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3 **Feeding behaviour and non-linear responses in dilution**
4 **experiments in a coastal upwelling system**

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7 I. G. Teixeira*, F. G. Figueiras

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9 Instituto de Investigaciones Mariñas (CSIC), Eduardo Cabello 6, 36208 Vigo, Spain

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12 *Corresponding author

13 Email: isabeltx@iim.csic.es

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19 RUNNING HEAD: Nonlinear feeding responses in dilution experiments

1 ABSTRACT: The occurrence of non-linear responses in several dilution experiments
2 conducted in the coastal upwelling system of the Ría de Vigo was examined in relation
3 to the possible violation of the basic assumptions of the technique. In addition to linear
4 responses, two types of non-linear responses, saturated and saturated-increased
5 responses, were obtained. Saturated responses are those showing constant net growth
6 rates at lower dilutions, while saturated-increased responses depict the increase of net
7 growth rates in low diluted bottles. Evidences relating these two non-linear responses to
8 nutrient limitation or changes in the microzooplankton community were not definitive.
9 In contrast, saturated and saturated-increased responses were frequent when the
10 percentage of microzooplankton was relatively low within a very abundant and diverse
11 plankton community. We suggest that saturated feeding responses were related to the
12 achievement of a maximum ingestion rate by microzooplankton and that saturated-
13 increased responses were associated with selective feeding by microzooplankton at
14 times when microzooplankton was feeding at its maximum ingestion rate. Simulated
15 dilution experiments incorporating these two assumptions, two consumers and three
16 preys were able of reproducing the three types of responses. The results indicate that
17 non-linear responses in dilution experiments must be expected in regions with high and
18 diverse food abundance, which should allow prey selection by microzooplankton.

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20 KEY WORDS: Non-linear feeding responses, dilution technique, microzooplankton,
21 plankton community, upwelling systems.

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INTRODUCTION

Since its introduction by Landry & Hassett (1982), the dilution technique, later adapted to accommodate conditions of nutrient limitation (e.g. Andersen et al. 1991, Landry 1993, 1994), has extensively been used to estimate the impact of nano- and microzooplankton (hereafter microzooplankton) on phytoplankton (Calbet & Landry 2004) and bacteria (e.g. Landry et al. 1984, Tremaine & Mills 1987, Worden & Binder 2003) in a wide variety of marine ecosystems. This technique, in which the seawater sample is diluted at several levels with filtered seawater from the same location to reduce the encounter rate of microzooplankton with their preys, relies on three fundamental assumptions regarding interactions among nutrients, phytoplankton and microzooplankton (Landry & Hassett 1982). First, it is assumed that phytoplankton grows exponentially. Second, the growth of a given individual phytoplankton is independent on the presence of other phytoplankton individuals and is not nutrient limited. This means that the specific phytoplankton growth rate does not differ between dilution treatments. Finally, the probability of a phytoplankton cell being grazed is directly related to its encounter rate with microzooplankton, which implies that the number of cells ingested by a given microzooplankton organism is linearly related to prey density. When these requirements are accomplished, a negative linear relationship between the net growth rate of phytoplankton (k , d^{-1}) and the fraction of unfiltered seawater (X) is obtained, with the slope representing the mortality rate of phytoplankton due to microzooplankton grazing (m , d^{-1}) and the y-axis intercept providing an estimate of the phytoplankton growth rate in the absence of predators (μ , d^{-1}):

$$k = \mu - mX \quad (1)$$

1 However, non-linear relationships are frequently reported (e.g. Gallegos 1989, Dolan
2 et al. 2000, Strom et al. 2001), with saturated feeding responses, those showing constant
3 net growth rates at low dilutions, being common in eutrophic systems. On occasions,
4 these types of non-linear relationships have been attributed to changes in the individual
5 feeding impact of microzooplankton in response to variations in food availability along
6 the dilution series (Gallegos 1989, Moigis 2006). Nevertheless, changes in feeding
7 impact can also be due to variations in the microzooplankton community (Dolan et al.
8 2000, Dolan & McKeon 2004, Agis et al. 2007). Thus, changes in growth and mortality
9 of the several microzooplankton species during incubation can induce nonlinearities in
10 their total and/or relative abundance within the dilution series and, consequently,
11 modify their feeding impact. In fact, microzooplankton dynamics during incubation was
12 used as a major criticism to the dilution technique, because it would cause mortality
13 patterns of phytoplankton quite different to those occurring in natural communities
14 (Dolan & McKeon 2004).

15 Here we analyse the results of 8 dilution experiments performed in a coastal
16 upwelling system in which linear and non-linear responses were obtained. The main
17 purpose was to investigate the causes for the occurrence of non-linear responses, taking
18 into account the possible violation of the assumptions on which the dilution technique
19 relies. Understanding the mechanisms behind the appearance of non-linear feeding
20 responses in dilution experiments is crucial to accept or reject this methodology as a
21 useful tool to estimate the microzooplankton impact in aquatic systems.

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MATERIALS AND METHODS

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Experimental setup. Net growth rates of plankton components $\leq 200 \mu\text{m}$ in the coastal upwelling system of the Ría de Vigo (NW Iberia) were estimated using the

1 dilution technique (Landry & Hassett 1982). A total of 8 experiments were done in
2 February, April, July and September 2002, two times each month. All experimental
3 containers, bottles, filters and tubing were soaked in 10% HCl and rinsed with Milli-Q
4 water before each experiment. Sampling took place at dawn in a station situated in the
5 main channel at the central part of the Ría de Vigo (Fig. 1) with a 30 l Niskin bottle that
6 was dipped twice at the surface. Water from the first dip was gravity filtered through a
7 0.2µm Gelman Suporcap filter. Although cell breakage during filtration may increase
8 the concentration of dissolved organic matter and inorganic nutrients, we did not
9 observe significant differences between filtered and raw seawater either in dissolved
10 organic carbon or dissolved inorganic nitrogen (t-test for paired samples $p = 0.94$ and
11 0.32 , respectively) using this filtration system. Measured volumes of filtered water and
12 unfiltered seawater obtained from the second dip were gently combined into carboys to
13 obtain dilution levels of ~10, 20, 40, 60, 80 and 100% of unfiltered seawater. The
14 dilution levels were checked from chlorophyll *a* (chl *a*) concentrations determined in
15 triplicate samples. Then, two clear polycarbonate bottles of 2.3 l were completely filled
16 from each dilution level and incubated for 24 hours at simulated *in situ* light and
17 temperature conditions. Carbon biomass (mg C m^{-3}) of autotrophic and heterotrophic
18 pico- ($\leq 2 \mu\text{m}$), nano- (2 to 20 μm) and microplankton (20 to 200 μm) were determined
19 in the initial unfiltered seawater and in all experimental bottles after incubation. The
20 initial concentrations for each dilution were estimated taking into account the dilution
21 factor. Chl *a* was also determined in all bottles after incubation.

22 **Analyses.** Chl *a* was determined by fluorometry after filtering subsamples of 250 ml
23 through 25mm Whatman GF/F filters. The filters were then stored frozen at -20°C until
24 pigments were extracted in 90% acetone at 4°C in the dark during 24 hours.

1 Pico- and nanoplankton were determined in subsamples of 10 ml fixed with buffered
2 0.2 μm filtered formaldehyde (2% final concentration) and stained with DAPI at 0.1 μg
3 ml^{-1} final concentration (Porter & Feig 1980). After 10 minutes in the dark, samples
4 were filtered through 0.2 μm black Millipore-Isopore filters. The filters were then
5 immersed in low fluorescence immersion oil and examined at x1000 magnification
6 using an epifluorescence microscope. Autotrophic organisms were enumerated under
7 blue light excitation and heterotrophic organisms were counted under excitation with
8 UV light. We realize that *Prochlorococcus* cannot be accurately counted with this
9 technique, but their abundance is not important in this coastal system (Rodriguez et al.
10 2003). Bacterial biomass was estimated according to Lee & Furhmann (1987).
11 Dimensions of several individuals of the other groups were taken and cell volumes were
12 calculated assuming spherical shape. Cell carbon was estimated following Verity et al.
13 (1992) for pico- and nanoflagellates and Bratbak & Dundas (1984) for *Synechococcus*-
14 type cyanobacteria.

