UNIVERSIDADE DO ALGARVE

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Declaração

Os resultados apresentados e discutidos nesta tese encontram-se incluídos nos seguintes artigos científicos, publicados ou submetidos:

Capítulo 2
Domingues RB, Barbosa A, Galvão H (2008) **Constraints on the use of phytoplankton as a biological quality element within the Water Framework Directive in Portuguese waters**. Marine Pollution Bulletin 56, 1389-1395.

Capítulo 3
Domingues RB, Anselmo TP, Barbosa AB, Sommer U, Galvão HM (2010) **Tidal variability of phytoplankton and environmental drivers in the freshwater tidal reaches of the Guadiana estuary (SW Iberia).** International Review of Hydrobiology 95, 352-369.

Capítulo 4
Domingues RB, Anselmo TP, Barbosa AB, Sommer U, Galvão HM (2010) **Nutrient limitation of phytoplankton in the freshwater tidal zone of a turbid, Mediterranean estuary.** Estuarine, Coastal and Shelf Science (in press).

Capítulo 5
Domingues RB, Anselmo TP, Barbosa AB, Sommer U, Galvão HM. **Effects of ammonium and nitrate on phytoplankton growth in the freshwater tidal estuarine zone of a turbid, Mediterranean estuary.** Aquatic Sciences (accepted).

Capítulo 6
Domingues RB, Anselmo TP, Barbosa AB, Sommer U, Galvão HM. **Light limitation and phytoplankton production in the freshwater tidal zone of the turbid Guadiana estuary.** Estuarine, Coastal and Shelf Science (accepted).

Capítulo 7
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A autora desta tese declara que foi responsável pela concepção e execução do trabalho experimental, pela interpretação dos resultados e pela redacção dos artigos.

Rita Isabel de Oliveira Soares Branco Domingues

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Why I did it (the Ph.D.):

"We must not forget that when radium was discovered no one knew that it would prove useful in hospitals. The work was one of pure science. And this is a proof that scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium a benefit for humanity."

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."

Why I'll keep doing it (science):

"One never notices what has been done; one can only see what remains to be done."

Quotes by Marie Curie (1867-1934)

ABSTRACT

Phytoplankton are key players in the aquatic environment and they can be effectively used to understand and predict the functioning and production of aquatic ecosystems. Given that phytoplankton is affected by natural and human-induced perturbations, such as eutrophication and global climate change, it is pressing to understand which factors regulate phytoplankton communities. The main goal of this work was, therefore, to understand how phytoplankton growth and production in the turbid Guadiana estuary, particularly in the freshwater tidal zone, is regulated by bottom-up factors, namely nutrients and light.

Enrichment bioassays were carried out to evaluate nutrient and light limitation of phytoplankton growth. Nutrient limitation, specifically by nitrogen, was observed during the productive period. Nitrogen, as nitrate, became limiting to phytoplankton growth at concentrations lower than 20 μM. Although nitrate was the main nitrogen source in the Guadiana estuary, an overall preference for ammonium was observed, mainly by cyanobacteria and green algae. Diatoms were the most nutrient-limited group, and they clearly preferred nitrate as their N-source. Regarding light availability, phytoplankton was not acclimated to the low light conditions that prevail in the Guadiana estuary and light limitation occurred throughout the year. Diatoms were the most light-limited group, whilst cyanobacteria seemed to be more acclimated to low light. Primary production was higher in the more turbid regions, where light availability was the lowest, but nutrient concentrations, although occasionally limiting, were the highest. Therefore, phytoplankton in such turbid regions were the most efficient in using limiting resources. River flow was a major regulator of nutrient and suspended matter inputs to the estuarine zone. Tidally-induced variability of phytoplankton and environmental drivers in the freshwater tidal estuarine zone was low and resulted from seasonal and fortnightly variability in river flow and tidal currents.

Keywords: phytoplankton, nutrients, light, primary production, regulation, limitation, Guadiana estuary.

Regulação da base para o topo do fitoplâncton no estuário do Guadiana

RESUMO

O fitoplâncton é um componente chave nos ecossistemas aquáticos. Além da sua função crítica como produtor primário, o fitoplâncton afecta a qualidade da água e desempenha importantes papéis em muitos processos aquáticos. O fitoplâncton é também um importante indicador de qualidade ecológica, podendo ser usado para prever o funcionamento e produção dos ecossistemas aquáticos e as suas respostas a perturbações naturais e antropogénicas. Para tal, é necessário compreender de que forma o fitoplâncton é ele próprio regulado pelas variáveis ambientais.

A variabilidade espacial e temporal do fitoplâncton reflecte a interacção entre processos que regulam o crescimento do fitoplâncton (regulação da base para o topo) e processos que regulam a sua biomassa (regulação do topo para a base). A regulação do topo para a base envolve processos de mortalidade e perda, através dos quais as células ou morrem ou são removidas do plâncton; estes processos incluem predação, lises virais, apoptose, advecção e sedimentação. Juntamente com estas fortes pressões do topo para a base, o fitoplâncton também compete entre si por recursos. Esta regulação da base para o topo é exercida pelos recursos que controlam o crescimento celular, como os nutrientes, a luz, a temperatura, o pH, a salinidade e a concentração de oxigénio.

