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RESEARCH ARTICLE

Ammonium, nitrate and phytoplankton interactions in a freshwater tidal estuarine zone: potential effects of cultural eutrophication

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Abstract Nitrate and ammonium are the most important nitrogen sources for phytoplankton growth. Differential utilization of inorganic nitrogenous compounds by phytoplankton has been observed and may have significant impacts on primary productivity at local scales. We used enrichment experiments with natural phytoplankton populations from the freshwater tidal zone of the Guadiana estuary, a coastal ecosystem increasingly subjected to anthropogenic influences, to study the effects of nitrate and ammonium on N-consumption and phytoplankton growth. In addition, we used combined additions of nitrate and ammonium to understand the inhibitory effect of ammonium over nitrate uptake. Ammonium concentrations in the freshwater tidal reaches of the Guadiana estuary throughout the sampling period were too low to exert an inhibitory effect on nitrate uptake or a toxic effect on phytoplankton growth. Nitrate was clearly the main nitrogen source for phytoplankton at the study site. Overall, nitrate seemed to become limiting at concentrations lower than 20 μM and N-limitation was particularly significant during summer. A trend of decreasing nitrate uptake with increasing ammonium concentrations and uptake suggested an overall preference for ammonium. However, preference for ammonium was group-specific, and it was observed mainly in green algae and cyanobacteria. In fact, cyanobacteria relied only on ammonium as their N-source. On the contrary, diatoms preferred nitrate, and did not respond to ammonium additions. The increasing eutrophication in the Guadiana estuary and particularly increased inputs of nitrogen as ammonium due to urban waste effluents may result in a shift in phytoplankton community composition, towards a dominance of cyanobacteria and green algae.

Keywords Phytoplankton · Nutrient limitation · Nitrogen · Nitrate · Ammonium · Guadiana estuary

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Introduction

Eutrophication has been increasing in estuarine ecosystems and is mainly associated with increased inputs of dissolved inorganic nitrogen and phosphorus that stimulate the growth of aquatic primary producers. Nitrate and ammonium are the main nitrogen forms associated with human influences. Nitrate derives from land clearing, production and applications of fertilizers, whilst ammonium derives from human waste discharge (Nixon 1995; Cloern 2001). Cultural eutrophication can therefore change the relative amounts of nitrate and ammonium available for primary producers. The knowledge on which form of nitrogen is preferentially assimilated by phytoplankton in nature and how the two nitrogen compounds interact is particularly important in view of the current eutrophication problem. In addition, the differential utilization of inorganic nitrogenous compounds may have significant impacts on primary productivity on local scales (e.g., Dugdale et al. 2007).

The differential utilisation of nitrate and ammonium by phytoplankton has been the subject of a significant number of studies for many decades, but a consensus on the interactions between ammonium and nitrate has yet to be reached. According to Dortch (1990), the classical apparent

negative effect of ammonium on nitrate uptake can be divided into two distinct processes, both strongly influenced by environmental conditions: (a) preference for ammonium, and (b) inhibition of nitrate uptake by ammonium. The relative preference for ammonium is related to the lower energetic costs associated with ammonium assimilation in relation to nitrate assimilation (Dugdale et al. 2007). Therefore, in the presence of high ammonium concentrations, phytoplankton productivity could be as high or even higher if the cells are using NH_4^+ rather than NO_3^- (Dugdale et al. 2007). Inhibition of nitrate uptake resulting directly from ammonium does occur, but it is a highly variable phenomenon, depending on environmental conditions, such as nitrogen and light availability, and species composition, and it is not as strong as usually considered (Dortch 1990). Conversely, it has been suggested that ammonium can exert a strong negative influence on phytoplankton production above a relatively low concentration (around 10 μM, Yoshiyama and Sharp 2006), contradicting the advantage to phytoplankton of preference for ammonium over nitrate.

The interactions between nitrate and ammonium uptake have been extensively studied in cultures and marine/brackish environments (e.g., Dortch et al. 1984; Torres-Valdés and Purdie 2006; Wilkerson et al. 2006; Dugdale et al. 2007; Tada et al. 2009), where simultaneous utilization of NH_4^+ and NO_3^- has been observed (Dortch 1990), as well as preference for ammonium and/or repression of nitrate uptake (Blasco and Conway 1982 and references therein). However, studies on freshwater tidal estuarine zones are rare (e.g., Carpenter and Dunham 1985; Pennock 1987; Twomey et al. 2005), and we are not aware of studies that have addressed the effects of nutrient enrichment on freshwater tidal zones of mesotidal, Mediterranean estuaries, given that most Mediterranean estuaries, including those not located in the Mediterranean basin (e.g., Swan River, Australia), are microtidal. Furthermore, most studies have evaluated the effect of nitrogenous compounds on uptake rates using ^{15}N-tracers, thus only providing information at the community level (e.g., Twomey et al. 2005). In our study, we used nutrient enrichment bioassays that allowed the quantification of the responses not only of the phytoplankton community, but also the responses of specific phytoplankton groups.

Additionally, Mediterranean climate estuaries are located in extremely vulnerable regions to climate change (IPCC 2001) and in view of the increasing human influences on estuaries and coastal zones, which include urban and agricultural runoffs and, consequently, nutrient enrichment, analyses of nutrient interactions and uptake by phytoplankton are particularly needed for these sensitive ecosystems. Knowledge on nitrate/ammonium interactions also represents an important contribution towards the understanding of new versus regenerated production, which is a crucial topic due to the increasing concern over the implication of global warming (Dugdale and Goering 1967; Flynn et al. 1997).

This study aims to (a) evaluate the effect of nitrate and ammonium enrichments on phytoplankton growth, and (b) the effect of variable ammonium concentrations on nitrate uptake and phytoplankton growth. This is a relevant subject given that the Guadiana estuary has been increasingly subjected to cultural eutrophication, but also to water and sediment trapping behind the recently built Alqueva dam.

Materials and methods

Study site

The Guadiana River (drainage area 67,039 km^2, length 810 km) estuary (Fig. 1) forms the border between Portugal and Spain. Located in a temperate Mediterranean climate area, it is a mesotidal, partially stratified estuary in its lower and middle sections, and is well mixed in the upper section. The upper, freshwater tidal zone represents the largest estuarine region in length, extending approximately from Álamo (25 km from the river's mouth) up to the tidal limit (>70 km from the river's mouth) (Morales 1995). Freshwater inputs to the estuarine zone used to vary sharply between dry and humid months (1995–2000: 333 ± 1,096 $m^3\ s^{-1}$: http://www.snirh.pt), but the recently built Alqueva dam (operating since 2002) has promoted a more regular freshwater flow throughout the year. The estuary also receives reduced freshwater inputs from some tributaries, whilst other inputs include sewage, mainly near the mouth.

Sampling strategy

Nitrate and ammonium addition experiments were undertaken throughout 2008 using water samples collected in the freshwater tidal reaches (upper estuary) of the Guadiana estuary, at station Alcoutim (see Fig. 1). Water samples for nitrate and ammonium enrichment experiments were collected near the surface (approx. 0.5 m depth), assuming that the whole water column was well mixed (confirmed by salinity and temperature profiles), during neap tides, immediately after high tide. Acid-cleaned 1 L polycarbonate bottles were used for sample collection and samples were kept in cold and dark conditions between collection and the experiment set-up (approx. 2 h).