15 Microplankton was determined in subsamples of 250-500 ml preserved in Lugol's
16 iodine. Depending on chl *a* concentration, a variable volume of 10-100 ml was
17 sedimented in composite sedimentation chambers and observed through an inverted
18 microscope. When needed, due to low abundances, additional volumes were
19 sedimented. The organisms were counted and identified to the species level when
20 possible. Phototrophic and heterotrophic species of dinoflagellates were differentiated
21 following Lessard & Swift (1986) and also using epifluorescence microscopy.
22 Dimensions were taken to calculate cell biovolumes after approximation to the nearest
23 geometrical shape (Hillebrand et al. 1999) and cell carbon was calculated following
24 Strathmann (1967) for diatoms and dinoflagellates, Verity et al. (1992) for flagellates
25 and Putt & Stoecker (1989) for ciliates.

1 Net growth rates k (d^{-1}) were estimated as:

$$k = \frac{1}{t} \cdot \ln\left(\frac{C_t}{C_0}\right) \quad (2)$$

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5 where t is the duration of the experiment (1 day) and C_0 and C_t are the initial and final
6 chl a concentration or carbon biomass, respectively.

9 RESULTS AND DISCUSSION

10 Feeding responses

11 Three types of feeding responses were found for total autotrophic (AC), total
12 heterotrophic (HC) plankton ≤ 200 μm , chl a and the several plankton groups considered
13 (Figs. 2 & 3, Tables 1 & 2). In addition to linear (Figs. 2a-c) and saturated feeding
14 responses (Figs. 2e-f), a third type of response (Figs. 2g-i) in which the net growth rates
15 increased in the low diluted bottles (hereafter named saturated-increased response) was
16 also obtained. To our knowledge, this third type of response was only reported by
17 Gallegos (1989) and Elser & Frees (1995). Linear relationships were common in
18 February and April, and saturated or saturated-increased responses were more frequent
19 in July (Fig. 3). September did not show a clear dominance of any of the three types of
20 responses.

21 According to the architecture of the dilution technique (Landry & Hassett 1982),
22 negative linear relationships between the net growth rate and the fraction of unfiltered
23 seawater occur when the specific growth rate keeps constant at all dilution levels and
24 the mortality increases proportionally with the increase in food abundance (represented

1 by the fraction of unfiltered seawater in eq. 1). Therefore, deviations from linearity
2 mean that any of these two conditions are not accomplished: i.e., (1) the specific growth
3 rate differs between dilution treatments and/or (2) mortality rate is not linearly related to
4 prey concentration. In the following sections, the occurrence of non-linear responses
5 will be examined in relation to possible variations in these 2 rates.

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Changes in the specific growth rate

8 As all bottles were incubated under the same conditions during each particular
9 experiment, the influence of physical variables prone to affect growth rates, as light and
10 temperature, should have been similar in all incubation bottles. In contrast, changes in
11 concentrations of some chemical components within the dilution bottles could occur.
12 Among all chemicals, nutrients are of main concern in these experiments, since it has
13 been shown that differences in nutrient concentrations along the dilution series can
14 induce changes in phytoplankton growth rates, whenever phytoplankton is nutrient-
15 limited (Andersen et al. 1991, Ayukai 1996, Gaul et al. 1999). Thus, high
16 concentrations of regenerated nutrients in low diluted bottles can result from increased
17 microzooplankton activity due to high food abundance, and this can enhance
18 phytoplankton specific growth rates. In contrast, highest diluted bottles would content
19 lower concentrations of regenerated nutrients, since microzooplankton consumption
20 should be less important. This raise in the specific growth rates in the low diluted
21 bottles would result in the increment of net growth rates and so lead to the appearance
22 of non-linear feeding responses.

23 To avoid the effect of nutrient limitation, it has been proposed to add nutrients to all
24 incubation bottles well in excess for phytoplankton growth (Landry & Hassett 1982,
25 Andersen et al. 1991, Landry 1994). However, the consequences of this experimental

1 step are controversial, because it can cause losses of oligotrichous ciliates (Gifford
2 1988) and also affect phytoplankton growth negatively (Lessard & Murrell 1998,
3 Worden & Binder 2003). Moreover, changes in microzooplankton behaviour and in the
4 shape of the functional response have also been reported when nutrients were added
5 (Worden & Binder 2003). Owing to these uncertainties and with the aim of maintaining
6 the plankton community as close as possible to *in situ* conditions, we did not add
7 nutrients to our incubation bottles. Consequently, this could have caused the appearance
8 of non-linear responses in our dilution experiments.

9 Initial nutrient concentrations (Table 3) were in some cases extremely low. Total
10 inorganic nitrogen was significantly lower ($p < 0.01$, t-test for two samples) in the two
11 experiments of April ($<1 \mu\text{mol l}^{-1}$) and in the experiment of July 18 ($1.38 \mu\text{mol l}^{-1}$).
12 Initial concentrations of phosphate were also significantly lower ($p < 0.05$) in both
13 experiments of April and in the experiment of September 19. If nutrient limitation was
14 responsible for the appearance of non-linear responses, a higher frequency of saturated
15 and saturated-increased responses should be expected under the most limiting
16 conditions of April. However, responses in April were linear whereas saturated and
17 saturated-increased responses were more frequent in July (Fig. 3). On September 19 the
18 three responses occurred (Fig. 3). Besides, saturated and saturated-increased responses
19 were also obtained for heterotrophic plankton organisms in July (Fig. 3), and although
20 heterotrophs can compete with phytoplankton for inorganic nutrients (e.g. Wheeler &
21 Kirchman 1986), their specific growth rates should be less affected. Therefore, we can
22 cautiously conclude that nutrient limitation was not the main factor causing saturated
23 and saturated-increased response in our experiments, despite nutrient influence was not
24 specifically assessed.

25

Changes in the mortality rate

The mortality rate at each dilution level (m_x , d^{-1}) is a function of microzooplankton clearance rate (c , $\mu l \text{ pg C}^{-1} \text{ d}^{-1}$) and microzooplankton abundance (Z_x , $\text{pg C } \mu\text{l}^{-1}$) (Landry & Hasset 1982, Gallegos 1989):

$$m_x = cZ_x \quad (3)$$

For the case of linear relationships, it is assumed that microzooplankton ingests preys at its maximum clearance rate. Therefore, the mortality rate along a dilution series should increase according to the increase in microzooplankton abundance, which in turn is proportional to the fraction of unfiltered seawater. Consequently, any change in the proportionality of the mortality rate (m_x) in a dilution experiment must be related to (a) changes in microzooplankton abundance and/or (b) changes in the clearance rate, during the incubation and along the dilution series.

Changes in microzooplankton abundance

Changes in microzooplankton growth and mortality within dilution series, which resulted in changes in microzooplankton abundance, have been reported (Dolan et al. 2000, Dolan & McKeon 2004, Agis et al. 2007). Thus, Dolan et al. (2000) observed that net growth rates of oligotrichs and tintinnids increased in the high diluted bottles following the increase in the abundance of potential nanoplankton preys. In contrast, net growth rates remained constant at the high prey abundance in the low diluted bottles, which coincided with saturation in the chl *a* response. Interestingly, Gallegos (1989) simulated a saturated-increased response assuming higher predation on microzooplankton in low diluted bottles, and concluded that predation on

1 microzooplankton would release preys from microzooplankton impact allowing them to
2 increase their net growth rates.