Os nutrientes são geralmente considerados os factores mais importantes na regulação do fitoplâncton. Aqueles que são necessários em maiores quantidades designam-se por macronutrientes, e a maior parte deles, como o carbono ou o oxigénio, ocorrem geralmente em quantidades suficientes nos sistemas aquáticos. Outros, como o azoto e o fósforo, existem geralmente em concentrações reduzidas, pelo que podem limitar o crescimento do fitoplâncton.

A disponibilidade luminosa condiciona o processo fotossintético, podendo, portanto, ser também um factor limitante do crescimento. A disponibilidade de luz nos ecossistemas aquáticos é altamente variável e depende sobretudo da radiação solar incidente, da profundidade da camada de mistura e do grau de atenuação da luz na coluna de água. Este trabalho focou-se no efeito dos nutrientes e da luz, uma vez que

estas variáveis são consideradas as mais importantes na regulação do crescimento fitoplanctónico.

A recente construção da barragem de Alqueva despoletou um grande interesse no estudo do ecossistema do estuário do Rio Guadiana. Contudo, os estudos publicados na área da dinâmica fitoplanctónica são meramente descritivos e baseados em observações no local. No entanto, estes estudos levantaram ainda mais questões acerca da dinâmica fitoplanctónica; especificamente, os factores que regulam a composição, crescimento e produção do fitoplâncton no estuário do Guadiana não são conhecidos. Dada a importância de um conhecimento sólido do funcionamento do ecossistema para avaliar, prevenir e/ou mitigar os impactos de perturbações naturais ou antropogénicas, estudos sobre a regulação fitoplanctónica neste ecossistema são imperativos. Assim, este trabalho teve como objectivos:

a) rever a importância do fitoplâncton em ecossistemas costeiros e o uso do fitoplâncton como elemento de qualidade biológica na avaliação da qualidade da água (Capítulo 2);

b) avaliar a variabilidade do fitoplâncton e seus factores reguladores induzida pelos ciclos de maré semidiurnos e quinzenais, na zona tidal de água doce do estuário do Guadiana (Capítulo 3);

c) determinar qual o nutriente limitante para o crescimento fitoplanctónico e a sua variabilidade sazonal, e compreender os efeitos de potenciais enriquecimentos antropogénicos em nutrientes na estrutura da comunidade fitoplanctónica na zona de água doce do estuário do Guadiana (Capítulo 4);

d) avaliar o efeito do nitrato e da amónia no crescimento do fitoplâncton, e o efeito de concentrações variáveis de amónia no consumo de nitrato na zona de água doce do Guadiana (Capítulo 5);

e) observar a ocorrência e intensidade de limitação por luz do crescimento fitoplanctónico ao longo do ciclo sazonal, e o papel de potenciais adaptações fisiológicas a ambientes de baixa disponibilidade luminosa na zona de água doce do estuário do Guadiana (Capítulo 6);

f) compreender a importância global da luz e dos nutrientes na sucessão e produção fitoplanctónicas (Capítulo 7).

A Directiva-Quadro da Água (DQA), a legislação comunitária que prevê a protecção e a gestão das águas naturais, refere o fitoplâncton como um dos elementos de qualidade biológica que deverão ser monitorizados regularmente e para o qual deverão ser estabelecidas condições de referência. No entanto, o uso do fitoplâncton como elemento de qualidade biológica em águas Portuguesas originará vários problemas, que são discutidos no Capítulo 2. Por exemplo, o estabelecimento de condições de referência para a comunidade fitoplanctónica poderá ser difícil em águas para as quais não existem dados históricos ou recentes. A frequência de amostragem para a monitorização do fitoplâncton (semestral) não parece ser a indicada para compreender a sucessão das comunidades e poderá impedir a detecção de florescências. Por fim, o uso da concentração de clorofila *a* como indicador de biomassa e mesmo abundância fitoplanctónicas tem sido proposto, o que pode negligenciar florescências de fitoplanctontes de menores dimensões (pico- e nanofitoplâncton) e sobrestimar a importância do microfitoplâncton. Adicionalmente, a maioria dos estudos de fitoplâncton em águas Portuguesas usa apenas a microscopia de inversão para a observação e identificação dos organismos. No entanto, este método não permite a distinção entre células auto- e heterotróficas, sobretudo em amostras preservadas com lugol, e não permite a observação de células picoplanctónicas e nanoplanctónicas de menores dimensões. Como o uso da microscopia em programas de monitorização não é financeira e temporalmente viável, outras técnicas, como a detecção remota e análises quimiotaxonómicas, são propostas como complementos em programas de monitorização do fitoplâncton.

Os efeitos das diferentes fases dos ciclos tidais semidiurnos e quinzenais na variabilidade do fitoplâncton e dos seus factores reguladores foram avaliados na zona de água doce do estuário do Guadiana e discutidos no Capítulo 3. Um método de amostragem Euleriano foi usado e as campanhas de amostragem cobriram diferentes estações do ano. Foram recolhidas amostras em situação de maré viva e maré morta, na preia-mar, vazante, baixa-mar e enchente. Várias variáveis físico-químicas foram avaliadas, assim como a abundância e biomassa fitoplanctónicas.