A fortnight sampling program was carried out in the Guadiana throughout 2008 with collection of water samples for determination of dissolved inorganic nutrient

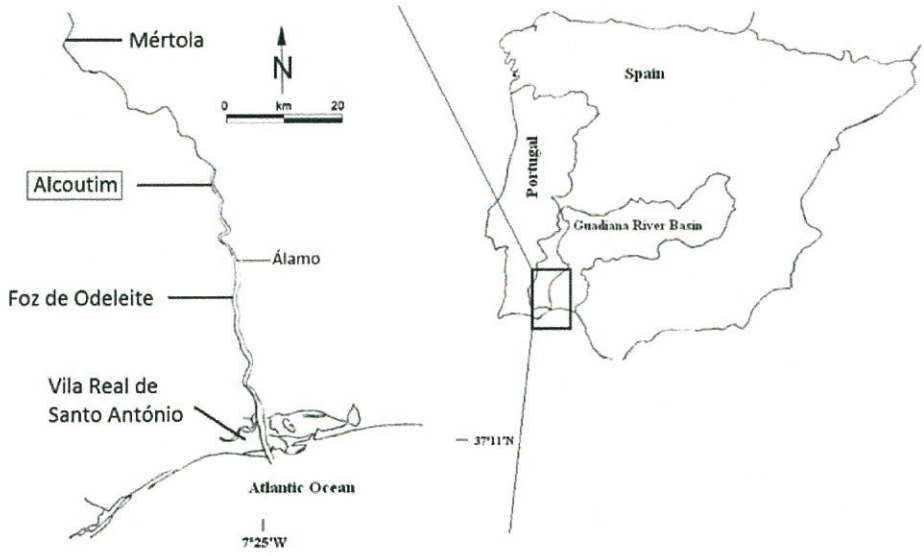

Fig. 1 Map of the Guadiana estuary and sampling station (Alcoutim)

concentrations and chlorophyll *a* concentration (See section "Laboratorial analysis"). Profiles of water temperature and salinity (measured as conductivity) were determined in situ using a YSI 556 MPS probe. Daily freshwater flow throughout 2008, measured at Pulo do Lobo hydrometric station, 85 km from the river mouth was obtained from http://www.snirh.pt.

Nitrate and ammonium addition experiments

Experiments were conducted throughout 2008 during representative seasons for phytoplankton growth: winter (7–11 February), spring (21–25 April), spring-summer transition (2–6 June), summer (1–5 August) and autumn (7–11 October). For each experiment, eight experimental treatments were prepared in duplicate in 1 L polycarbonate bottles and ran for 4 days. Potassium nitrate (KNO_3, hereafter referred as NIT) and ammonium chloride (NH_4Cl, hereafter referred as AMM) were added to the experimental treatments at day 0, in a single pulse, according to Table 1. The effects of ammonium and nitrate enrichments on phytoplankton were evaluated using separate additions of each nutrient ("control NIT", "AMM") and a control with no additions ("control"). The effect of ammonium on nitrate uptake was evaluated by adding a fixed concentration of nitrate (100 μM) and increasing ammonium concentrations ("1AMM + NIT", "10AMM + NIT", "20AMM + NIT", "50AMM + NIT", "100AMM + NIT"); treatment "control NIT" was the control for this experiment.

The bottles were incubated inside a plant growth chamber under in situ temperature and in situ light–dark cycle at approximately 110 μmol photons m^{-2} s^{-1}, which is slightly higher than the mean light intensity in the mixed layer at the time of sampling. However, phytoplankton cells are exposed to this light intensity throughout the day in their natural environment, given that sampling was conducted in the early morning when solar irradiance was lower. The bottles were opened daily and gently shaken twice a day. Nutrient consumption rates were determined by following the disappearance of NO_3^- and NH_4^+ from solution at days 0, 1, 2, and 4. Chlorophyll *a* and phytoplankton composition and abundance were evaluated at days 0 and 4, given that daily in vivo fluorescence measurements confirmed exponential growth of phytoplankton until day 4 (data not shown).

Table 1 Concentrations (μM) of nutrients added to the experimental

	NO_3^-	NH_4^+
Control	–	–
NIT	100	–
AMM	–	100
1 AMM + NIT	100	1
10 AMM + NIT	100	10
20 AMM + NIT	100	20
50 AMM + NIT	100	50
100 AMM + NIT	100	100

Nitrate was added as potassium nitrate (KNO_3) and ammonium as ammonium chloride (NH_4Cl)

Laboratory analysis

Subsurface (approx. 0.5 m) water samples for determination of dissolved inorganic macronutrients were collected and immediately filtered through cellulose acetate filters (Whatman, pore diameter = 0.2 μm). Ammonium (NH_4^+) was determined immediately after sample collection, whilst samples for nitrate (NO_3^-) were frozen (−20°C)

until analysis. All nutrients were determined in triplicate, according to the spectrophotometric methods described by Grasshoff et al. (1983), using a spectrophotometer Hitachi U-2000 for ammonium and an autoanalyzer Skalar for nitrate.

Chlorophyll *a* concentration was measured according to Parsons et al. (1984), using glass fibre filters (Whatman GF/F, pore diameter = 0.7 μm). Chlorophyll *a* was extracted overnight at 4°C with 90% acetone. After centrifugation, absorbance of the supernatant was measured spectrophotometrically (Hitachi U-2000) at 750 and 665 nm, before and after addition of HCl 1 M.

Epifluorescence and inverted microscopy were used to determine phytoplankton abundance and composition, following the methods of Haas (1982) and Utermöhl (1958), respectively. Samples for enumeration of cyanobacteria were preserved with glutardialdehyde (final concentration 2%), stained with proflavine and filtered onto black polycarbonate membrane filters (Whatman, pore diameter = 0.45 μm). Preparations were made within 24 h of sampling, using glass slides and non-fluorescent immersion oil (Cargille type A), and then frozen (−20°C) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5× magnification using an epifluorescence microscope (Leica DM LB). Samples for enumeration of other phytoplankton groups were preserved with acid Lugol's solution, settled in sedimentation chambers and observed at 400× magnification using an inverted microscope (Zeiss Axiovert 100). A minimum of 50 random visual fields, at least 400 cells in total and 50 cells of the most common genus, were counted. Assuming that the cells were randomly distributed, the counting precision was ±10% (Venrick 1978).

Data analysis

The relative preference index (RPI) for nitrate (NO_3^-RPI) utilization was calculated according to McCarthy et al. (1977) as:

$$NO_3^- RPI = \frac{(Nit0 - Nit4)}{(Nit0 - Nit4) + (Amm0 - Amm4)} x \frac{Nit0 + Amm0}{Nit0}$$

where Nit0, Nit4, Amm0 and Amm4 are nitrate (Nit) and ammonium (Amm) concentrations at days 0 and 4. RPI values higher than 1 indicate preference for nitrate, whilst RPI < 1 indicate preference for ammonium.