3 Changes in the microzooplankton community also occurred in some of our
4 experiments (Table 4). However, these changes were linear, with microzooplankton
5 increasing (slope > 1) or decreasing (slope < 1) during incubations. For the cases with
6 not significant regressions ($0.37 \leq r^2 \leq 0.66$) between initial and final microzooplankton
7 abundance, changes were also linear. There was no case showing a regular decrease at
8 lower dilutions that could be related to the appearance of saturated-increased responses.
9 Moreover, most of the not significant regressions occurred on February 28 and in the
10 two experiments of April, when the responses in dilution experiments were linear (Fig.
11 3).

12 According to equation (3) linear changes in the microzooplankton abundance without
13 variations in the clearance rate during the incubation can cause modifications in the
14 slope defining mortality rates, but not in the shape of the response. This can be
15 appreciated comparing the real responses obtained in the experiments with the expected
16 responses assuming that microzooplankton abundance varied linearly while clearance
17 rate was maintained constant. The results show that real and simulated responses were
18 different (Fig. 4). Saturated-increased responses occurred when microzooplankton
19 biomass did not change during the experiment (Fig. 4a, Table 4), as well as when there
20 was an increase (Fig. 4b) or decrease (Fig. 4c) in the microzooplankton biomass.
21 Consequently, we can conclude that the non-linear responses that we found in our
22 dilution experiments were not apparently related to changes in the microzooplankton
23 community. Instead, they would be attributed to changes in the clearance rate.

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1 *Changes in feeding behaviour*

2 The carbon specific ingestion rate of a given microzooplankton organism (I , pg
3 Cprey pg Cmicrozoo⁻¹ d⁻¹) is a function of the specific clearance rate (c , μl pgC⁻¹ d⁻¹)
4 and prey concentration (P , pgC μl⁻¹) (e.g. Frost 1972, Landry & Hasset 1982):

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$$I = cP \quad (4)$$

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8 In linear responses it is supposed that microzooplankton ingests preys at its
9 maximum clearance rate, and so ingestion rates increase in proportion to the increase in
10 prey abundance, represented by the fraction of unfiltered seawater. However, it has been
11 observed that microzooplankton may reach a maximum ingestion rate at high food
12 concentration. For example, Heinbokel (1978) showed that the ingestion rate of
13 tintinnids increased up to a maximum value following the availability of preys. Then,
14 the maximum ingestion rate remained constant in spite of further increases in prey
15 abundance. In these cases, as food availability increases there is a proportional decrease
16 in the microzooplankton clearance rate to maintain the ingestion at its maximum
17 constant value.

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$$I_{max} = cte = cP \quad (5)$$

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21 This feeding behaviour is an argument often used to explain the occurrence of
22 saturated feeding responses in dilution experiments (e.g. Gallegos 1989, Moigis 2006).
23 However, the occurrence of saturated-increased responses necessarily implies the
24 decrease in the ingestion rate when food increases, which means that the decrease in the

1 clearance rate should be more abrupt and not proportional to the increase in food
2 availability.

3 Total carbon biomass in our experiments (Table 3) was significantly higher in July
4 and September than in February and April ($p < 0.05$, t-test for two samples). In July,
5 when only two responses were linear (Fig. 2), initial chl *a* concentrations were also
6 higher ($p < 0.05$) and percentages of microzooplankton biomass lower ($p < 0.001$) than
7 in the other experiments (Table 2). From this, we can infer that saturated and saturated-
8 increased responses were more frequent in the experiments where there was a low
9 percentage of microzooplankton within a very abundant plankton community. The
10 excess of food can saturate the ingestion of microzooplankton and therefore cause
11 saturated feeding responses. The reasons for the decrease in the ingestion rate that lead
12 to the occurrence of saturated-increased responses are not clear, but we hypothesize
13 they could result from active or passive food selection by microzooplankton when prey
14 availability is high. Active prey selection is a well documented process and it has been
15 attributed to several factors, as morphological characteristics, presence of metabolites or
16 nutritive value of preys (e.g. Verity 1991, Wolfe et al. 1997, John & Davidson 2001).
17 This selectivity is more important in situations with high food availability (Heinbokel
18 1978, Jürgens & DeMott 1995, John & Davidson 2001), when the concentration of
19 preferred preys is sufficient to satisfy the nutritional needs of the predator. Passive
20 selection could be due to different encounter rates between microzooplankton and its
21 preys, which can release the less abundant preys from predation in situations of high
22 food availability, because the maximum ingestion rate of microzooplankton is satisfied
23 with the more abundant preys. Both types of selection would explain why saturated-
24 increased responses in our dilution experiments were more frequent in July, when food
25 was abundant (Table 3).

1 This hypothesis on prey selectivity was assessed simulating 2 dilution experiments.
2 The 2 simulations contained 3 preys (A, B and C) and two microzooplankton species
3 (M1 and M2) with initial biomasses ($\text{pg C } \mu\text{l}^{-1}$) proportional to the dilution levels (prey
4 A = 30X, prey B = 100X, prey C = 80X, M1 = 20X and M2 = 20X, X is the fraction of
5 unfiltered seawater). In the first simulation, the maximum ingestion rate of both M1 and
6 M2 was set up at $5 \text{ pg C pg C}^{-1} \text{ d}^{-1}$. M1 ingests prey A and B but prefers prey A, and
7 this preference establishes when its maximum ingestion rate is satisfied. Note that this
8 preference of M1 for the less abundant prey A can be ascribed to active selection. M2
9 only ingests prey C. With these assumptions the ingestion rates of each prey at each
10 dilution level were calculated with equation (4) assuming a maximum clearance rate of
11 $0.11 \mu\text{l pg C}^{-1} \text{ d}^{-1}$ for both consumers. Then, once the available food (prey A + prey B) in
12 the low diluted bottles allows M1 to ingest $\geq 5 \text{ pg C pg C}^{-1} \text{ d}^{-1}$, the ingestion of prey B
13 was calculated as the difference between this maximum ingestion rate and the ingestion
14 calculated for prey A. This implies that the clearance rate of M1 for prey B diminishes.
15 With these ingestions calculated for each prey and dilution level, the corresponding
16 clearance rates for each prey and dilution were estimated with equation (4). Clearance
17 rates were then used in equation (3) to estimate mortality rates at each dilution level,
18 which were used to estimate the net growth rates of each prey as the difference between
19 the specific growth rate ($\mu = 0.7 \text{ d}^{-1}$ for the 3 preys) and the mortality rates. In the
20 second simulation, which corresponded to passive selection of the more abundant prey,
21 the maximum ingestion rate of M1 was set at $10 \text{ pg C pg C}^{-1} \text{ d}^{-1}$ with no prey
22 preference. All the other parameters and rates were equal to those used in the first
23 simulation.

24 The outputs of the 2 simulations (Fig. 5) show the 3 types of responses. Feeding on
25 prey C was in the two cases saturated, because consumption of this prey remained

1 constant after M2 reached its maximum ingestion rate, despite further increases in food
2 concentration. In contrast, the responses of prey A and B changed according to the
3 restriction imposed. For the case of active selection (Fig. 5a), the release of pressure on
4 the more abundant prey B allows it to grow, whereas for the case of passive selection
5 (Fig. 5b) the release of pressure occurs on the less abundant prey A. The biomass of the
6 other preys (A and B respectively) was not enough to saturate the ingestion rate of M1,
7 and this resulted in linear responses for both.

