrophic dinoflagellates and micrometazoans was similar in both sites. In addition, the density of mixotrophic ciliates in the sewage-affected site was higher, and this group represented 2 to 56% of total microzooplankton abundance (Fig. 3). In particular, the mixotrophic ciliate, *L. oviformis*, was a ubiquitous species in CV (> 400 cell l^{-1}) and its density was consistently higher than in BM (Mann–Whitney U-test, $p < 0.05$).
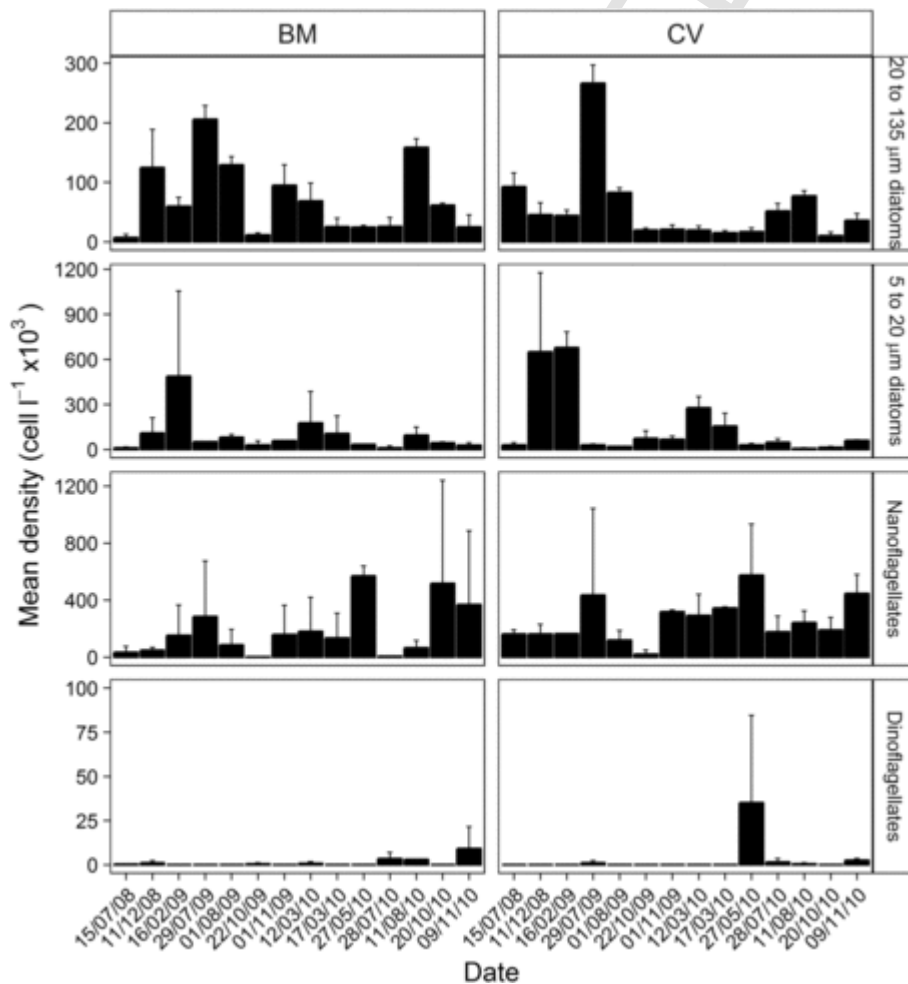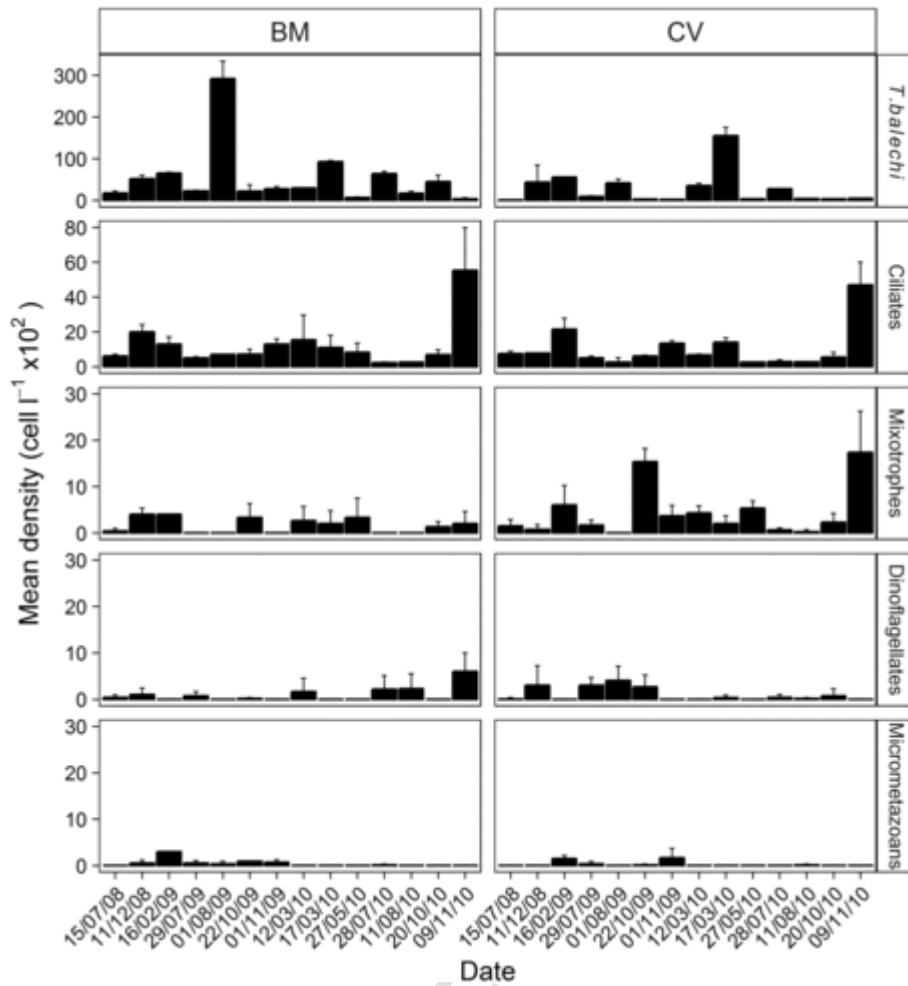