A salindade foi maior em preia-mar e a concentração de matéria particulada em suspensão foi maior em maré viva e na enchente, devido a uma maior mistura vertical na coluna de água e à ressuspensão de sedimentos. A concentração de clorofila *a* no Inverno e no Verão foi maior em situação de maré morta que em maré viva, enquanto a abundância de diatomáceas pinuladas foi superior durante as marés vivas de Inverno e Primavera, reflectindo provavelmente diferenças a nível do caudal fluvial. No geral, a variabilidade induzida pela maré na zona de água doce do estuário do Guadiana não é tão significativa como a observada na zona marinha do estuário. No entanto, a ocorrência de variabilidade induzida pela maré em estações do ano específicas aponta para a importância de uma amostragem frequente em programas de monitorização do fitoplâncton. Amostragens ocasionais não irão reflectir a variabilidade típica deste tipo de ecossistemas altamente dinâmicos.

A identificação do nutriente limitante para o crescimento fitoplanctónico é fundamental para o controle sustentado da eutrofização. No Capítulo 4 é apresentada a primeira evidência experimental da ocorrência de limitação por nutrientes e a sua variação sazonal na zona de água doce do estuário do Guadiana. Para tal, realizaram-se experiências em microcosmos com comunidades naturais de fitoplâncton do alto estuário do Guadiana. Efectuaram-se adições de nitratos, fosfatos e silicatos aos tratamentos experimentais, e a resposta da comunidade fitoplanctónica foi avaliada através de alterações na abundância e biomassa de grupos específicos.
No geral, o crescimento do fitoplâncton, em particular as clorofíceas e as diatomáceas, esteve limitado por azoto ao longo do período produtivo. No verão de 2008, as cianobactérias e o dinoflagelado tóxico *Kryptoperidinium foliaceum* responderam significativamente ao enriquecimento em azoto na ausência de sílica. A presença de *Kryptoperidinium foliaceum* foi observada pela primeira vez na zona de água doce do estuário do Guadiana, local onde geralmente não são observados dinoflagelados. O aumento significativo das taxas de crescimento dos dinoflagelados e cianobactérias em resposta a adições de azoto na ausência de sílica é preocupante, visto que os enriquecimentos antropogénicos são de azoto e fósforo, e não de sílica. Adicionalmente, concentrações relativamente altas de nitrato, até 22 μM, revelaram-se limitantes para o crescimento do fitoplâncton.

O azoto é consumido pelo fitoplâncton sobretudo na forma de iões inorgânicos, o nitrato e a amónia. A utilização diferencial destes compostos azotados inorgânicos pelo fitoplâncton, que tem sido observada quer em culturas quer em comunidades naturais, pode ter impactos significativos na produtividade primária a nível local. No Capítulo 5 são apresentadas e discutidas experiências de enriquecimento em nutrientes com comunidades naturais de fitoplâncton da zona de água doce do estuário do Guadiana que tiveram como objectivo avaliar o consumo diferencial de amónia e nitrato, e também o efeito inibitório da amónia no consumo de nitrato e no crescimento do fitoplâncton. A resposta do fitoplâncton foi avaliada em termos de abundância e biomassa, usando microscopia de epifluorescência e microscopia de inversão.

As concentrações de amónia na zona de água doce do estuário foram demasiado baixas para exercerem qualquer efeito inibitório no consumo de nitrato ou um efeito tóxico no crescimento do fitoplâncton. O nitrato foi claramente a principal fonte de azoto no estuário. No geral, o nitrato tornou-se limitante para o crescimento para concentrações inferiores a 20 μM, como tinha sido já observado no capítulo 4, e essa limitação foi particularmente intensa durante os meses de Verão. Um decréscimo no consumo de nitrato com o aumento da concentração e do consumo de amónia foi observado nas experiências, o que sugere uma preferência geral por amónia como fonte de azoto. No entanto, essa preferência não foi igual em todos os grupos de fitoplâncton, e foi observada sobretudo nas cianobactérias e clorofíceas. Pelo contrário, as diatomáceas preferiram o nitrato, não respondendo às adições de amónia. A eutrofização crescente no estuário do Guadiana e sobretudo o aumento do enriquecimento em amónia pode assim resultar em alterações na composição específica da comunidade fitoplanctónica, em direcção a uma dominância de cianobactérias e clorofíceas.

A luz é geralmente o principal factor que regula o crescimento do fitoplâncton em estuários de elevada turbidez, mas tem recebido muito menos atenção que os nutrientes como factor regulador da base para o topo. No Capítulo 6 são apresentadas evidências experimentais da ocorrência de limitação por luz e sua variabilidade sazonal na zona de água doce do estuário do Guadiana.