8

9 **Some considerations on viral influence**

10 It must be noted that in the present analysis the virus influence was not specifically
11 assessed. Regarding the dilution technique, two basic concerns can be distinguished.
12 The first refers to the use of filtered water through 0.2 μm pore size, which is grazer-
13 free but not virus-free. Thus, virus concentration should be equal at all dilution levels,
14 and if viral infection is density dependent, mortality due to virus should be constant and
15 hence lead to the sub-estimation of specific growth rates (Evans et al. 2003, Baudoux et
16 al. 2006). Although an accurate estimate of rates is the objective of dilution
17 experiments, a constant mortality factor due to virus at all dilution levels should not
18 induce the appearance of non-linear responses. The second concern relates to enhanced
19 viral infection at high grazing activity (Šimek et al. 2001, Sime-Ngando & Pradeep Ram
20 2005, Weinbauer et al. 2007), which would lead to higher viral infection in low diluted
21 bottles. This can be relevant concerning non-linear responses, because the enhanced
22 virus infection would cause a significant increase in lysis mortality during the
23 incubation time, enhancing nutrient and DOM concentrations in those bottles and so
24 affect specific growth rates. To what extent viruses are responsible for the appearance of
25 non-linear responses in dilution experiments is a topic that needs further assessment.

Feeding behaviour in the Ría de Vigo

1
2 Whilst definitive conclusions on the occurrence of food selection cannot be attained
3 from the output of dilution experiments, because incubations are done with all
4 populations interacting at the same time, the different responses obtained (Fig. 3a,
5 Tables 1 & 2) point at its occurrence in the Ría de Vigo. Non-linear responses were
6 more frequent in summer (July) and at the beginning of autumn (September), when
7 typically upwelling brings nutrients to the system and plankton diversity is high
8 (Figueiras et al. 2002). At that time, several heterotrophic (ciliates, *Protoperidinium*
9 spp.) and mixotrophic species (*Ceratium* spp., *Dinophysis* spp.) of different size coexist
10 with a very abundant and diverse autotrophic community composed of several diatom
11 species and ANF. This situation would be suitable for the appearance of different
12 trophic relationships in which passive or active selection of food could be possible.
13 Winter blooms of diatoms, like that recorded in February (Álvarez-Salgado et al. 2005),
14 may also be appropriate for this type of relationships (Fig. 3a), because grazing on
15 diatoms can release of predation to other species. In situations with relatively low
16 abundance of food, such as that in April, selection of food should be less feasible (Fig.
17 3a). In general, HB and HPF appear to be a suitable food for microzooplankton, because
18 responses (with the exception of HB on February 21) were linear or saturated (Fig. 3a).
19 In contrast, *Synechococcus* frequently showed saturated-increased responses, mainly in
20 summer and autumn (Fig. 3a). This suggests that *Synechococcus* was not a preferred
21 prey, which agrees with previous results reported by other authors. Thus, Caron et al.
22 (1991) observed one case of active selection against *Synechococcus*, which was not a
23 good food source for protozoa when compared with bacteria. The results obtained by
24 Fonda Umani & Beran (2003) also suggest this type of negative selection on
25 *Synechococcus*.

1 Chl *a* and AC not always showed the same type of response and rates (Fig. 3b, Table
2 1), which might be attributed to the different carbon:chl_a ratios of the several
3 phytoplankton species and then result in different feeding responses for these two bulk
4 properties.

5

6 **Concluding remarks**

7 Although not clearly observed, there is evidence that non-linear feeding responses in
8 this work occurred due to a non-linear feeding behaviour of microzooplankton.
9 Furthermore, we suggest that the non-linear feeding behaviour of microzooplankton
10 may be consequence of the complexity of the microbial food web, where a diverse
11 plankton community can lead to the occurrence of numerous trophic interactions.
12 Microzooplankton can feed on a wide variety of preys, from phytoplankton to bacteria
13 and other heterotrophic organisms (e.g. Rassoulzadegan & Sheldon 1986, Jeong 1999).
14 Consumers may hence modify their food preference depending on the abundance and
15 quality of preys and so release or hold pressure on some plankton components,
16 producers or consumers, which would cause an increase or decrease in their
17 populations. Moreover, mixotrophic organisms are relatively abundant in plankton
18 communities (Bockstahler & Coasts 1993, Stoecker 1999, Zubkov & Tarran 2008), and
19 they often regulate their nutrition mode according to food and nutrient accessibility,
20 changing to heterotrophy under conditions of enough food or nutrient limitation
21 (Sanders et al. 1990, Arenovski et al. 1995). Therefore, feeding behaviour should be
22 especially relevant in aquatic systems with high availability and diversity of preys.

23 Non-linear feeding behaviour of microzooplankton implies the violation of the
24 assumption that the consumption rate is linearly related to the dilution factor. However,
25 this must not invalidate the use of the dilution technique. Non-linear feeding behaviour

1 of microzooplankton is an intrinsic property of these organisms and it should also occur
2 in the environment. The main concern under the occurrence of non-linear feeding
3 responses in dilution experiments is the accurate calculation of the rates. If we assume
4 that the net growth rate in the undiluted bottle integrates all growth and mortality
5 processes operating in the environment, the critical point is to obtain accurate estimates
6 of specific growth rates in the absence of predators. In our experiments the specific
7 growth rates calculated as the y-axis intercept of the regression of the linear part of the
8 non-linear responses (Strom et al. 2001, Moigis 2006) do not differ from the specific
9 growth rates estimated from simulated responses as in figure 4, that is, using final
10 microzooplankton biomasses and assuming maximum clearance rates (Fig. 6). This
11 suggests that estimates of specific growth rates using the linear part of the responses are
12 robust. However, this may not be the rule and therefore it seems appropriate to increase
13 the number of highly diluted bottles to ensure that these regressions are obtained where
14 processes are more similar to those occurring in a sample without predators (Gallegos
15 1989). This would avoid the use of the intermediate dilutions which carry higher
16 uncertainties in the accomplishment of the requirements of the method. The specific
17 growth rates thus calculated can be used to estimate the mortality due to
18 microzooplankton in the natural sample (Gallegos 1989, Strom et al. 2001).

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9

LITERATURE CITED

- 1
2 Agis M, Granda A, Dolan JR (2007) A cautionary note: Examples of possible microbial
3 community dynamics in dilution grazing experiments. J Exp Mar Biol Ecol 341:176-
4 183
- 5 Álvarez-Salgado XA, Nieto-Cid M, Piedracoba S, Crespo BG, Gago J, Brea S, Teixeira
6 IG, Figueiras FG, Garrido JL, Rosón G, Castro CG, Gilcoto M (2005) Origin and
7 fate of a bloom of *Skeletonema costatum* during a winter upwelling/downwelling
8 sequence in the Ría de Vigo (NW Spain). J Mar Res 63:1127-1149
- 9 Andersen T, Schartau AKL, Paasche E (1991) Quantifying external and internal
10 nitrogen and phosphorus pools, as well as nitrogen and phosphorus supplied through
11 remineralization, in coastal marine plankton by means of a dilution technique. Mar
12 Ecol Prog Ser 69:67-80
- 13 Arenovski AL, Lim EL, Caron DA (1995) Mixotrophic nanoplankton in oligotrophic
14 surface waters of the Sargasso Sea may employ phagotrophy to obtain major
15 nutrients. J Plankton Res 17:801-820
- 16 Ayukai T (1996) Possible limitation of the dilution technique for estimating growth and
17 grazing mortality rates of picoplanktonic cyanobacteria in oligotrophic tropical
18 waters. J Exp Mar Biol Ecol 198:101-111
- 19 Baudoux AC, Noordeloos AAM, Veldhuis MJW, Brussaard CPD (2006) Virally
20 induced mortality of *Phaeocystis globosa* during two spring blooms in temperate
21 coastal waters. Aquat Microb Ecol 44:207-217
- 22 Bockstahler KR, Coats DW (1993) Spatial and Temporal Aspects of Mixotrophy in
23 Chesapeake Bay Dinoflagellates. J Eukaryot Microbiol 40:49-60
- 24 Bratbak G, Dundas I (1984) Bacterial dry matter content and biomass estimation. Appl
25 Environ Microb 48:755-757