Fig. 2. Mean density (+ 1 SD) of two different size fractions of diatoms, nanoflagellates and autotrophic dinoflagellates at the beginning of dilution experiments during the sampling dates in both sites.

Fig. 3. Mean density (+ 1 SD) of microzooplankton at the beginning of dilution experiments during the sampling dates in both sites. Microzooplankton were grouped into five categories: i) *Tintinnidium balechi*, ii) ciliates (other tintinnid species and aloricated ciliates), iii) heterotrophic dinoflagellates, iv) mixotrophes (*Cyrtostrombidium longisomum*, *Lohmaniella oviformis* and *Strombidium capitatum*), and v) micrometazoans (rotifers and crustacean nauplii).

3.3. Dilution experiments

A total of 27 dilution experiments were performed (13 in BM and 14 in CV), from which, 15 resulted in significant grazing effect (Table 2). Phytoplankton growth rate was not related to the concentration of chlorophyll *a* at the beginning of the experiments in BM, however, in CV, both variables were negatively related (Fig. 4). Nitrogen limitation was detected in two experiments conducted in the non-affected site during November 2009 and August 2010, which warns on the potential underestimation of phytoplankton growth rate and overestimation of microzooplankton grazing rate during these experiments.

The results from all dilution experiments and the suggested functional response are given in Table 2. In BM, 7 experiments showed linear response and 1 experiment showed a concave curve, while in CV, dilution experiments yielded 1 linear plot, 8 with different curvatures and 3 with positive slopes. Mean phytoplankton growth and mortality rate were not significantly different between sites. The average primary productivity consumed by microzooplankton (μ:m) was 87% (ranged from 0 to 298%) in BM and 70% in CV (ranged from 0 to 220%). The relationship between phytoplankton growth

and mortality rate in BM showed a linear trend, and evidenced that microzooplankton grazing rate can eventually exceed the daily growth rate of phytoplankton (Fig. 5). In turn, phytoplankton mortality rate under severe eutrophication, responded non-linearly to phytoplankton growth rate (Fig. 5).

The index of error and squared correlation coefficients used for the selection between the linear and the non-linear model are shown in Table 3. Based on the experiments that showed saturated feeding, the concentration of prey that saturated the feeding of microzooplankton (Ps) was computed. The saturating prey concentration (estimated through chlorophyll *a*) was on average, 1.82 μg l^{-1}, and showed a linear relationship with the mean concentration of chlorophyll *a* in the incubation bottles (Fig. 6). The occurrence of saturated feeding did not coincide with the highest concentration of chlorophyll *a* recorded in this study, and no relationship was found between Ps and the concentration of microzooplankton. Highly diluted treatments applied in the set of experiments conducted during July 2008, did not produce significant changes on the estimation of *m* in BM. In contrast, in CV the grazing constant calculated from the first pair of dilution points (5%–25%) was 5.20 d^{-1}, while the constant obtained using the second pair of dilution points (25%–50%) was consistently lower (3.21 d^{-1}).

Table 2

Summary of experimental results. P_0: initial chlorophyll *a* concentration (μg l^{-1}), Z: initial concentration of consumers (cell l^{-1}), μ: phytoplankton net growth rate (d^{-1}), *m*: phytoplankton mortality rate (d^{-1}), Arctan (*m*:μ): transformed (arctangent) value of the proportion of primary production consumed by microzooplankton. The grazing effect detected in every dilution plot is also shown, as well as the reasonable cause of non-linear feeding responses after evaluating the composition of plankton samples at the beginning of experiments.

Site	Date	P_0	Z	μ	*m*	Arctan (μ:*m*)	Grazing effect in dilution plots	Suggested functional response
BM	Jul-08	3.90	2440.0	1.97	4.32	1.14	Concave curve	Saturated feeding
	Dec-08	10.75	7700.0	0.48	1.43	1.25	Lineal	
	Feb-09	8.67	8500.0	1.22	1.89	1	Lineal	
	Jul-09	8.49	2875.0	0	0	0	No effect	
	Aug-09	3.02	29,933.3	0.02	0	0	No effect	
	Oct-09	14.61	3353.3	0.51	0.7	0.94	Lineal	
	Nov-09	7.11	4100.0	0.28	0	0	No effect	
	Mar-10	8.13	4933.3	1.55	0.89	0.52	Lineal	
	Mar-10	3.57	10,550.0	1.61	1.21	0.64	Lineal	
	May-10	5.58	1833.3	1.2	1.16	0.77	Lineal	
	Jul-10	8.03	6813.3	0.19	0.02	0.09	No effect	
	aug-10	5.28	2124.4	0.5	0.44	0.73	Lineal	
	Nov-10	2.26	6680.0	0.3	0	0	No effect	
CV	Jul-08	3.7	1025.0	2.36	5.2	1.14	Concave curve	Saturated feeding
	Dec-08	7.47	5533.3	0.29	0.1	0.33	No effect	
	Feb-09	6.2	8450.0	0	0	0	Positive slope	Trophic cascade
	Jul-09	13.46	1933.3	0	0	0	Positive slope	Trophic cascade
	Aug-09	6.05	4803.3	0	0	0	Positive slope	Trophic cascade
	Oct-09	7.74	2693.3	1.53	1.49	0.77	Convex curve	Feeding threshold
	Nov-09	5.94	2066.7	2.09	2.71	1.22	Convex curve	Feeding threshold
	Mar-10	4.5	4633.3	1.75	2.86	1.02	Concave curve	Saturated feeding
	Mar-10	4.9	13,062.5	0.21	0	0	No effect	
	May-10	2.09	1250.0	2.12	3.62	1.04	Concave curve	Saturated feeding
	Jul-10	6.68	3146.7	0.36	0	0	No effect	
	Aug-10	4.44	746.7	3.83	3.79	0.78	Convex curve	Feeding threshold
	Oct-10	1.7	1212.5	1.38	0.98	0.62	Lineal	
	Nov-10	2.01	6900.0	1.16	0	0	Positive slope	Mixotrophy