For each experimental treatment, nutrient concentrations, chlorophyll *a* and phytoplankton abundances within duplicates were statistically compared using a *t* test or a Mann–Whitney rank sum test when the Kolmogorov–Smirnov normality test failed. Since no significant differences were found between replicates, the values of all replicates were combined for subsequent data analysis. Nutrient net consumption rates and phytoplankton net growth rates were estimated using GraphPad Prism 5 software. Nutrient net consumption rates for each treatment were estimated as the slope of a linear or exponential function adjusted to the data points ($n = 8$). Phytoplankton community net growth rate and group specific net growth rates for each experimental treatment ($n = 4$) (μ, d^{-1}) were estimated as the slope of ln N(t) versus time (4 days), where N(t) represents chlorophyll *a* concentration or phytoplankton abundance at day t, respectively, assuming exponential growth (confirmed by in vivo Chl*a* fluorescence). Slopes and associated standard errors were then compared across experimental treatments using a one-tailed *t* test, according to Fowler and Cohen (1990) to assess significant differences between nutrient consumption and phytoplankton growth rates of the controls and the treatments.

In respect to nutrient consumption, we determined nutrient disappearance rates that result from different processes such as uptake, excretion, nutrient regeneration, etc., and which can be different from uptake rates (inward nutrient transport through the cell membrane). For nitrate, it is probable that disappearance rates were similar to uptake rates, given that it is unlikely that nitrification had occurred inside the microcosms. On the contrary, ammonium in the medium may increase as a result of animal excretions and bacterial decomposition of organic nitrogenous compounds (Toscas 2008). However, given that ammonium concentrations decreased in most treatments throughout the experiments, it is unlikely that significant ammonium enrichment to the medium had occurred during the experiments.

Results and discussion

Ammonium and nitrate availability in the Guadiana estuary

In the Guadiana estuary, ammonium concentrations ranged between undetectable values (<0.05 μM) and 8.6 μM, but remained mostly below 3 μM throughout 2008 (2.7 ± 2.2 μM) (Fig. 2), which can be considered low concentrations, compared to other estuaries (e.g., <0.2 to 41.5 μM, Southampton Water: Torres-Valdés and Purdie 2006; >2 μM, Delaware estuary: Yoshiyama and Sharp 2006; >4 μM, San Francisco Bay: Dugdale et al. 2007). It has been suggested that ammonium concentrations higher than a certain threshold, usually around 1–4 μM, inhibit nitrate uptake (see Dortch 1990), or that nitrate only becomes available to phytoplankton when ammonium concentration is <4 μM (Dugdale et al. 2007). Therefore,

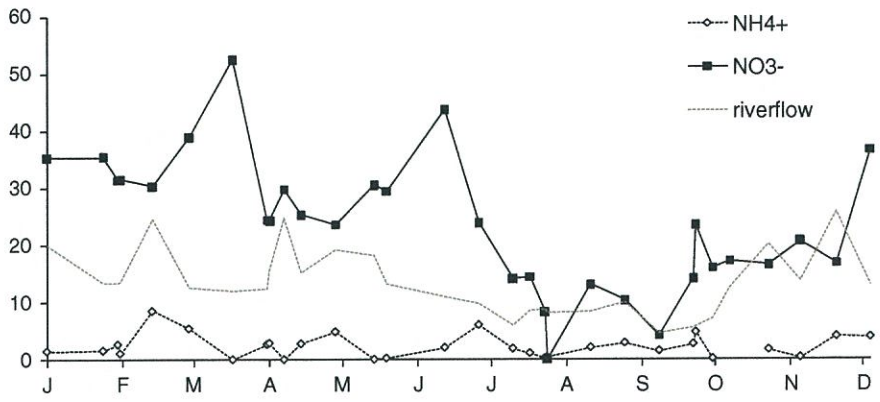

Fig. 2 Variation of river flow ($m^3\ s^{-1}$), and nitrate and ammonium concentration (μM) in Alcoutim throughout 2008

the inhibitory effect of these ammonium concentrations on nitrate uptake was most likely minimal in the Guadiana estuary, as well as the potential toxic/inhibitory effect of ammonium on phytoplankton production.

Nitrate was the predominant nitrogenous compound throughout 2008 (24.2 ± 12.7 μM) (Fig. 2). Nitrate concentration was always higher than 10 μM, except for three sampling dates in summer. River flow (14 ± 9 $m^3\ s^{-1}$) was positively correlated to nitrate concentration ($r = 0.5$, $p < 0.01$, $n = 31$), indicating that the river itself was the main source of nitrate to the estuary (Fig. 2), as previously observed the Guadiana estuary (Domingues and Galvão 2007; Barbosa et al. 2010) and for other estuarine systems (Borsuk et al. 2004). Ammonium, on the contrary, was not related to river flow or rainfall, reflecting the importance of biological sources and sedimentary fluxes (Barbosa et al. 2010).

Nitrate represented, on average, 82–88% of the total dissolved inorganic nitrogen, so the phytoplankton community was probably fuelled by nitrate, in contrast to other estuarine systems where ammonium is the dominant form of nitrogen taken up (e.g., Twomey et al. 2005; Torres-Valdés and Purdie 2006). The dominance of micro- and larger nano-sized (>10 μm) phytoplankton species in the Guadiana estuary (e.g., Domingues et al. 2005) is most likely a consequence of this nutritional environment dominated by nitrate, given that smaller cells (<10 μm) usually prefer ammonium as their N-source (Probyn 1985; Wafar et al. 2004; Maguer et al. 2009). Indeed, in estuaries such as San Francisco Bay, larger phytoplankton blooms depend mostly on nitrate whilst smaller phytoplankton blooms are fuelled by ammonium (Wilkerson et al. 2006).

Significant negative correlations between nitrate and phytoplankton biomass (Barbosa et al. 2010) further support the pivotal role of nitrate on bloom development in the freshwater tidal reaches of this estuarine system. In the last years, and probably due to the regulation of freshwater flow by the Alqueva dam that started in 2004, nitrate availability has been lower than before (1996–2003 annual means between 56.2 and 73.6 μM; 2008 annual mean = 23.7 μM). The decrease in the availability of nitrate and other nutrients simultaneously with a lower turbidity and higher light availability is expected to promote a shift from a potentially light-limited environment to a more nutrient-limited one in the freshwater tidal reaches of the Guadiana estuary (Barbosa et al. 2010).

Effects of ammonium on nitrate uptake

In general, nitrate consumption decreased with increasing ammonium concentrations and increasing ammonium uptake (Figs. 3a, 4a, 5a, 6a; Table 2), which could be attributed to inhibition of nitrate uptake by ammonium and/or preference for ammonium. These results were more obvious during the productive period. For instance, nitrate uptake rates in the spring experiment decreased from 7.0 μM d^{-1} in treatment 1AMM + NIT to 0.5 μM d^{-1} in treatment 100AMM + NIT, whereas ammonium uptake rates increased from 0.3 to 8.3 μM d^{-1} with increasing ammonium concentrations (Fig. 4a). The same was observed in the spring-summer transition (Fig. 5a, Table 2) and in summer (Fig. 6a, Table 2). In the autumn experiment, variability in nitrate consumption rates within replicates was too large (Fig. 7a) to draw any conclusions. Nitrate uptake in estuarine systems can indeed be suppressed by low ammonium concentrations (<2 μM: Pennock 1987; <4 μM: Dugdale et al. 2007). Although initial (ambient) ammonium concentrations were low (between undetectable values and 4 μM, Fig. 2), ammonium additions in the treatments (up to 100 μM) were high enough to exert an inhibitory effect on nitrate uptake. Nitrate uptake that occurs only when ammonium concentrations are low is a frequently observed phenomenon in enrichment experiments (e.g., Balode et al. 1998), thus following the classical dogma of preference for ammonium over nitrate.