Comunidades naturais de fitoplâncton foram expostas a diferentes intensidades luminosas. Incubações de curto período com adição de isótopos radioactivos de carbono permitiram estimar os parâmetros fotossintéticos da comunidade fitoplanctónica, ao passo que incubações mais longas permitiram avaliar os efeitos de diferentes intensidades luminosas na composição e crescimento do fitoplâncton.
Durante o período estudado, foi observada uma constante limitação por luz na zona de água doce do estuário, ao passo que a fotoinibição da fotossíntese não ocorreu para intensidades luminosas iguais ou inferiores a 615 μmol fotões m^{-2} s^{-1}. No Verão ocorreu co-limitação por nutrientes, o que evitou que a comunidade fitoplanctónica respondesse positivamente ao aumento de intensidade luminosa. As diatomáceas foram o grupo mais limitado por luz, enquanto que as cianobactérias pareceram mais bem adaptadas a baixas luminosidades. Os parâmetros fotossintéticos estimados, com valores elevados de intensidade luminosa saturante e taxa fotossintética máxima e uma baixa eficiência fotossintética, indicam que de facto a comunidade fitoplanctónica não se encontra fisiologicamente adaptada às condições de baixa luminosidade à qual está sujeita no estuário do Guadiana.

Os nutrientes e a luz são considerados geralmente os mais importantes factores reguladores do crescimento fitoplanctónico em estuários. O Capítulo 7 teve como objectivo compreender a importância relativa da luz e dos nutrientes na sucessão e produção fitoplanctónicas no estuário do Guadiana. Para tal, realizaram-se campanhas de amostragem quinzenais em várias localidades do estuário, cobrindo as regiões alta (zona de água doce), média e baixa do estuário. Várias variáveis abióticas e bióticas, incluindo a disponibilidade luminosa e nutricional, e a composição, abundância e biomassa do fitoplâncton, foram determinadas quinzenalmente ao longo de dois anos de amostragem.
Durante 2007 e 2008, o caudal fluvial controlou o fornecimento de nitratos e matéria particulada em suspensão para o estuário. O azoto foi limitante para o crescimento do fitoplâncton durante 2008, quando as concentrações de nitrato foram geralmente inferiores a 20 μM. Adicionalmente, a abundância e biomassa do fitoplâncton foram inferiores em 2008, apesar de ter sido observado o mesmo padrão sazonal. A típica sucessão fitoplanctónica de sistemas temperados de água doce foi observada no alto

e médio estuários, com uma florescência de diatomáceas no fim da Primavera, seguida de um máximo de biomassa de clorofíceas, e por fim, florescências de cianobactérias durante os meses de Verão. As diatomáceas foram o principal componente da biomassa da comunidade, enquanto as cianobactérias dominaram em termos de abundância. A luz foi limitante durante todo o período de estudo, e o fitoplâncton das zonas de maior turbidez não estava adaptado às condições de baixa luminosidade. A produção primária foi mais elevada nas regiões mais túrbidas, onde a disponibilidade luminosa é menor, mas onde as concentrações de nutrientes, se bem que por vezes limitantes, são maiores. Assim, o crescimento do fitoplâncton no estuário do Guadiana, sobretudo nas zonas do alto e médio estuário, não é apenas regulado pela luz, como descrito para outros estuários semelhantes, mas sim pela interacção entre a luz e os nutrientes.

Finalmente, as conclusões dos capítulos anteriores estão resumidas no Capítulo 8, onde também são recapitulados os principais objectivos do trabalho. Relativamente aos factores que regulam o crescimento do fitoplâncton da base para o topo, este trabalho permitiu concluir que a limitação por luz é constante no alto e médio estuários do Guadiana, enquanto a limitação por nutrientes, especificamente por azoto, ocorre sobretudo nos meses de Primavera e Verão. Apesar de a amónia ser a fonte preferida de azoto pela comunidade fitoplanctónica, o nitrato é o nutriente azotado que ocorre em maiores concentrações no estuário, sobretudo no alto e médio estuários, pois a principal fonte de nitrato para a zona estuarina é o caudal do Rio. O Rio transporta também matéria particulada em suspensão, que por seu turno controla a atenuação da luz na coluna de água e, portanto, a disponibilidade luminosa. Relativamente à variabilidade induzida pelos ciclos tidais, concluiu-se que ocorre em períodos específicos do ano, e é observada sobretudo na concentração de clorofila *a* e na concentração de matéria particulada em suspensão.

Por fim, são propostas futuras linhas de investigação na área da dinâmica fitoplanctónica no estuário do Guadiana, das quais se destaca o estudo dos efeitos das alterações globais na sucessão e crescimento do fitoplâncton. Especificamente, a avaliação dos efeitos de potenciais aumentos de CO_2 atmosférico, radiação ultravioleta e temperatura na dinâmica do ecossistema é de crucial importância,

tendo em conta que a bacia do Guadiana está localizada numa área considerada altamente vulnerável às alterações climáticas.

Palavras-chave: fitoplâncton, nutrientes, luz, produção primária, regulação, limitação, estuário do Guadiana.

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Chapter 1

General Introduction

1.1 Phytoplankton

1.1.1 Phytoplankton roles and importance

Phytoplankton are a heterogeneous group of prokaryotic and eukaryotic photosynthetic organisms whose powers of locomotion are such that they drift freely in the water column. The first phytoplankton evolved in the Archaean oceans, more than 2.8 billion years ago (Bidle and Falkowski, 2004). Since then, phytoplankton has undergone dramatic diversification and numerous extinction events, and conquered the freshwater realm (Litchman and Klausmeier, 2008). Downsizing their paramount importance in the world's aquatic ecosystems, phytoplankton are basically the producers of original autochthonous organic material that will fuel aquatic food webs. Today they account for approximately 50% of the Earth's primary productivity (Falkowski et al., 2004).