4. Discussion

4.1. Seasonal variation in microzooplankton grazing

Primary productivity consumed by microzooplankton ranged from 0 to 298% and averaged 79% in both sites. This result was similar to that found in other estuaries (Murrell et al., 2002; Calbet and Landry, 2004; Juhl and Murrell, 2005; Schmoker et al., 2013). The lowest percentage of primary production grazed occurred in winter. This may be the direct consequence of the strong dependence of heterotrophic protists on temperature and the specific composition of phytoplankton during cold months. For instance, the prevalence of a lower proportion of edible prey during winter, may reduce the impact of microzooplankton grazing. The winter bloom is a recurring productivity event in the Bahía Blanca Estuary (Guinder et al., 2010), dominated by chain-forming diatom species (*Thalasiossira* spp. and *Chaetoceros* spp.). These species are likely excluded from ciliate diet given its size range and morphological features (Hansen et al., 1994). In addition, the shallow depth of the Bahía Blanca Estuary promotes a great annual variability of temperature, which may range between 23 °C in summer to less than 7 °C in winter (Freije et al., 2008). Below 15 °C, the biomass of microzooplankton in the estuary and their ability to synchronize with phytoplankton maximal growth rate are rapidly reduced (Barría de Cao et al., 2005; Pettigrosso and Popovich, 2009; Rose and Caron, 2007). Overall, the low standing stock of microzooplankton, temperature-related uncoupling of consumers and prey growth rate and the prevalence of inedible prey during cold months, favor the build-up of the estuarine winter bloom and the lower proportion of phytoplankton biomass removal. Conversely, during summer, phytoplankton was also dominated by diatoms (*T. minima*), however, the abundance of nano-size phytoplankton and nanoflagellates was higher than in winter. Thus, a higher proportion of edible prey as well as higher consumer density during summer, contribute to increase the fraction of daily primary productivity grazed. The highest percentage of daily primary production grazed by microzooplankton was found in spring and fall, in concurrence with the highest concentration of nanoflagellates. These results suggest that, regardless of the eutrophication level, nanoflagellates are preferential food items of microzooplankton and that temperature and the seasonal succession of phytoplankton are first-order determinants of microzooplankton grazing pressure in the Bahía Blanca Estuary.