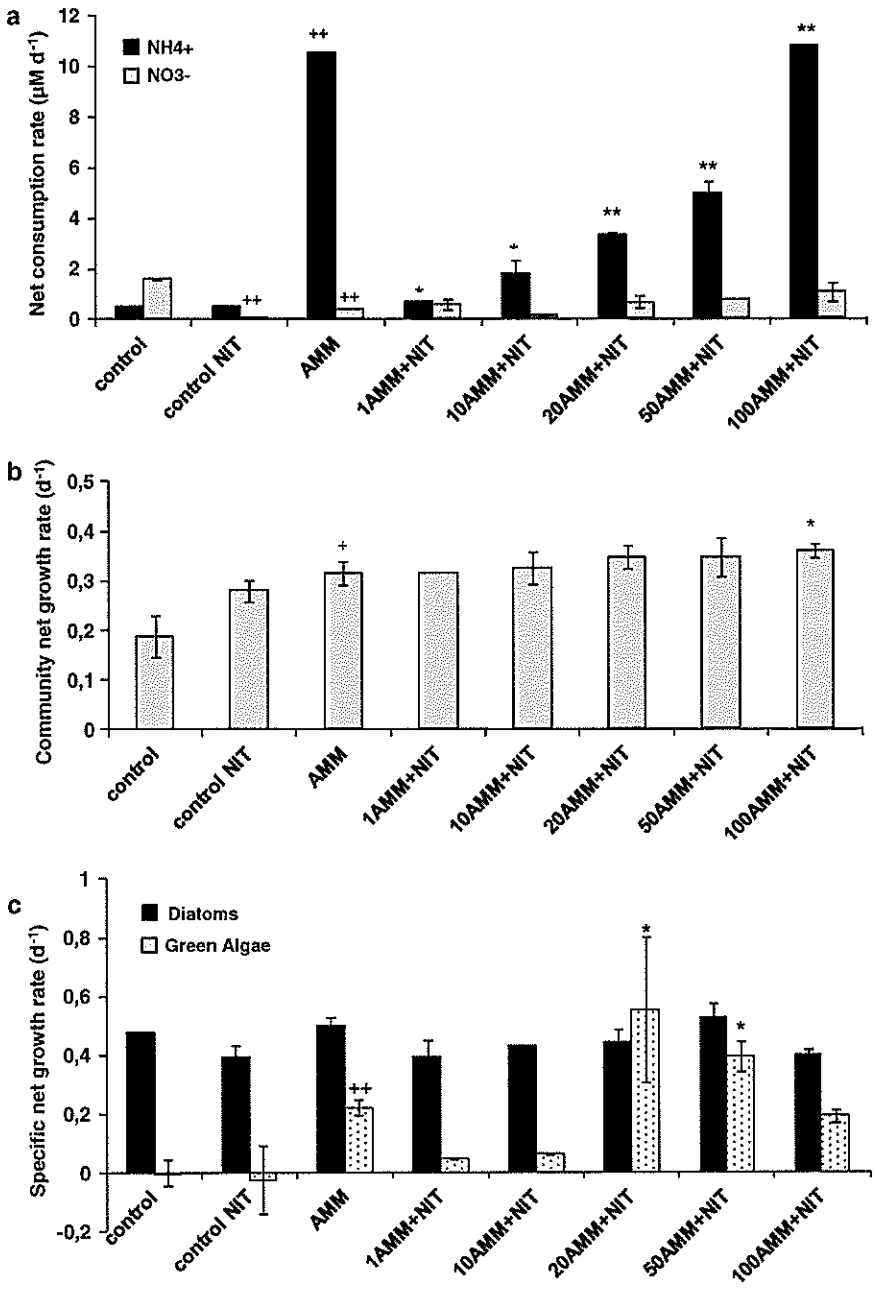

Fig. 3 a Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), b community net growth rate (d^{-1}), and c specific net growth rates (d^{-1}) of diatoms and green algae during the winter experiment. *Vertical lines* represent ± 1 SD. Significant differences in treatments "control NIT" and "AMM" in relation to the "Control" are denoted by $^{+}p < 0.05$ or $^{++}p < 0.01$ over the corresponding *bar*. Significant differences in the other treatments in relation to the "Control NIT" are denoted by $^{*}p < 0.05$) or $^{**}p < 0.01$ over the corresponding *bar*

Effects of ammonium and nitrate on the phytoplankton community

The phytoplankton community from the freshwater tidal reaches of the Guadiana estuary responded differentially to nitrate additions. In the summer experiment, nitrate added alone promoted a significantly higher net consumption rate (26.0 µM d^{-1}) than in the control (3.6 µM d^{-1}; Fig. 6a), resulting in significant increases in community biomass (Fig. 6b). Phytoplankton growth rates in all the N-enriched treatments (0.23–0.32 d^{-1}) were significantly higher than in the control (0.04 d^{-1}; Fig. 6b). Thus, we may assume that the community was indeed N-limited when the initial nitrate concentration was 15.5 µM, and that the nitrogenous nutrients were used for growth and not for storing in internal pools.

In the other experiments (initial nitrate concentrations between 22.2 and 35.4 µM), nitrate-alone additions had no significant effects on uptake and growth rates (Figs. 3, 4, 5, 7, Table 2), indicating that nitrate concentrations higher than ~ 20 µM were not limiting to phytoplankton (see Domingues et al. 2010); otherwise, cells would have taken