Phytoplankton are key players on aquatic systems' functioning. Besides their critical function of primary production, they have significant impacts on water quality and play vital roles in many ecosystem processes, such as in biogeochemical processes, mediating cycling, sequestration and exportation of inorganic and organic compounds. In addition, phytoplankton are excellent model systems to address fundamental ecological questions (Litchman and Klausmeier, 2008), and they are also widely used for paleoenvironmental reconstructions (Barbosa, 2009). Overall, phytoplankton are a vital gauge of ecological condition and change, and they are effectively used to understand and predict the functioning and production of aquatic ecosystems and the responses to natural and anthropogenic-induced changes (e.g., Cloern and Dufford, 2005; Smetacek and Cloern, 2008).

Phytoplankton communities are composed by an array of different species, with distinct biochemical contents and cell sizes spanning six orders of magnitude (Cloern and Dufford, 2005). Therefore, the composition of phytoplankton communities impacts the functioning of aquatic ecosystems, determining the pathways and efficiencies of energy transfer to aquatic food webs (see reviews by Cloern and Dufford, 2005, and Litchman and Klausmeier, 2008). Given that phytoplankton community composition is easily altered by many natural and human-induced perturbations, such as eutrophication and global climate change, it is pressing to

understand what factors regulate the growth and composition of phytoplankton communities.

1.1.2 Phytoplankton regulation

The spatial and temporal variability of phytoplankton in aquatic ecosystems basically reflects the interaction between many environmental factors that regulate phytoplankton growth (bottom-up regulation) and phytoplankton loss (top-down regulation).

Top-down regulation of phytoplankton biomass involves mortality and loss processes, by which phytoplankton cells either die or are removed from the plankton to die elsewhere (Reynolds, 1997). These processes include grazing, cell lyses, viral lyses, cell apoptosis, advection and sinking. Although all these top-down pressures may have significant impacts on phytoplankton biomass during specific periods, it is now widely recognized that the major mortality source of phytoplankton is grazing by phagotrophic protists; these unicellular protists can ingest, on average, 67% of phytoplankton daily production (Calbet and Landry, 2004).

In addition to the strong pressures phytoplankton face from the top-down, cells also compete among each other for resources. This bottom-up regulation includes the resources that control cell replication, such as nutrients, light, temperature, pH, salinity and oxygen concentration.

Nutrients are classically considered the most important factor regulating phytoplankton growth. A variety of elements are needed for cell growth, some in relatively large amounts, the macronutrients (e.g., C, H, O, N, P, Si, Mg, K, Ca), and others in much smaller quantities, the micronutrients or trace elements (e.g., Fe, Mn, Cu, Zn, Ba, Na, Mo, Cl, V, Co) (Parsons et al., 1984a). Most of these elements are available in sufficient amounts in marine and freshwaters, but others, particularly nitrogen (N), phosphorus (P) and silicon (Si, required only by Si-containing cells such as diatoms), may occur in natural waters in extremely low concentrations for phytoplankton growth. Therefore, these elements, which are taken up by cells mostly in their inorganic form, will often limit phytoplankton growth.

Nitrogen (N) is essential for the synthesis of amino acids and proteins. Although atmospheric nitrogen (N_2) is the most abundant gas in the atmosphere, it can not be used by most autotrophs (N_2-fixing cyanobacteria are the exception) in its elemental form, but only in ionic forms such as ammonium (NH_4^+) and nitrate (NO_3^-). As the concentrations of these ions in natural surface waters are usually low, nitrogen may be a limiting factor for phytoplankton growth. Sources of nitrogen to estuarine ecosystems include inputs from surface and groundwaters, atmospheric deposition and N recycling in the water column and sediments (Paerl et al., 2002), but dominant inputs of N are strongly linked to freshwater inputs from rivers (Bouwman et al., 2005). Many of these inputs have increased in the last decades as a direct result of human activities and have lead to enhanced primary production, which can result in harmful algal blooms, hypoxia and even anoxia.

Phosphorus (P) is an essential constituent of genetic material (DNA, RNA), cellular membranes (phospholipids) and energy-transforming molecules (e.g., ATP). Phosphorus availability in aquatic systems depends largely upon P speciation, since it can occur in inorganic and organic forms, either dissolved or particulate. Reactive phosphorus includes the potentially bioavailable phosphorus, that is mostly composed by $H_2PO_4^-$ in freshwaters and HPO_4^{2-} in marine waters (Morel, 1983). Phosphorus enters rivers due to the weathering of terrestrial rock materials and anthropogenic inputs; in ecosystems not strongly impacted by anthropogenic activities, freshwater inputs from rivers are the main source of P to estuaries.