The role of mesozooplankton within the planktonic food web has been evaluated by fatty acid markers in the same sites of this study (Dutto et al., 2014). The results showed that the main differences on mesozooplankton feeding were seasonal; in the colder season, mesozooplankton showed active feeding on terrestrially-derived compounds while during the warmer season, feeding switched to phytoplankton, in particular diatoms, and microzooplankton. Dietary shifts were ascribed to changes in mesozooplankton structure from the dominance of detritivore cirriped larvae in the colder season, to the dominance of omnivorous calanoid copepod, in the warmer season. Considering that the dilution method does not incorporate the effect of mesozooplankton grazing on heterotrophic protists, summer microzooplankton herbivory calculated here was likely overestimated. Despite this caveat, however, microzooplankton grazing showed a tight coupling with phytoplankton growth during the whole period, suggesting that microzooplankton rapidly responded to an increase of newly produced food, and represented a primary factor curbing biomass accumulation after bloom formation. These findings provide quantitative data on the temporal pattern of consumers control on
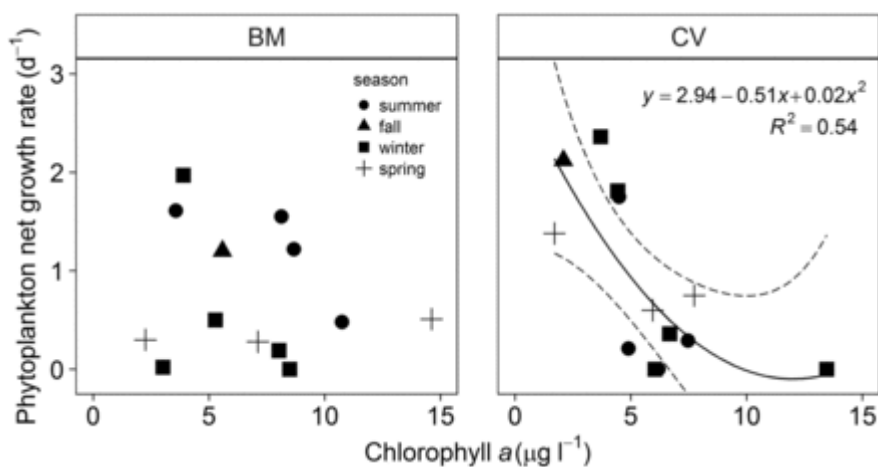
Fig. 4. Relationship between phytoplankton net growth rate and chlorophyll *a* content in both sampling sites in all seasons. Dashed lines represent confidence interval at 95%.
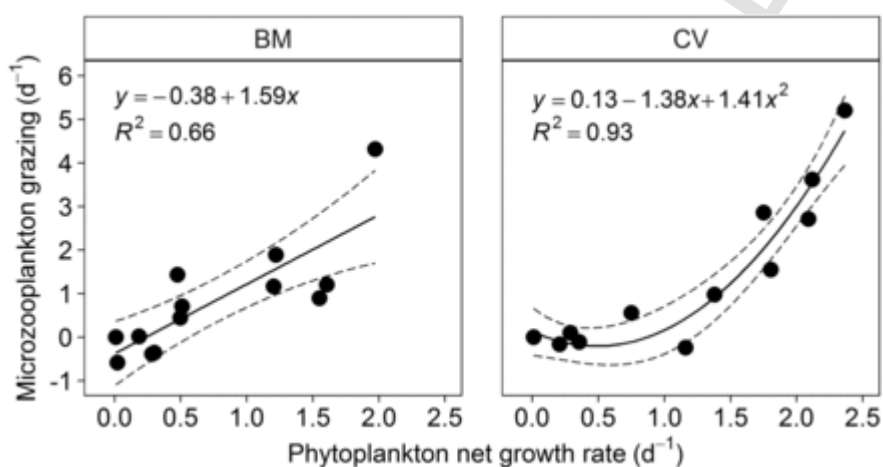

Fig. 5. Microzooplankton grazing rate as a function of phytoplankton net growth rate in both sites. Rate estimates showed linear relationship in the site with low exposure to anthropogenic eutrophication (BM), while in the sewage-affected site (CV), the relationship was quadratic, denoting low coupling of rates at low μ-values. Dashed lines represent confidence interval at 95%.

Table 3
Index of error ($\sigma^2_{y'}$) and squared correlation coefficient (r^2_{xy}) for the linear (L) and non-linear (NL) models in dilution experiments in which saturated feeding was detected.

Site, date	$\sigma^2_{y'}$(L)	$\sigma^2_{y'}$(NL)	r^2_{xy}(L)	r^2_{xy}(NL)
BM, 15–07-08	0.424	0.061	0.677	0.995
CV, 15–07-08	0.381	0.054	0.494	0.982
CV, 11–03-10	0.722	0.026	0.024	0.992
CV, 26–05-10	1.190	0.072	0.333	0.957

phytoplankton, and shed light on the main pathways of organic carbon within the estuarine network.