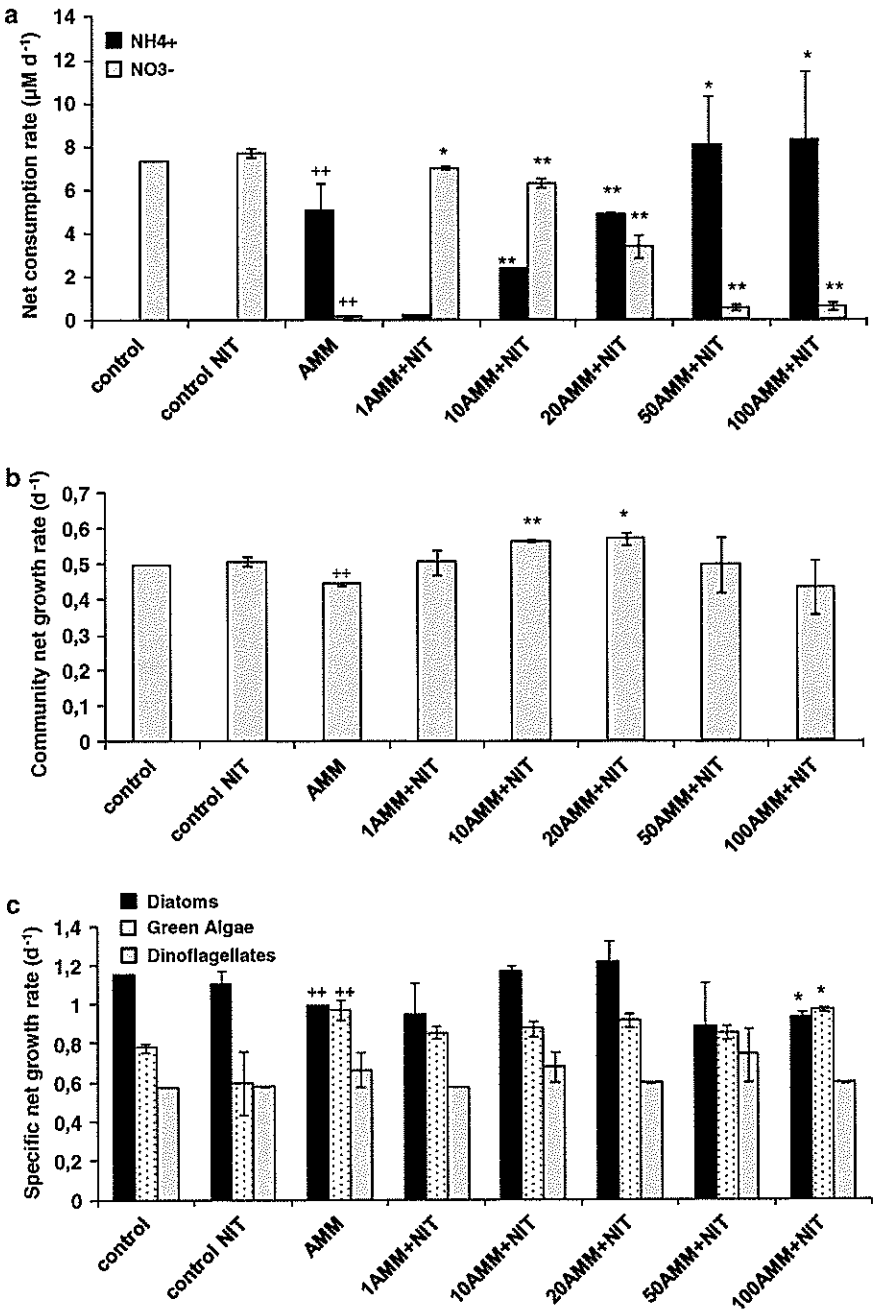

Fig. 4 a Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates ($\mu M\ d^{-1}$), b community net growth rate (d^{-1}), and c specific net growth rates (d^{-1}) of diatoms, green algae and dinoflagellates during the spring experiment. *Vertical lines* represent ± 1 SD. Significant differences in treatments "control NIT" and "AMM" in relation to the "Control" are denoted by $^{+}p < 0.05$ or $^{++}p < 0.01$ over the corresponding bar. Significant differences in the other treatments in relation to the "Control NIT" are denoted by $*p < 0.05$ or $**p < 0.01$ over the corresponding *bar*

up the available nitrate. However, in the ammonium-enriched treatments, significant increases in net consumption rates were observed (spring-summer transition and autumn, Figs. 5a, 7a) and could be attributed to luxury consumption or consumption by cells other than phytoplankton (e.g., heterotrophic bacteria). Significant increases in uptake and community biomass were also observed in the ammonium-enriched treatments (winter, Fig. 3b), indicating growth limitation by N. Considering the specific composition of the phytoplankton community, it is clear that ammonium was the preferred N-source for both green algae and cyanobacteria (see below), so it is probable that these ammonium-preferring groups were N-limited and the nitrate-preferring groups were not. Therefore, nutrient limitation should be evaluated in terms of specific groups or even species, rather than the whole phytoplankton community, composed of different species with highly diverse nutritional requirements.

Increased ammonium uptake that did not result in cell growth was observed in the spring-summer transition and

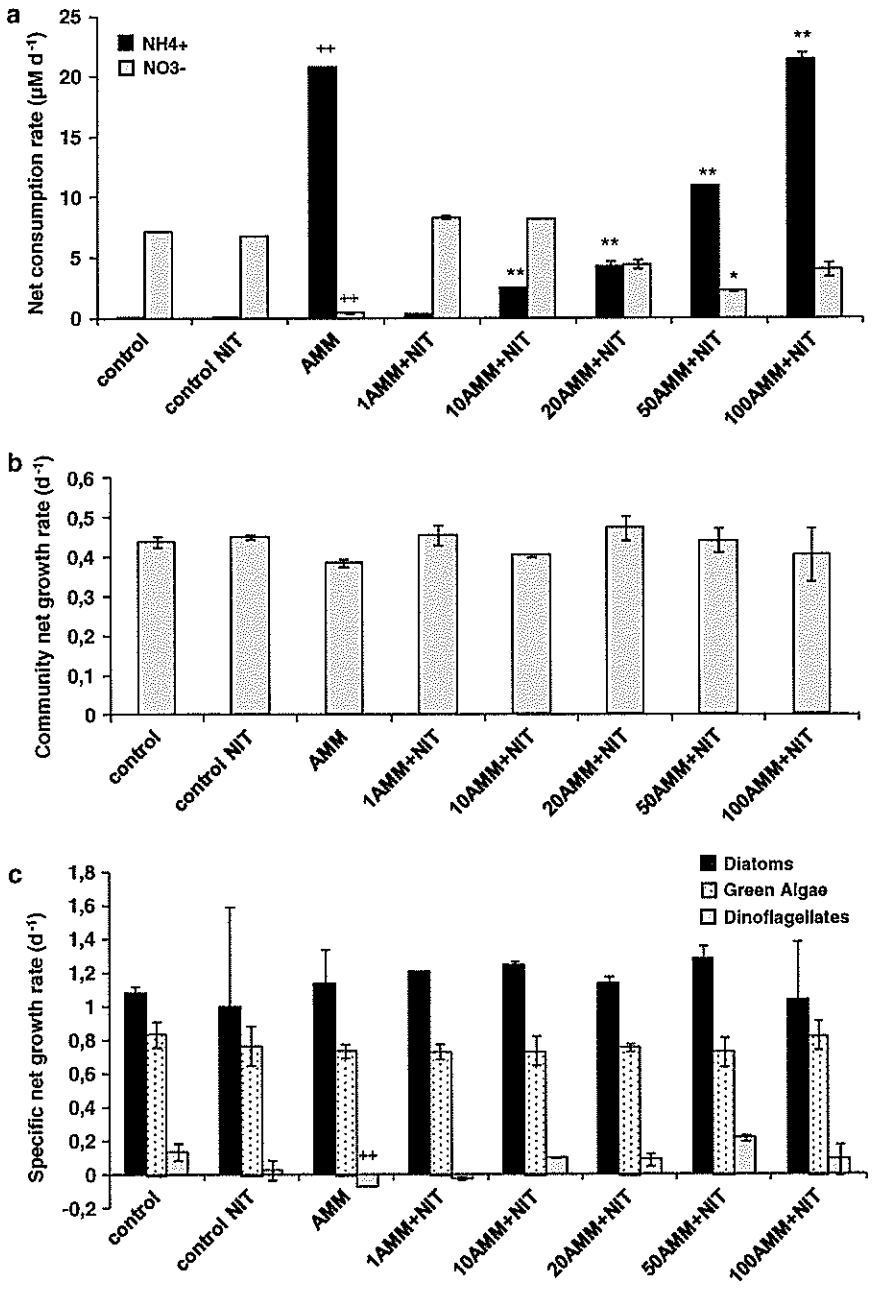

Fig. 5 a Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), b community net growth rate (d^{-1}), and c specific net growth rates (d^{-1}) of diatoms, green algae and dinoflagellates during the spring-summer transition experiment. *Vertical lines* represent ± 1 SD. Significant differences in treatments "control NIT" and "AMM" in relation to the "Control" are denoted by $^{+}p < 0.05$ or $^{++}p < 0.01$ over the corresponding bar. Significant differences in the other treatments in relation to the "Control NIT" are denoted by $*p < 0.05$ or $**p < 0.01$ over the corresponding *bar*

autumn experiments (Figs. 5, 7; Table 2). The accumulation of nitrogen in transient or permanent internal pools is a common response to N-pulses that will induce cells to take up nitrogen faster than they can assimilate it, and therefore they will store it. The ability to store nitrogen is a way by which phytoplankton growth is buffered from the effects of a changing, and sometimes growth-limiting, nitrogen supply in the environment (Dortch 1982).