- 1 Calbet A, Landry MR (2004) Phytoplankton growth, microzooplankton grazing, and
2 carbon cycling in marine systems. *Limnol Oceanogr* 49:51-57
- 3 Caron DA, Lim EL, Miceli G, Waterbury JB, Valois FW (1991) Grazing and utilization
4 of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory
5 cultures and a coastal plankton community. *Mar. Ecol Prog Ser* 76:205-217
- 6 Dolan JR, Gallegos CL, Moigis A (2000) Dilution effects on microzooplankton in
7 dilution grazing experiments. *Mar Ecol Prog Ser* 200:127-139
- 8 Dolan JR, McKeon K (2004) The reliability of grazing rate estimates from dilution
9 experiments: Have we over-estimated rates of organic carbon consumption? *Ocean*
10 *Science Discussions* 1:21-36
- 11 Elser JJ, Frees DL (1995) Microconsumer grazing and sources of limiting nutrients for
12 phytoplankton growth: Application and complications of a nutrient-deletion/dilution-
13 gradient technique. *Limnol Oceanogr* 40:1-16
- 14 Evans C, Archer SD, Jacquet S, Wilson WH (2003) Direct estimates of the contribution
15 of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp.
16 population. *Aquat Microb Ecol* 30:207-219
- 17 Figueiras FG, Labarta U, Fernández Reiriz MJ (2002) Coastal upwelling, primary
18 production and mussel growth in the Rías Baixas of Galicia. *Hydrobiologia* 484:121-
19 131
- 20 Fonda Umani S, Beran A (2003) Seasonal variations in the dynamics of microbial
21 plankton communities: first estimates from experiments in the Gulf of Trieste,
22 Northern Adriatic Sea. *Mar Ecol Prog Ser* 247:1-16
- 23 Frost BW (1972) Effects of size and concentration of food particles on the feeding
24 behavior of the marine copepod *Calanus pacificus*. *Limnol Oceanogr* 17:805-815

- 1 Gallegos CL (1989) Microzooplankton grazing on phytoplankton in Rhode River,
2 Maryland: nonlinear feeding kinetics. *Mar Ecol Prog Ser* 57:23-33
- 3 Gaul W, Antia AN, Koeve W (1999) Microzooplankton grazing and nitrogen supply of
4 phytoplankton growth in the temperate and subtropical northeast Atlantic. *Mar Ecol*
5 *Prog Ser* 189:93-104
- 6 Gifford DJ (1988) Impact of grazing by microzooplankton in the Northwest Arm of
7 Halifax Harbour, Nova Scotia. *Mar Ecol Prog Ser* 47:249-258
- 8 Heinbokel JF (1978) Studies on the functional role of tintinnids in the southern
9 California Bight. I. Grazing and growth rates in laboratory cultures. *Mar Biol*
10 47:177-189
- 11 Hillebrand H, Dürselen C, Kirschtel D, Pollingher U, Zohary T (1999) Biovolume
12 calculation for pelagic and benthic microalgae. *J Phycol* 35:403-424
- 13 Jeong HJ (1999) The ecological roles of heterotrophic dinoflagellates in marine
14 planktonic community. *J Eukaryot Microbiol* 46:390-396
- 15 John EH, Davidson K (2001) Prey selectivity and the influence of prey carbon:nitrogen
16 ratio on microflagellate grazing. *J Exp Mar Biol Ecol* 260:93-111
- 17 Jürgens K, DeMott WR (1995) Behavioral flexibility in prey selection by bacterivorous
18 nanoflagellates. *Limnol Oceanogr* 40:1503-1507
- 19 Landry MR (1993) Estimating rates of growth and grazing mortality of phytoplankton
20 by the dilution method. In: Kemp PF, Sherr BF, Sherr EB (eds) *Handbook of*
21 *methods in aquatic microbial ecology*. Lewis Publishers, p 715-722
- 22 Landry MR (1994) Methods and controls for measuring the grazing impact of
23 planktonic protists. *Marine Microbial Food Webs* 8:37-57
- 24 Landry MR, Haas LW, Fagerness VL (1984) Dynamics of microbial plankton
25 communities: experiments in Kaneohe Bay, Hawaii. *Mar Ecol Prog Ser* 16:127-133

- 1 Landry MR, Hassett RP (1982) Estimating the grazing impact of marine
2 microzooplankton. *Mar Biol* 67:283-288
- 3 Lee S, Fuhrmann JA (1987) Relationships between biovolume and biomass of naturally
4 derived marine bacterioplankton. *Appl Environ Microb* 53:1298-1303
- 5 Lessard EJ, Murrell MC (1998) Microzooplankton herbivory and phytoplankton growth
6 in the northwestern Sargasso Sea. *Aquat Microb Ecol* 16:173-188
- 7 Lessard EJ, Swift E (1986) Dinoflagellates from the North Atlantic classified as
8 phototrophic or heterotrophic by epifluorescence microscopy. *J Plankton Res*
9 8:1209-1215
- 10 Moigis AG (2006) The clearance rate of microzooplankton as the key element for
11 describing estimated non-linear dilution plots demonstrated by a model. *Mar Biol*
12 149:743-762
- 13 Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic
14 microflora. *Limnol Oceanogr* 25:943-948
- 15 Putt M, Stoecker DK (1989) An experimental determined carbon:volume ratio for
16 marine "oligotrichous" ciliates from estuarine and coastal waters. *Limnol Oceanogr*
17 34:1097-1103
- 18 Rassoulzadegan F, Sheldon RW (1986) Predator-prey interactions of nanozooplankton
19 and bacteria in an oligotrophic marine environment. *Limnol Oceanogr* 31:1010-1021
- 20 Rodríguez F, Pazos Y, Maneiro J, Zapata M (2003) Temporal variation in
21 phytoplankton assemblages and pigment composition at a fixed station of the Ría of
22 Pontevedra. *Estuar Coast Shelf S* 58:499-515
- 23 Sanders RW, Porter KG, Caron DA (1990) Relationship between phototrophy and
24 phagotrophy in the mixotrophic Chrysophyte *Poterioochromonas malhamensis*.
25 *Microbial Ecol* 19:97-109

1 Šimek K, Pernthaler J, Weinbauer MG, Hornák K, Dolan JR, Nedoma J, Mašín M,
2 Amann R (2001) Changes in bacterial community composition and dynamics and
3 viral mortality rates associated with enhanced flagellate grazing in a mesoeutrophic
4 reservoir. *Appl Environ Microb* 67:2723-2733

5 Sime-Ngando T, Pradeep Ram AS (2005) Grazer effects on prokaryotes and viruses in a
6 freshwater microcosm experiment. *Aquat Microb Ecol* 41:115-124

7 Stoecker DK (1999) Mixotrophy among dinoflagellates. *J Eukaryot Microbiol* 46:397-
8 401

9 Strathmann R (1967) Estimating the organic carbon content of phytoplankton from cell
10 volume or plasma volume. *Limnol Oceanogr* 12:411-418

11 Strom SL, Brainard MA, Holmes JL, Olson MB (2001) Phytoplankton blooms are
12 strongly impacted by microzooplankton grazing in coastal North Pacific waters. *Mar*
13 *Biol* 138:355-368