4.2. Eutrophication effects on community composition and phytoplankton growth

Severe eutrophication in the Bahía Blanca Estuary became apparent through higher producers' biomass rather than higher overall growth rate. This appears to be the general rule in eutrophic environments, as phytoplankton biomass continues to increase with nutrient supply while the growth rate increases until the species-specific maximum (Chen et al., 2009). The effect of anthropogenic eutrophication

on phytoplankton was also perceived as the shift towards the dominance of nanoflagellates. The stimulation of nano-sized, non-siliceous algae, is the anticipated result from variations in nutrient stoichiometry, i.e. excess N and P with respect to Si (Ragueneau et al., 2006; Billen and Garnier, 2007; Yunev et al., 2007; Suikkanen et al., 2013). Likewise, the negative relationship between phytoplankton growth and the concentration of chlorophyll *a* in polluted environments has been attributed to the dominance of nanophytoplankton in dilution bottles (Calbet et al., 2011; Stoecker et al., 2013). The results showed that fast growing, smaller sized phytoplankton drive the annual productivity pattern under severe eutrophication.

The sewage-affected site featured a higher proportion of mixotrophic ciliates in the planktonic community. The co-occurrence of autotrophic and heterotrophic nutrition, allows mixotrophic organisms to gain certain independence from specific ambient resources (Tittel et al., 2003), and provides a competitive advantage under highly variable conditions occurring in disturbed areas. In contrast, cell density of *T. balechi* was lower under severe eutrophication. This tintinnid species largely dominates microzooplankton abundance in the Bahía Blanca Estuary during the entire annual cycle (Barría de Cao et al., 2005). Although the dimension of its lorica is within the micro-sized planktonic fraction, the cell is relatively small (~ 20 μm).
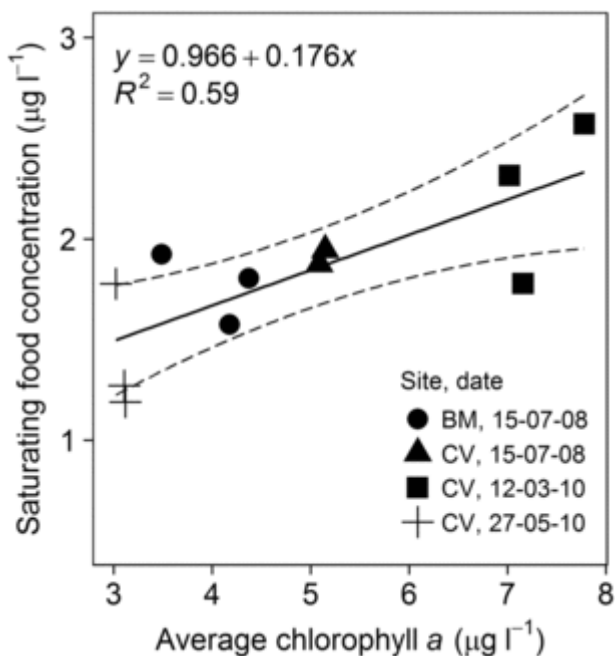
Fig. 6. Link between saturating food concentration and average chlorophyll *a* within the incubation bottles. Saturating food concentration was calculated from dilution experiments that yielded concave curves (listed in the lower right corner of the plot), as described by Redden et al. (2002).

Considering the scalar ratio between ciliates and their prey (Wirtz, 2012), the optimal prey size of *T. balechi* is below 2 μm, suggesting that a great proportion of its diet consist on bacteria. This could partially explain why the lower concentration of *T. balechi* in the sewage-affected site had no impact on the percentage of primary production grazed.