Overall, ammonium seemed to be the preferred nitrogenous nutrient by phytoplankton, according to the RPI, which is also a common observation in other estuarine systems (e.g., McCarthy et al. 1977; Carpenter and Dunham 1985; Balode et al. 1998). RPI values were slightly higher than 1, indicating a relative preference for nitrate only when ammonium concentrations were undetectable (Fig. 8).

Effects of ammonium and nitrate on specific phytoplankton groups

Green algae showed the most consistent responses to nitrate and ammonium additions. Throughout the winter

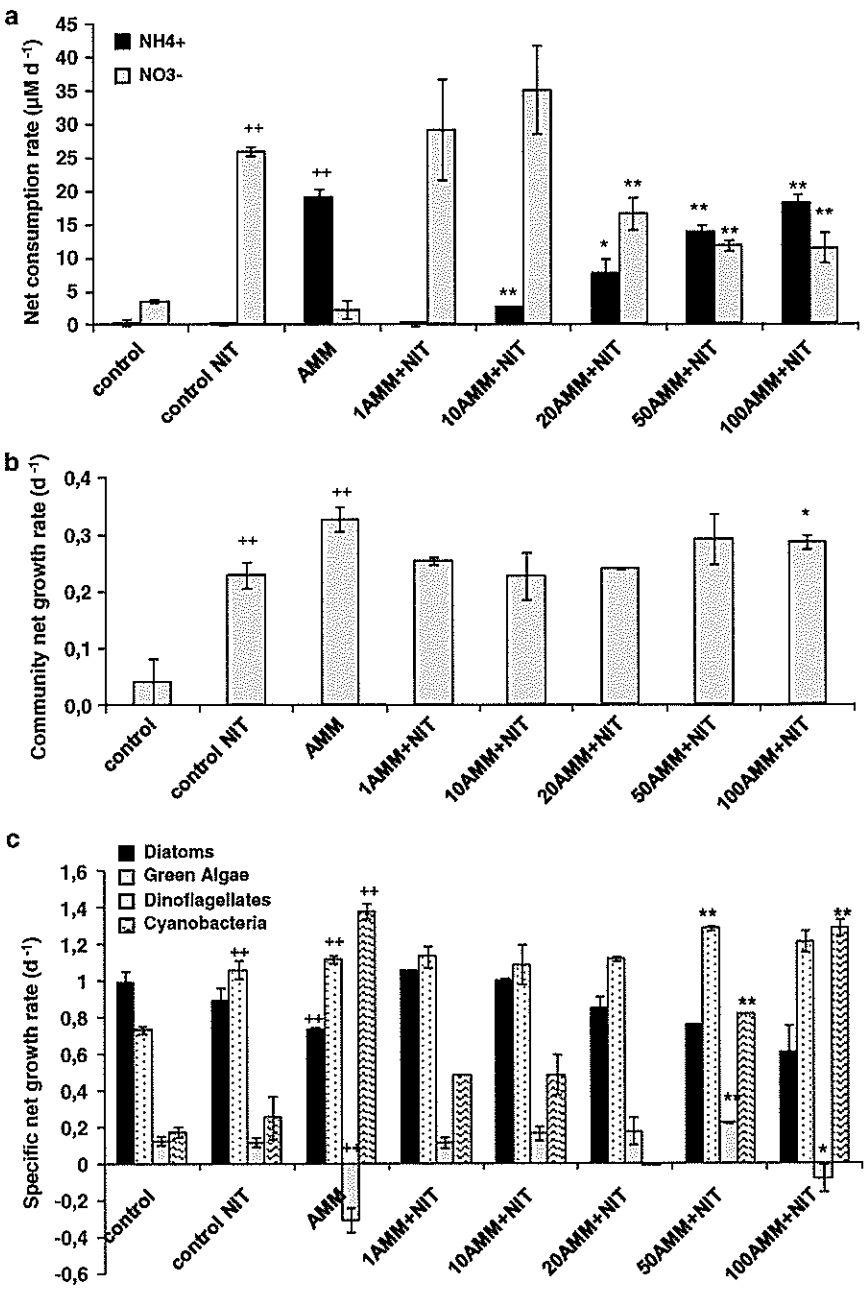

Fig. 6 a Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), **b** community net growth rate (d^{-1}), and **c** specific net growth rates (d^{-1}) of diatoms, green algae, dinoflagellates and cyanobacteria during the summer experiment. *Vertical lines* represent ± 1 S.D. Significant differences in treatments "control NIT" and "AMM" in relation to the "Control" are denoted by $^{+}p < 0.05$ or $^{++}p < 0.01$ over the corresponding *bar*. Significant differences in the other treatments in relation to the "Control NIT" are denoted by $*p < 0.05$ or $**p < 0.01$ over the corresponding *bar*

(Fig. 3c), spring (Fig. 4c) and summer (Fig. 6c), green algae responded significantly to ammonium additions with initial DIN (nitrate + ammonium) concentrations ranging between 2.0 and 37.4 µM (Table 3). Green algae also responded positively to nitrate additions in summer (Fig. 6c) when initial DIN concentrations were <20 µM (Table 3). Whenever nitrate concentrations were higher than approx. 20 µM, green algae relied only on ammonium as their N-source. Although a preference for ammonium seemed to exist, green algae could grow efficiently on both N-sources under N-limitation (nitrate < 20 µM), most likely due to a reduced internal pool of regulatory N-compounds at the beginning of the experiments, as a result of the low DIN concentration in the medium. Indeed, nutrient uptake rates are determined not only by external nutrient concentrations but also by intracellular pools of regulatory compounds (Dortch et al. 1984). A highly N-starved cell would therefore take up and assimilate or store any form of nitrogen added to the medium. Other studies, however, indicate that under non-limiting conditions, green algae, namely *Scenedesmus*, *Ankistrodesmus* and *Selenastrum*, may reach similar densities growing on