Although silicon (Si) is the second most abundant element on the Earth's surface, its importance on biogeochemical cycles is rather limited (Conley, 2002). Si is only needed by siliceous phytoplankton such as diatoms, but since diatoms are a major component of phytoplankton biomass, Si plays a major role on phytoplankton community structure. Silicon appears in surface freshwaters as a result of chemical weathering of sedimentary and crystalline rocks, and freshwater inputs from rivers are the only source of Si to estuaries and coastal areas (Turner et al., 2003 and references therein). A decrease in dissolved and particulate Si inputs to estuarine zones has been observed in the last decades due to water and sediment retention behind dams. This change in nutrient supply, accompanied by increased anthropogenic inputs of N and P, may promote changes in phytoplankton biomass

and species composition (Smayda, 1980), and may even lead to the development of nuisance algal blooms (Flynn, 2002). The most remarkable example of ecological problems associated to decreased Si inputs occurred in the Black Sea, where a diatom-based phytoplankton community was replaced by flagellates and other non-siliceous organisms, due to water and sediment trapping behind the Iron Gates Dam in the Danube River (Humborg et al., 1997).

Light has not yet received the same attention as nutrients as an environmental driver of phytoplankton, but light availability is of paramount importance for photosynthesis, the process by which phytoplankton produce their own organic material. Light availability in aquatic systems is extremely heterogeneous in space and time and is highly dependent on the incident solar radiation, the depth of the mixed layer and the degree of light attenuation in the water column. Light attenuation is mostly a function of the quantity and quality of dissolved and particulate materials in the medium, resulting in a pronounced vertical gradient in intensity and spectral distribution (Kirk, 1994). Photosynthesis is highly dependent on light intensity; the rate of photosynthesis is high at intermediate light levels and decreases as the light intensity either decreases or increases. The variability of light availability has significant impacts on phytoplankton community structure and seasonal succession, given that the optimum light intensities for photosynthesis vary between different phytoplankton groups and species.

1.1.3 Estuarine phytoplankton

Estuaries are among the most productive ecosystems in the world and their importance in terms of carbon fixation, fisheries habitat, nutrient assimilation, water storage and sediment stabilisation has long been recognized (Baban, 1997). These coastal ecosystems are characterized by strong environmental gradients, due to the dilution of seawater with freshwater derived from land drainage. The interplay between river flow and tidal regime affects the physical-chemical environment, particularly water column stability, water residence time, and nutrient and light availability, resulting in an extreme and complex ecosystem to phytoplankton. Estuarine phytoplankton is thus subjected to rapid spatial and temporal changes in

growth limiting resources. Light limitation may occur seasonally, especially in the winter, or throughout the whole year, being more common in the upper estuarine reaches and in the maximum turbidity zone. Nutrient limitation varies tremendously across estuaries, but the general observed trend is P limitation during winter and N limitation during summer, whilst Si may also limit diatom growth in the spring. Additionally, the lower estuary, at the seaward end, is more likely to be N-limited, whilst the upper estuary may be more P-limited (e.g., D'Elia et al., 1986; Fisher et al., 1999; Kocum et al., 2002).

Estuaries can be longitudinally divided in three sections, the lower, the middle and the upper estuaries. The upper estuary, or freshwater tidal zone, represents an extreme environment to phytoplankton, characterized by salinity conditions similar to the river, but subjected to a strong tidal influence. Resuspension of bottom sediments, increased turbidity, potential light limitation, high nutrient concentrations and occasional salt water intrusion are common characteristics of freshwater tidal estuarine environments (Morris et al., 1978; Cole et al., 1992; Muylaert et al., 1997). Yet, dense phytoplankton communities are usually found in these regions (Muylaert et al., 2000 and references therein), and they are also important sources of nutrients and biomass to downriver estuarine reaches and adjacent coastal areas (Rocha et al., 2002; Domingues and Galvão, 2007). However, freshwater tidal zones have been neglected in both limnological studies, due to the presence of oceanic tidal influence, and estuarine studies, because they are bathed by freshwater and inhabited primarily by freshwater organisms (Odum, 1988).

1.2 Rationale and Objectives

The Guadiana River arises in Spain, at Campo de Montiel, province of Ciudad Real, flows for 810 km, and drains into the Atlantic Ocean, between Vila Real de Santo António, in Portugal, and Ayamonte, in Spain. It has the fourth largest drainage basin in the Iberian Peninsula, with an area of 67,039 km^2, and its last 70 km are the estuarine zone. The Guadiana estuary is located in a Mediterranean climate area, subjected to hot, dry summers and temperate, wet winters. The estuary extends from the river mouth to the village of Mértola (approx. 70 km upriver), where the

semidiurnal, mesotidal regime is still detected. The upper estuary, or freshwater tidal zone, where most of the studies presented in this thesis were conducted, represents the largest estuarine region in length, extending from Álamo (25 km from the river mouth) up to the tidal limit (see Fig. 7.1, Chapter 7, p. 147).