14 Tremaine SC, Mills AL (1987) Tests of the critical assumptions of the dilution method
15 for estimating bacterivory by microeucaryotes. *Appl Environ Microb* 53:2914-2921

16 Verity PG (1991) Feeding in Planktonic Protozoans: Evidence for Non-Random
17 Acquisition of Prey. *J Protozool* 38:69-76

18 Verity PG, Robertson CY, Tronzo CR, Andrews MG, Nelson JR, Sieracki ME (1992)
19 Relationships between cell volume and the carbon and nitrogen content of marine
20 photosynthetic nanoplankton. *Limnol Oceanogr* 37:1434-1446

21 Weinbauer MG, Hornák K, Jezbera J, Nedoma J, Dolan JR, Šimek K (2007) Synergistic
22 and antagonistic effects of viral lysis and protistan grazing on bacterial biomass,
23 production and diversity. *Environ Microbiol* 9:777-788

24 Wheeler PA, Kirchman DL (1986) Utilization of inorganic and organic nitrogen by
25 bacteria in marine systems. *Limnol Oceanogr* 31:998-1009

- 1 Wolfe GV, Steinke M, Kirst GO (1997) Grazing-activated chemical defence in a
2 unicellular marine algae. *Nature* 387:894-897
- 3 Worden AZ, Binder BJ (2003) Application of dilution experiments for measuring
4 growth and mortality rates among *Prochlorococcus* and *Synechococcus* populations
5 in oligotrophic environments. *Aquat Microb Ecol* 30:159-174
- 6 Zubkov MV, Tarran GA (2008) High bacterivory by the smallest phytoplankton in the
7 North Atlantic Ocean. *Nature* 455:224-2

Table 1. Growth (μ , d^{-1}) and mortality (m , d^{-1}) rates obtained from dilution experiments for autotrophic plankton. APF, autotrophic picoflagellates; ANF, autotrophic nanoflagellates; AC, total autotrophic carbon; chl *a*, chlorophyll *a*; ns, not significant; ^asaturated response; ^bsaturated-increased response. For saturated and saturated-increased responses r^2 and μ were obtained by regression of the linear part of the responses and m was estimated as the difference between μ and the net growth rate (k) in the undiluted sample. See text (concluding remarks) and Fig. 6 for more details. $p < 0.05$ for linear responses. The significance of saturated and saturated-increased responses was in some occasions > 0.05 owing to the few points included in the regression.

Date	<i>Synechococcus</i>			APF			ANF			Diatoms			AC			chl <i>a</i>		
	m	μ	r^2	m	μ	r^2	m	μ	r^2	m	μ	r^2	m	μ	r^2	m	μ	r^2
21 Feb	0.11	0.56	0.95 ^b	0.70	1.47	0.96 ^b	ns	ns	ns	0.36	1.32	0.68	0.35	1.27	0.77	0.35	1.36	0.87
28 Feb	ns	ns	Ns	ns	ns	ns	0.75	1.03	0.78 ^b	0.10	0.70	0.93	0.21	0.73	0.81	0.23	0.72	0.69
11 Apr	1.18	2.08	0.81	1.58	0.73	0.97	0.85	0.37	0.89	ns	ns	ns	0.77	0.42	0.93	0.61	0.07	0.81
18 Apr	0.39	0.31	0.95	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.39	0.31	0.95	0.56	0.09	0.99 ^a
18 Jul	0.88	1.13	0.94	1.39	1.39	0.98 ^b	0.33	1.46	0.99 ^b	1.13	1.33	0.92 ^a	1.10	1.33	0.92 ^a	0.58	1.57	0.98 ^b
26 Jul	0.28	1.63	0.89 ^b	2.55	2.86	0.99 ^a	0.0	0.85	0.97 ^b	0.73	1.33	0.99 ^b	0.74	1.35	0.99 ^b	2.22	2.15	0.99 ^a
19 Sep	0.24	0.75	0.92 ^b	1.27	3.14	0.97 ^b	0.79	0.86	0.99 ^a	ns	ns	ns	0.49	0.87	0.77	0.24	0.37	0.87
26 Sep	1.09	1.78	0.99 ^b	1.76	3.04	0.91	0.32	1.49	0.89	ns	ns	ns	0.58	1.75	0.70	0.25	1.31	0.73

Table 2. Growth (μ , d^{-1}) and mortality (m , d^{-1}) rates obtained from dilution experiments for heterotrophic plankton. HB, heterotrophic bacteria; HPF, heterotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HC, total heterotrophic carbon; ns, not significant; ^asaturated response; ^bsaturated-increased response. For saturated and saturated-increased responses r^2 and μ were obtained by regression of the linear part of the responses and m was estimated as the difference between μ and the net growth rate (k) in the undiluted sample. See text (concluding remarks) and Fig. 6 for more details. $p < 0.05$ for linear responses. The significance of saturated and saturated-increased responses was in some occasions > 0.05 owing to the few points included in the regression.

Date	HB			HPF			HNF			HC		
	m	μ	r^2	m	μ	r^2	m	μ	r^2	m	μ	r^2
21 Feb	0.94	1.42	0.97 ^b	2.66	2.24	0.97	ns	ns	ns	0.88	0.73	0.74
28 Feb	1.31	2.24	0.92	1.41	1.25	0.66	ns	ns	ns	1.05	1.57	0.87
11 Apr	1.54	2.44	0.90	1.27	1.97	0.87	0.87	0.74	0.86	0.79	1.06	0.99
18 Apr	1.48	1.24	0.96	2.00	1.78	0.88	0.63	0.11	0.98	0.99	0.48	0.95
18 Jul	1.92	1.56	0.98 ^a	1.99	1.98	0.98 ^a	0.22	1.58	0.68 ^b	0.81	1.40	0.96 ^b
26 Jul	1.68	2.25	0.96 ^a	1.70	2.70	0.96 ^a	1.12	1.81	0.74	1.27	1.82	0.90 ^a
19 Sep	1.82	2.01	0.99	1.88	1.39	0.90	ns	ns	ns	0.63	0.62	0.93
26 Sep	0.97	1.14	0.92 ^a	2.15	1.95	0.91	ns	ns	ns	0.46	0.80	0.81

Table 3. Initial conditions for each experiment. DIN, dissolved inorganic nitrogen; TC, total carbon of plankton community, Microzoo %, percentage of microzooplankton biomass in the plankton community.

Date	Salinity psu	Temperature °C	DIN $\mu\text{mol l}^{-1}$	HPO_4^{2-} $\mu\text{mol l}^{-1}$	Chl <i>a</i> mg m^{-3}	TC mg m^{-3}	Microzoo %
21 Feb	35.5	13.2	5.41	0.41	3.3	200	25
28 Feb	35.4	13.2	4.38	0.34	2.7	84	19
11 Apr	34.6	13.4	0.20	0.17	4.2	143	27
18 Apr	35.3	13.8	0.68	0.18	6.2	341	37
18 Jul	35.2	15.3	1.38	0.37	6.7	819	7
26 Jul	35.3	16.6	3.51	0.51	5.8	333	10
19 Sep	34.8	16.9	2.13	0.31	5.3	407	29
26 Sep	35.0	17.8	3.66	0.41	3.4	460	19

Table 4. Slopes \pm standard error of the initial *versus* final carbon biomass of microzooplankton in the eight dilution experiments. Microzoo, total microzooplankton; HNF, heterotrophic nanoflagellates; HDF, heterotrophic dinoflagellates. For all significant relationships, the y-axis intercept was not significantly different from zero.

* $p < 0.05$; ** $p < 0.01$; ns, not significant.