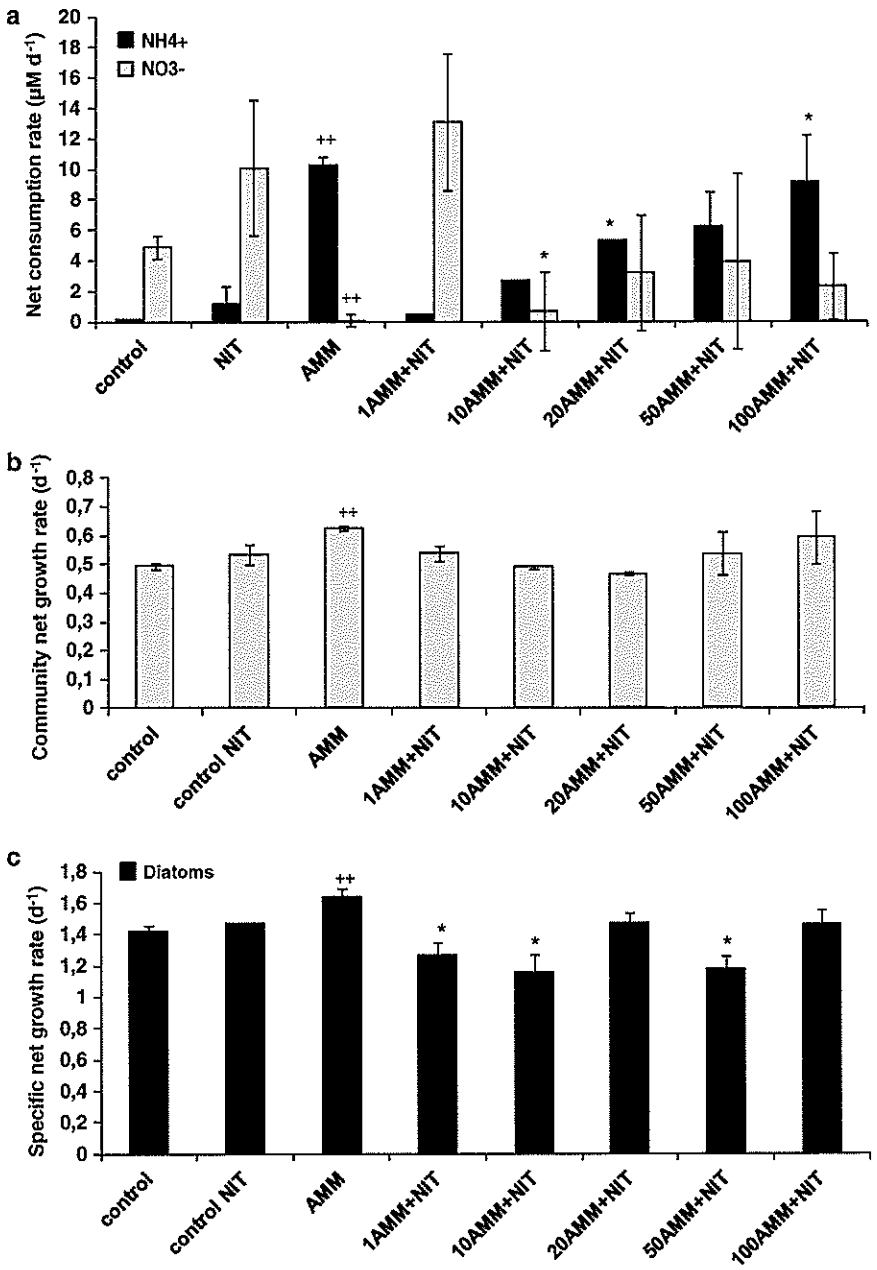

Fig. 7 a Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), **b** community net growth rate (d^{-1}), and **c** specific net growth rates (d^{-1}) of diatoms during the winter experiment. *Vertical lines* represent ± 1 SD. Significant differences in treatments "control NIT" and "AMM" in relation to the "Control" are denoted by $^+p < 0.05$ or $^{++}p < 0.01$ over the corresponding *bar*. Significant differences in the other treatments in relation to the "Control NIT" are denoted by $*p < 0.05$ or $**p < 0.01$ over the corresponding *bar*

both nitrate and ammonium (Taub 2009). Furthermore, both green algae and cyanobacteria are able to use organic N-sources, such as urea, in an extremely efficient manner (Balode et al. 1998).

Nitrate uptake is a light-dependent process, i.e., nitrate uptake will occur only if light intensity is high enough to support the consumption of reductive power necessary to assimilate nitrate (e.g., Hyenstrand et al. 2000). Since light limitation in the experiments was alleviated during incubation, green algae were energetically able to take up both ions. Therefore, green algae demonstrated a preference for ammonium when nitrate was plentiful, but were able to use both N-sources when nitrate concentration was at limiting concentrations. Furthermore, competition for nitrogen between green algae and other phytoplankton groups, namely cyanobacteria, probably played an important role on the specific responses to N enrichments. Green algae are commonly favoured by high N:P ratios and cyanobacteria by low N:P ratios (see Domingues et al. 2005). It is likely that the increased N:P ratios induced by

Table 2 Ammonium (NH_4^+) and nitrate (NO_3^-) net consumption rates (μM d^{-1}), community net growth rates (Chl*a*, d^{-1}) and specific net growth rates (d^{-1}) of diatoms (DIA), green algae (GA), dinoflagellates (DINO) and cyanobacteria (CYA) in the different nutrient enrichment experiments

Winter	NH_4^+	NO_3^-	Chl*a*	DIA	GA	DINO	CYA
Control	0.5	1.6	0.19	0.48	0.00		
Control NIT	0.5	0.1	0.28	0.39	-0.03		
AMM	10.5	0.4	0.32	0.50	0.22		
1 AMM + NIT	0.7	0.6	0.32	0.39	0.05		
10 AMM + NIT	1.8	0.2	0.33	0.43	0.06		
20 AMM + NIT	3.3	0.7	0.35	0.44	0.55		
50 AMM + NIT	5.0	0.8	0.35	0.53	0.39		
100 AMM + NIT	10.8	1.1	0.36	0.40	0.19		
Spring							
Control	0.0	7.3	0.50	1.15	0.78	0.58	
Control NIT	0.0	7.7	0.51	1.10	0.60	0.58	
AMM	5.1	0.2	0.44	0.99	0.97	0.66	
1 AMM + NIT	0.3	7.0	0.51	0.94	0.85	0.58	
10 AMM + NIT	2.4	6.3	0.56	1.17	0.87	0.67	
20 AMM + NIT	4.8	3.4	0.57	1.22	0.91	0.60	
50 AMM + NIT	8.0	0.6	0.50	0.89	0.85	0.74	
100 AMM + NIT	8.3	0.6	0.43	0.93	0.97	0.60	
SS							
Control	0.1	7.2	0.44	1.08	0.84	0.14	
Control NIT	0.1	6.7	0.45	1.01	0.77	0.03	
AMM	20.7	0.5	0.38	1.14	0.74	-0.07	
1 AMM + NIT	0.3	8.3	0.46	1.21	0.73	-0.02	
10 AMM + NIT	2.4	8.1	0.40	1.25	0.74	0.10	
20 AMM + NIT	4.3	4.4	0.47	1.14	0.76	0.09	
50 AMM + NIT	10.9	2.2	0.44	1.28	0.73	0.22	
100 AMM + NIT	21.4	4.0	0.40	1.04	0.83	0.09	
Summer							
Control	0.4	3.6	0.04	0.99	0.73	0.13	0.17
Control NIT	0.2	26.0	0.23	0.89	1.06	0.12	0.25
AMM	19.0	2.2	0.33	0.73	1.12	-0.31	1.38
1 AMM + NIT	0.2	29.2	0.25	1.06	1.13	0.12	0.49
10 AMM + NIT	2.5	35.1	0.23	1.00	1.09	0.17	0.48
20 AMM + NIT	7.7	16.6	0.24	0.85	1.12	0.18	-0.01
50 AMM + NIT	13.8	11.8	0.29	0.76	1.29	0.23	0.82
100 AMM + NIT	18.2	11.4	0.29	0.61	1.21	-0.08	1.29
Autumn							
Control	0.1	4.9	0.50	1.42			
Control NIT	1.1	10.1	0.53	1.47			
AMM	10.3	0.1	0.62	1.63			
1 AMM + NIT	0.5	13.1	0.54	1.27			
10 AMM + NIT	2.7	0.7	0.49	1.15			
20 AMM + NIT	5.3	3.2	0.47	1.47			
50 AMM + NIT	6.3	3.9	0.54	1.17			
100 AMM + NIT	9.1	2.3	0.59	1.46			

nitrogen additions have favoured green algae throughout the experiments.