4.3. Microzooplankton feeding response to nutrient loading

The overall impact of microzooplankton grazing on daily primary production was similar in both sites, denoting a close trophic link regardless of differences in their exposure to pollution. The resilience of plankton community exposed to severe eutrophication is partially given by the stoichiometric flexibility of autotrophic plankton (Geider and La Roche, 2002; Sterner and Elser, 2002; Grob et al., 2013), and efficient homeostatic mechanisms of microzooplankton that offsets abrupt changes on resource element composition (Persson et al., 2010; Golz et al., 2015). In addition, the ability of microzooplankton to respond almost immediately to increased resource supply (Fenchel and Finlay, 1983), promotes the trophic coupling in highly disturbed environments characterized by discrete and less predictable productivity events.

The close link between phytoplankton growth and microzooplankton grazing in both sites supports the notion that phytoplankton net growth rate, is a powerful trigger of microzooplankton grazing in productive areas (York et al., 2011; Sanderson et al., 2012). In the sewage-affected site, however, microzooplankton were activated at higher phytoplankton growth rates than in the site with low exposure to organic pollution. The transient uncoupling of rates at low μ-values, evidences that phytoplankton production must exceed a higher threshold in order to activate microzooplankton feeding. Thus, besides the high resilience of plankton community, results warn on the lower reactivity potential of micrograzers exposed to sewage pollution.

Experiments conducted in the sewage-affected site showed high prevalence of non-linear feeding response. High instability of plankton assemblages as a result of continuously shifting nutrient ratio and the higher prey density in CV, may be the main factors underlying non-linear responses. In addition, low consumer to prey ratio poses serious difficulties on the calculation of in situ microzooplankton grazing by the traditional dilution protocol. Such uncertainties may be reduced by the incorporation of highly diluted treatments to the dilution series and the monitoring of plankton density and composition during the incubation time (Gallegos, 1989; Dolan et al., 2000; First et al., 2007). In fact, during the experiments conducted in July 2008, the addition of a highly diluted treatment to the dilution series, resulted in higher rate estimates in the site exposed to sewage pollution, suggesting that phytoplankton growth and mortality in this site were most likely underestimated.

Saturating prey concentration, P_S, showed a linear relationship with chlorophyll *a* content at the beginning of the incubation, P_0. Experiments with concave curves, however, did not coincide with the highest chlorophyll *a* concentrations recorded during the study, which implies that the concentration of autotrophic prey is not the exclusive factor leading to saturated feeding response. Instead, saturated feeding occurred in concurrence with the highest values of μ and m. It has been suggested that the positive correlation between P_S and P_0 results from the adjustment of microzooplankton feeding in response to newly produced algal biomass, and that this phenomenon is a functional mechanism that stabilizes phytoplankton populations (Sanderson et al., 2012). On the other hand, saturating prey concentration showed no relationship with the initial concentration of microzooplankton, Z. The lack of interrelation between P_S and Z, has been ascribed to the difficulty of obtaining accurate estimation of Z (Sanderson et al., 2012). The results support this idea and emphasize that the identification of the trophic position of species within food chains is fundamental to improve grazing formulations. An additional V-shaped functional response (Elser and Frees, 1995; Teixeira and Figueiras, 2009) was obtained during March 2010 in CV. This feeding behavior was attributed to changes in the clearance rate of microzooplankton due to food saturation and the stimulus of certain prey in undiluted treatments due to selective feeding (Teixeira and Figueiras, 2009).

The main difficulties emerging in non-linear plots are associated to the inaccurate identification of the trophic role of species. As the interconnectivity of species increases, the interpretation of dilution plots turns uncertain. Mixotrophy, intraguild predation and prey switching may increase the connectivity of species, leading to a vast range of potential trophic configurations (Boyce et al., 2015). The occurrence and prevalence of non-linear behavior in dilution experiments conducted in eutrophic habitats, evidences the need for developing an analytical complement to precisely quantify feeding rates.

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