The recent construction of the Alqueva dam, a multipurpose hydrotechnical infrastructure located approx. 150 km from the river mouth, was the catalyst for an enhancement of research in different fields of ecosystem ecology in the Guadiana estuary. Published studies on phytoplankton in this estuarine system are descriptive and focused on the effects of the Alqueva dam on phytoplankton succession (Domingues et al., 2005, 2007; Domingues and Galvão, 2007), long-term trends (Barbosa et al., 2010) and cyanobacteria blooms (Caetano et al., 2001; Rocha et al., 2002; Sobrino et al., 2004; Galvão et al., 2008). These studies have answered some questions on phytoplankton dynamics in the Guadiana estuary, but have asked even more. Specifically, the regulating mechanisms of phytoplankton in this estuarine system are still not clear. Given the importance of a sound knowledge on ecosystem functioning to properly assess, prevent and/or mitigate the impacts of natural or human-induced perturbations, studies on phytoplankton regulation are imperative. Therefore, the main goals of this thesis are:

a) to review the importance of phytoplankton in coastal ecosystems and its use as a biological quality element for water quality assessment (Chapter 2);

b) to analyse tidal variability of phytoplankton and their environmental drivers (salinity, temperature, nutrients, light), along semidiurnal and fortnightly time scales, in the freshwater tidal reaches of the Guadiana estuary (Chapter 3);

c) to determine the limiting nutrient for phytoplankton growth and its seasonal variation, and to understand the effects of potential anthropogenic nutrient enrichments on phytoplankton community structure in the freshwater tidal zone of the Guadiana estuary (Chapter 4);

d) to evaluate the effect of nitrate and ammonium on phytoplankton growth, and the effect of variable ammonium concentrations on nitrate uptake in the freshwater tidal zone of the Guadiana estuary (Chapter 5);

e) to observe the occurrence and intensity of light limitation of phytoplankton growth throughout the seasonal cycle, and the role played by potential physiological adaptations to a low light environment in the freshwater tidal zone of the Guadiana estuary (Chapter 6);
f) to understand the overall importance of light and nutrients on phytoplankton succession and production in the Guadiana estuary (Chapter 7).

1.3 Thesis outline

A general introduction to phytoplankton is presented in Chapter 1, followed by the rationale behind this study and its main goals. Chapter 2 is an extended introduction on the importance of phytoplankton and its use as an indicator of ecological quality. Chapter 3 analyses the tidally-induced variability of phytoplankton and some important environmental variables, including bottom-up factors, in the freshwater tidal reaches of the Guadiana estuary. Chapters 4, 5 and 6 focus on specific bottom-up factors. Nutrient and light limitation of phytoplankton in the freshwater tidal zone of the Guadiana estuary are discussed in these chapters and results on nutrient and light enrichment bioassays are used to infer about growth limitation and phytoplankton community structure. Specifically, Chapter 4 deals with the effects of nitrogen (as nitrate), phosphorus and silicon, and Chapter 5 analyses the interactive effects of two nitrogen compounds, ammonium and nitrate, on phytoplankton composition and growth. Chapter 6 describes the effects of light enrichments on phytoplankton composition and growth, and also on primary production. Chapter 7 evaluates the overall importance of nutrients and light as bottom-up factors regulating phytoplankton, and the analysis is extended to the estuarine salinity gradient. Finally, general conclusions are presented in Chapter 8.

Chapter 2

Constraints on the use of phytoplankton as a biological quality element within the Water Framework Directive in Portuguese waters

Constraints on the use of phytoplankton as a biological quality element within the Water Framework Directive in Portuguese waters

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Abstract

The European Union Water Framework Directive (WFD), a new regulation aiming to achieve and maintain a clean and well-managed water environment, refers phytoplankton as one of the biological quality elements that should be regularly monitored and upon which reference conditions of water quality should be established. However, the use of phytoplankton as a biological quality element will produce several constrains, which are analysed in this article with examples from Portuguese waters. Specifically, the establishment of reference conditions of water quality may be difficult in some water bodies for which no historical data exists. The sampling frequency proposed for phytoplankton monitoring does not seem suitable to assess phytoplankton succession and may preclude the detection of algal blooms. Finally, the use of chlorophyll *a* as a proxy of phytoplankton biomass and abundance has been proposed by some authors, but it may overlook blooms of pico- and small nanophytoplankton and overestimate the importance of large microphytoplankton. Furthermore, most studies in Portugal have used only inverted microscopy for phytoplankton observation and quantification; this method does not permit the distinction between autotrophic and heterotrophic cells, especially in samples preserved with Lugol's solution, and does not allow the observation of smaller-sized cells. Finally, some techniques, such as remote sensing and chemotaxonomic analysis, are proposed to be used as supplements in phytoplankton monitoring programs.

Keywords: Water Framework Directive, phytoplankton, biomass, abundance, chlorophyll a, Portuguese waters

2.1 Introduction

Phytoplankton has largely been used as a gauge of ecological condition and change. Besides its critical ecological function of primary production that directly and indirectly fuels food webs, it has tremendous impacts on water quality and plays a number of other major roles in many ecosystem processes. For instance, phytoplankton is a fundamental actor in global biogeochemical processes, participating in the transformation and cycling of key elements. Additionally, phytoplankton affects turbidity, oxygen depletion and the total productivity of the system (Los and Wijsman, 2007). Although phytoplankton has been mostly used as an indicator of changes in nutrient loads, it is also effective in evaluating responses to many other environmental stressors, due to its fast population responses to changes in water quality, hydrology or climate. The effect of alterations in the nutritional environment, namely nutrient enrichment, on phytoplankton composition and succession has been addressed for a long time (e.g., Schindler, 1977), but it gained a new meaning due to the global eutrophication problem and the new European guidelines for surface waters quality. The European Union Directive 2000/60/EC (EC, 2000), also known as Water Framework Directive (WFD), aims to achieve and maintain a clean and well-managed water environment, through the establishment of reference conditions of water quality, based on the evaluation of several biological and chemical quality elements. Phytoplankton is the only planktonic element referred by the WFD. Several phytoplankton-related variables, namely phytoplankton composition, abundance and biomass, as well as the composition, frequency and intensity of phytoplankton blooms, which are fundamental to define/classify the ecological status of surface waters, are required to be evaluated by Member States. This article aims to analyze the use of phytoplankton as a biological quality element in Portuguese surface waters. It is our belief that with the application of the WFD several constrains will emerge, mainly related to reference conditions, sampling frequency, phytoplankton variables and methodology.