Date	Microzoo	HNF	HDF	Ciliates
21 Feb	$0.93 \pm 0.19^{**}$	$0.89 \pm 0.17^{**}$	ns	$4.15 \pm 0.86^{**}$
28 Feb	Ns	ns	$2.10 \pm 0.60^*$	$5.09 \pm 1.48^*$
11 Apr	Ns	ns	$1.05 \pm 0.17^{**}$	$1.02 \pm 0.29^*$
18 Apr	Ns	ns	$0.18 \pm 0.03^{**}$	$1.11 \pm 0.34^*$
18 Jul	$2.06 \pm 0.61^*$	$2.99 \pm 0.91^*$	$1.13 \pm 0.26^*$	ns
26 Jul	$1.19 \pm 0.19^{**}$	$1.50 \pm 0.49^*$	$0.54 \pm 0.06^{**}$	ns
19 Sep	$0.99 \pm 0.11^{**}$	$1.63 \pm 0.49^*$	$0.86 \pm 0.10^{**}$	$0.73 \pm 0.23^*$
26 Sep	$1.41 \pm 0.30^{**}$	$2.35 \pm 0.56^*$	$0.56 \pm 0.15^*$	ns

Figure legends

Fig. 1. Map of the Ría de Vigo with the location of the sampled station.

Fig. 2. Examples of the 3 types of responses obtained in the 8 dilution experiments made in the Ría de Vigo. (a to c) linear, (d to f) saturated, (g to i) saturated-increased responses (see text for more details). HB, heterotrophic bacteria; ANF, autotrophic nanoflagellates.

Fig. 3. Distribution of the 3 types of responses found in the 8 dilution experiments for (a) plankton groups and (b) bulk plankton properties. ANF, autotrophic nanoflagellates; APF, autotrophic picoflagellates; HNF, heterotrophic nanoflagellates; HPF, heterotrophic picoflagellates; HB, heterotrophic bacteria; AC, total autotrophic carbon; HC, total heterotrophic carbon. Y-axis is dimensionless.

Fig. 4. Real (black circles) and simulated (open circles) responses of 3 dilution experiments. Simulated responses were made with the microzooplankton biomass at the end of the experiments and assuming a constant maximum clearance rate. These maximum clearance rates were estimated, using the equation (3) in the text, from the mortality rates observed at highest dilution levels in real responses. In (a) and (b) simulated responses are for total microzooplankton; in (c) for heterotrophic dinoflagellates. Note that the impact of changes in microzooplankton biomass is minimal at the highest dilutions.

Fig. 5. Output of the simulations made with dilution experiments considering (a) active and (b) passive selection of preys. See text for details.

Fig. 6. Relationship between the specific growth rates estimated from the regressions of the linear part in non-linear responses of dilution experiments (μ) and the specific growth rates estimated considering the final concentration of microzooplankton (μ') (see also Fig. 4 and Table 4).