Cyanobacteria growth rates increased with increasing ammonium concentrations, indicating N-limitation (Fig. 6c). However, no response was observed to nitrate-only additions (control NIT). Cyanobacteria growth rates can even decrease following nitrate additions (Domingues et al. 2010). Although cyanobacteria usually have a preference for ammonium (Dokulil and Teubner 2000), they can take up a variety of N-sources, such as nitrate, nitrite, ammonium, urea, and, in some cases, atmospheric nitrogen and amino acids such as arginine and glutamine (Flores and Herrero 2005). In these experiments, ammonium seemed to be the preferred N-source, whilst nitrate apparently was not taken up, even under N-deficiency. Although ammonium concentrations higher than 100 μM can inhibit nitrate uptake in some cyanobacteria (Incharoensakdi and Wangsupa 2003), NH_4^+ in the beginning of the experiments (<4 μM, Table 3) was too low to exert any inhibitory effect on nitrate uptake.

Diatoms did not respond in most treatments, which could be attributed to co-limitation by N and P, as suggested by Domingues et al. (2010) for the freshwater tidal reaches of the Guadiana estuary during spring/early summer. In addition, growth rates of diatoms in the spring (Fig. 4c) and summer (Fig. 6c) even decreased significantly in the treatments with ammonium >50 μM (see Table 2), suggesting a toxic/inhibitory effect of ammonium on this group. Inhibition of diatom growth has been observed at different ammonium concentrations, for instance, >35 μM for benthic diatoms (Admiraal 1977) and >200 μM for *Pseudonitzschia pungens* (Hillebrand and Sommer 1996). However, stimulatory effects of ammonium on diatoms have also been observed, with increases on diatom abundance following ammonium additions and no responses to nitrate additions (Takeda et al. 1995), and higher growth rates when ammonium was the N-source (Tada et al. 2009).

Dinoflagellates, mainly represented by the harmful species *Kryptoperidinium foliaceum*, were clearly inhibited in the treatments with the highest ammonium concentrations (100 μM, Fig. 6c). However, the effect of ammonium on the growth of dinoflagellates may vary tremendously. For instance, inhibition of growth has been observed in cultures at concentrations >20 μM–N NH_4^+ for *Ceratium furca* (Baek et al. 2008) and >50 μM–N NH_4^+ for *Alexandrium tamarense* (Leong and Taguchi 2004). Conversely, *Alexandrium minimum* had the highest growth rates at 25 μM–N NH_4^+, and started to decrease at concentrations > 50 μM–N NH_4^+ (Chang and McLean 1997). Overall, growth of *K. foliaceum* in the freshwater tidal zone of the Guadiana estuary seemed strongly dependent on the

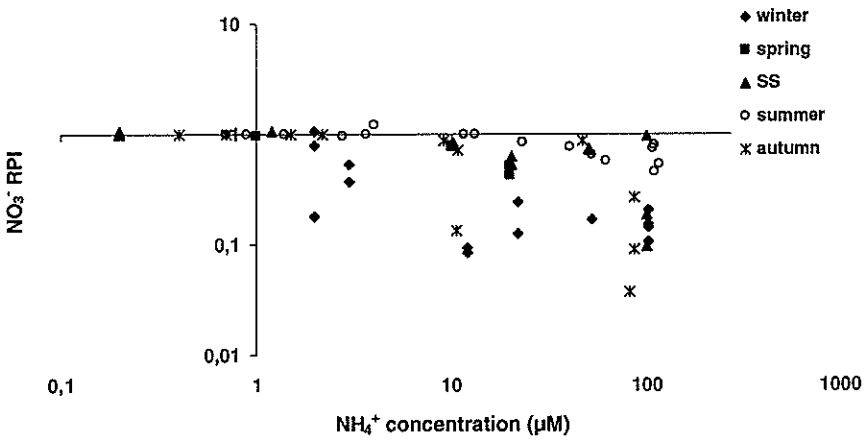

Fig. 8 Relative preference index (*RPI*) for nitrate uptake as a function of ammonium concentration, for each treatment and each experiment. *SS* spring-summer transition. RPI values >1 indicate preference for nitrate, whilst RPI <1 indicate preference for ammonium

Table 3 Initial nutrient concentrations (μM) and molar ratios, potential limiting nutrient according to the Redfield ratio at the time of sampling, phytoplankton abundance (cells L^{-1}) and chlorophyll *a* concentration (μg L^{-1}) at the time of sampling

	Winter	Spring	SS tran.	Summer	Autumn
NO_3^-	35.4	29.8	29.4	15.5	22.2
NH_4^+	2.0	Nd	0.2	4.0	0.4
N:P	19.7	13.0	15.6	7.0	7.8
Si:N	1.3	2.6	2.4	2.8	1.5
Pot. Lim.	N, P	N	N	N	N
DI	1.1×10^5	6.2×10^5	2.8×10^5	5.1×10^5	6.2×10^4
GA	3.5×10^5	7.3×10^4	1.2×10^5	8.9×10^4	nd
DINO	nd	5.6×10^3	9.4×10^4	1.9×10^5	nd
CYA	nd	nd	nd	2.4×10^7	nd
Chl*a*	6.4	10.1	11.7	10.7	5.9

NO_3^- nitrate, NH_4^+ ammonium, *DT* diatoms, *GA* green algae, *DINO* dinoflagellates, *CYA* cyanobacteria, *Chla* chlorophyll *a* concentration, *nd* not detected

form and concentration of N. It reached extremely high growth rates in nitrate-enriched waters (Domingues et al. 2010) and was inhibited by high concentrations of ammonium.

Conclusions

In the freshwater tidal reaches of the Guadiana estuary, ammonium concentrations throughout 2008 were most likely too low to exert any inhibitory effect on nitrate uptake or a toxic effect on phytoplankton growth. Indeed, nitrate has been the main nitrogen source for phytoplankton in the Guadiana upper estuary. Considering the nutrient enrichment experiments that have been undertaken with natural phytoplankton assemblages (this study and Domingues et al. 2010), nitrogen seems to become limiting at nitrate concentrations lower than approx. 20 μM. The interactions between nitrate and ammonium, namely a decrease on nitrate consumption with increasing ammonium concentrations and increasing ammonium consumption, point towards an overall preference of phytoplankton for ammonium. However, preference for ammonium was group-specific. Green algae and cyanobacteria preferred ammonium, whilst nitrate was preferred by diatoms and dinoflagellates. Green algae showed the most consistent responses to nitrogen additions. Ammonium was clearly preferred, but nitrate was also used by green algae under severe N-limitation (<20 μM). Cyanobacteria, in contrast, relied only on ammonium as their N-source. Lastly, future scenarios of water and sediment retention in dams leading to reduced nitrate inputs to the estuary and increases in anthropogenic ammonium inputs to the Guadiana estuary will most likely promote a shift of the phytoplankton community towards dominance of small-sized, ammonium-preferring groups such as green algae and cyanobacteria, which can have significant impacts on higher trophic levels and water quality.

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