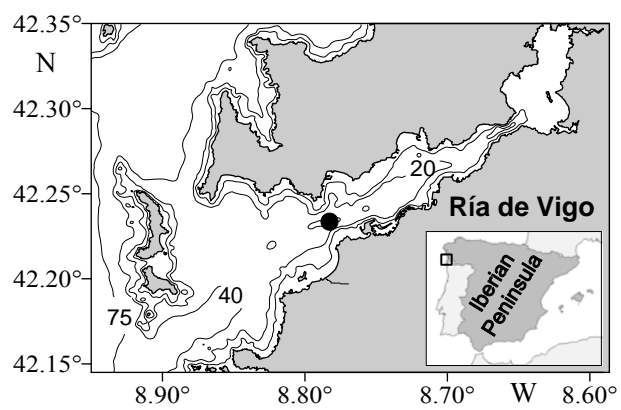


Fig. 1
Teixeira & Figueiras

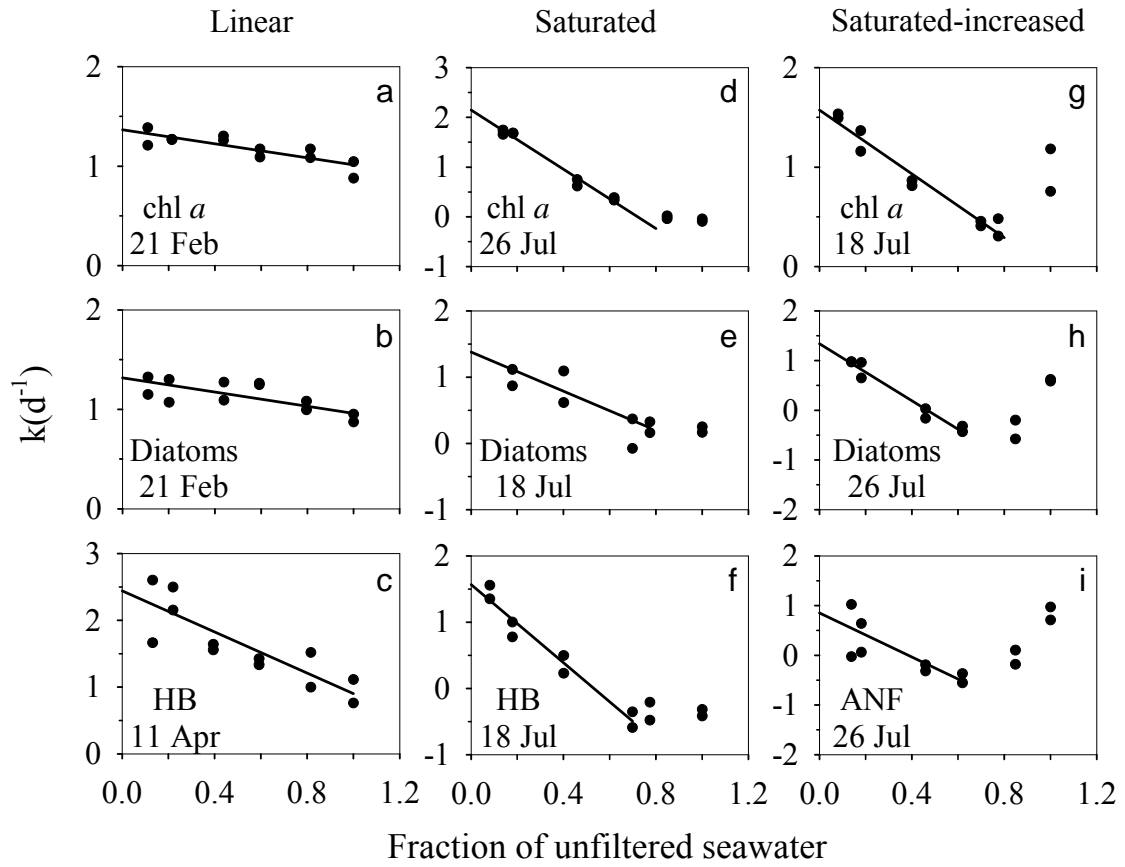


Fig. 2
Teixeira & Figueiras

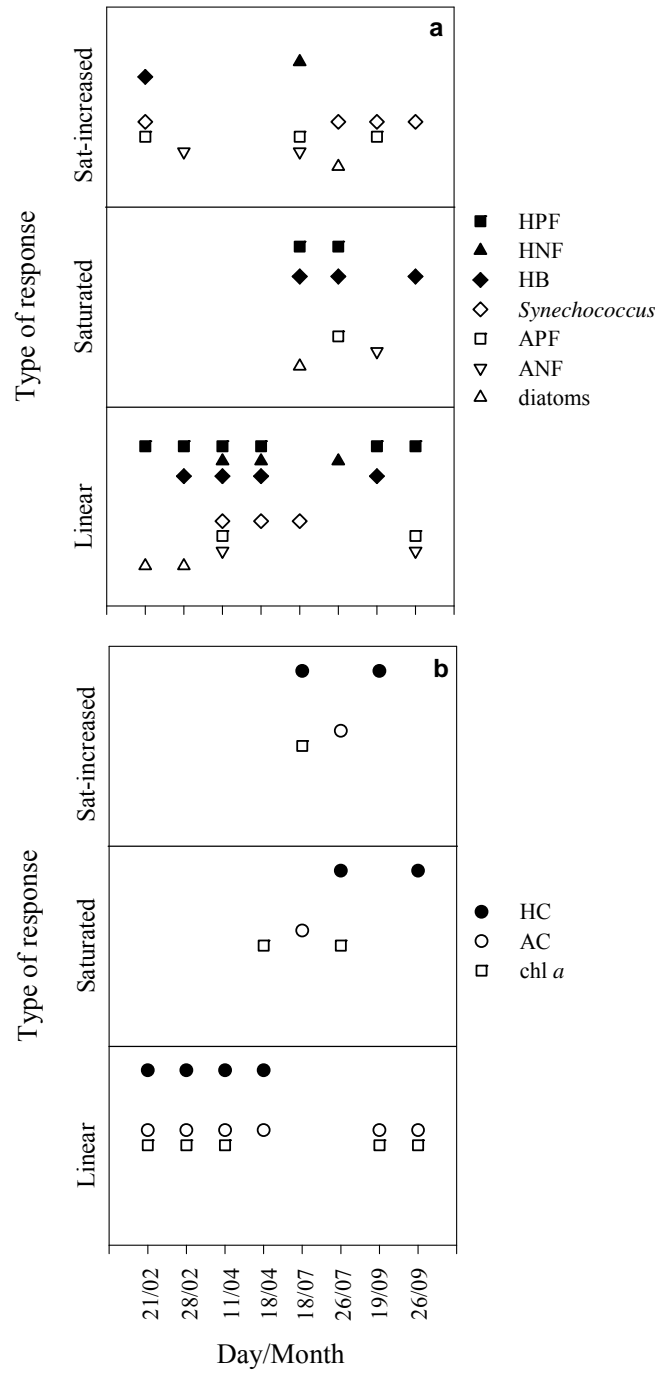


Fig. 3
Teixeira & Figueiras

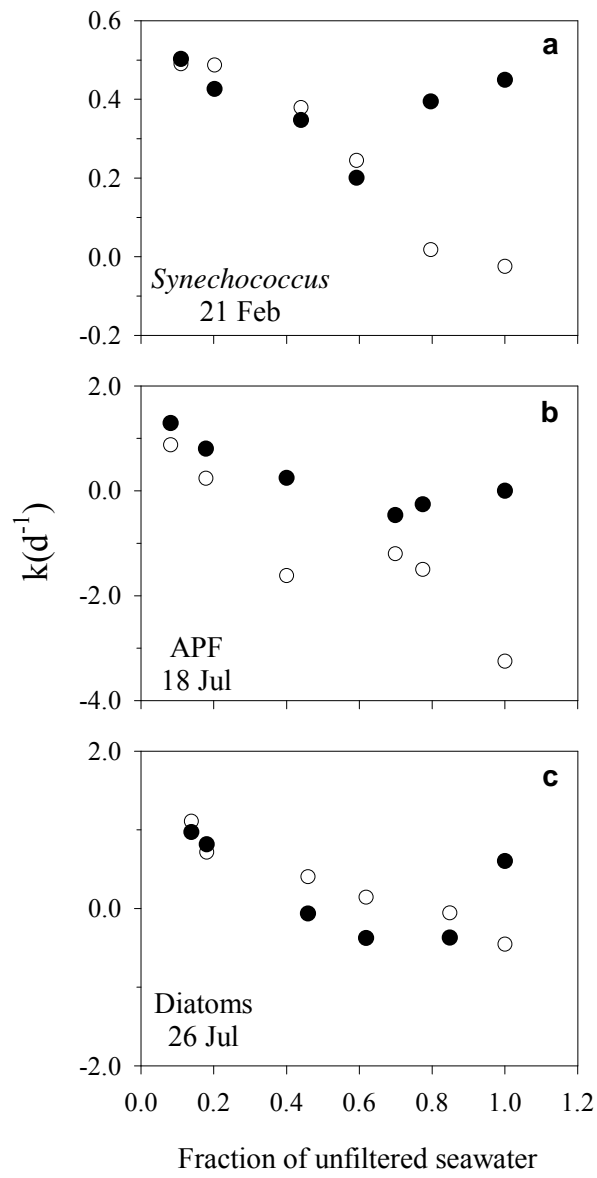


Fig. 4
Teixeira & Figueiras

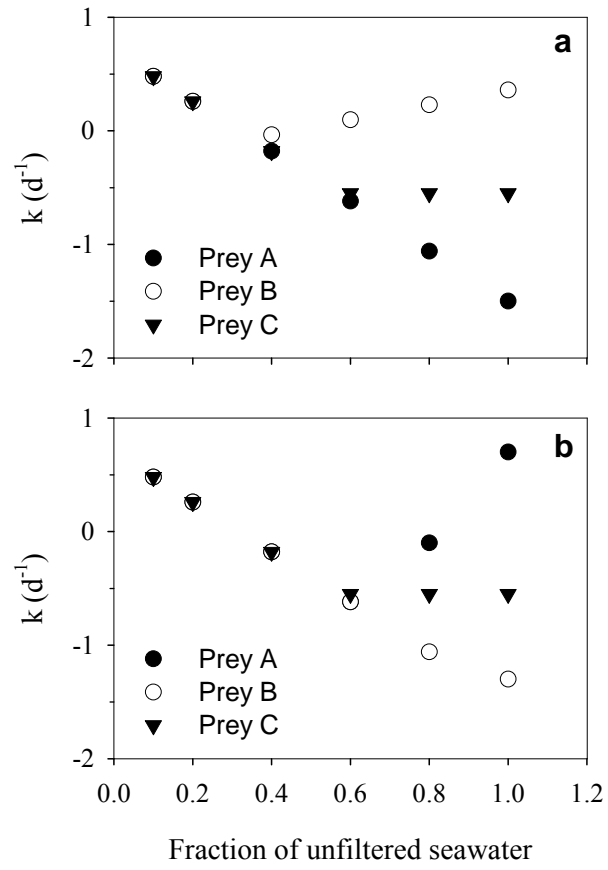


Fig. 5
Teixeira & Figueiras

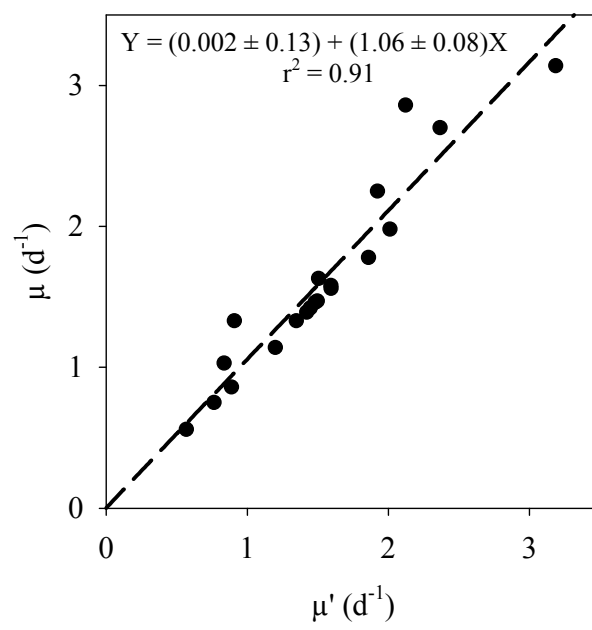


Fig. 6
Teixeira & Figueiras