2.2 Constraints

2.2.1 Reference conditions

The establishment of reference conditions, i.e., a description of the quality elements that correspond to totally or nearly totally undisturbed conditions, that is, with no, or with only a very minor impact of human activities (EC, 2000), is a fundamental step for the implementation of the Water Framework Directive. Comparison with an existing undisturbed site, historical data, models or expert judgement are the options for deriving reference conditions for each body of water (EC, 2000). Ecological modelling is in fact the only tool to determine reference conditions in water bodies such as dams and reservoirs, where non-disturbed conditions never existed (e.g., Cabecinha et al., 2007). However, it is our belief that there are no sufficient historical and recent data for the establishment of reference conditions for phytoplankton composition, abundance and biomass in most Portuguese surface waters. Although several sets of phytoplankton data can be found in grey literature, such as project reports and thesis (graduation, masters and doctoral), most of these sources are not usually publicized and/or are not generally available for consultation, thus constraining the identification of data holders. In addition, some data can be found in technical reports published by Portuguese governmental research institutes, between late 1940's and 1980's. These studies were mainly focused on estuaries (Guadiana, Sado, Tagus), coastal lagoons (Ria Formosa, Óbidos Lagoon) and bays (Sesimbra, Cascais, S. Martinho do Porto). Conversely, data published and discussed in scientific articles are scarce. Published data on phytoplankton community structure are relatively recent and do not cover all Portuguese surface waters. The aquatic systems studied are several lakes (Lake Vela: Abrantes et al., 2006; de Figueiredo et al., 2006), mesotidal well-mixed estuaries (Guadiana estuary: Rocha et al., 2002; Domingues et al., 2005, 2007; Chícharo et al., 2006; Domingues and Galvão, 2007; Tagus estuary: Gameiro et al., 2004, 2007, Brogueira et al., 2007), mesotidal shallow lagoons (Ria Formosa coastal lagoon: Loureiro et al., 2006), mesotidal semi-enclosed lagoons (Quinta do Lago lake: Morais et al., 2003; Foz de Almargem: Coelho et al., 2007; Santo André coastal lagoon: Macedo et al., 2001; Duarte et al., 2006) and

mesotidal Atlantic coast (Loureiro et al., 2005a; Silva et al., 2008) (Fig. 2.1, Table 2.I). Other articles present data on chlorophyll and/or primary production (e.g., Linhos Lake: Pereira et al., 2002; Ria de Aveiro: Almeida et al., 2002, 2005; Mondego estuary: Lillebø et al., 2005; Douro estuary: Azevedo et al., 2006) in other Portuguese ecosystems. However, the establishment of reference conditions based on these variables, particularly chlorophyll *a*, is questionable, and will be discussed below.

In addition to the lack of spatial and temporal coverage of phytoplankton community structure in Portugal, most of the published data is based only on inverted microscopy, thus neglecting picophytoplankton and many nano-sized organisms (0.2-20 μm) (see section 2.3). Furthermore, some studies classified phytoplankton only into main groups, providing no information on species composition. Overall, the approaches used so far in phytoplankton studies in Portuguese waters will inevitably constrain the establishment of reference conditions based on this biological quality element.

In conclusion, this scenario makes it difficult to establish reference conditions representing a non-disturbed situation, particularly in systems where the human impact has increased significantly and promoted drastic changes on phytoplankton communities. For instance, alterations in nutrient ratios, specifically decreases in Si:N and Si:P, have been driving changes in phytoplankton biomass and composition, from diatom-based communities to dominance of non-siliceous forms. These shifts in phytoplankton composition have already been observed with dam construction (e.g., Black Sea due to the Iron Gates dam in the Danube River: Humborg et al., 1997) and cultural eutrophication of surface waters (e.g., German Bight: Radach et al., 1990). The introduction of exotic herbivores may also promote community changes; for instance, an introduced suspension-feeding clam (*Potamocorbula amurensis*) in San Francisco Bay (USA), is presumably responsible for the disappearance of the summer phytoplankton biomass maximum (Alpine and Cloern, 1992). Increased turbidity associated to dredging for harvesting the bivalve *Tapes philippinarum* in Venice Lagoon (Italy) induced a large decrease in phytoplankton biomass and changes in phytoplankton composition (Facca et al., 2002). Additionally, phytoplankton communities have experienced long-term changes unrelated to human impact. Indeed, changes in phytoplankton community structure, production and the

occurrence of phytoplankton blooms associated to climatic changes (e.g., Howarth et al., 2000; Paerl et al., 2003; Cloern et al., 2005) have also been documented.

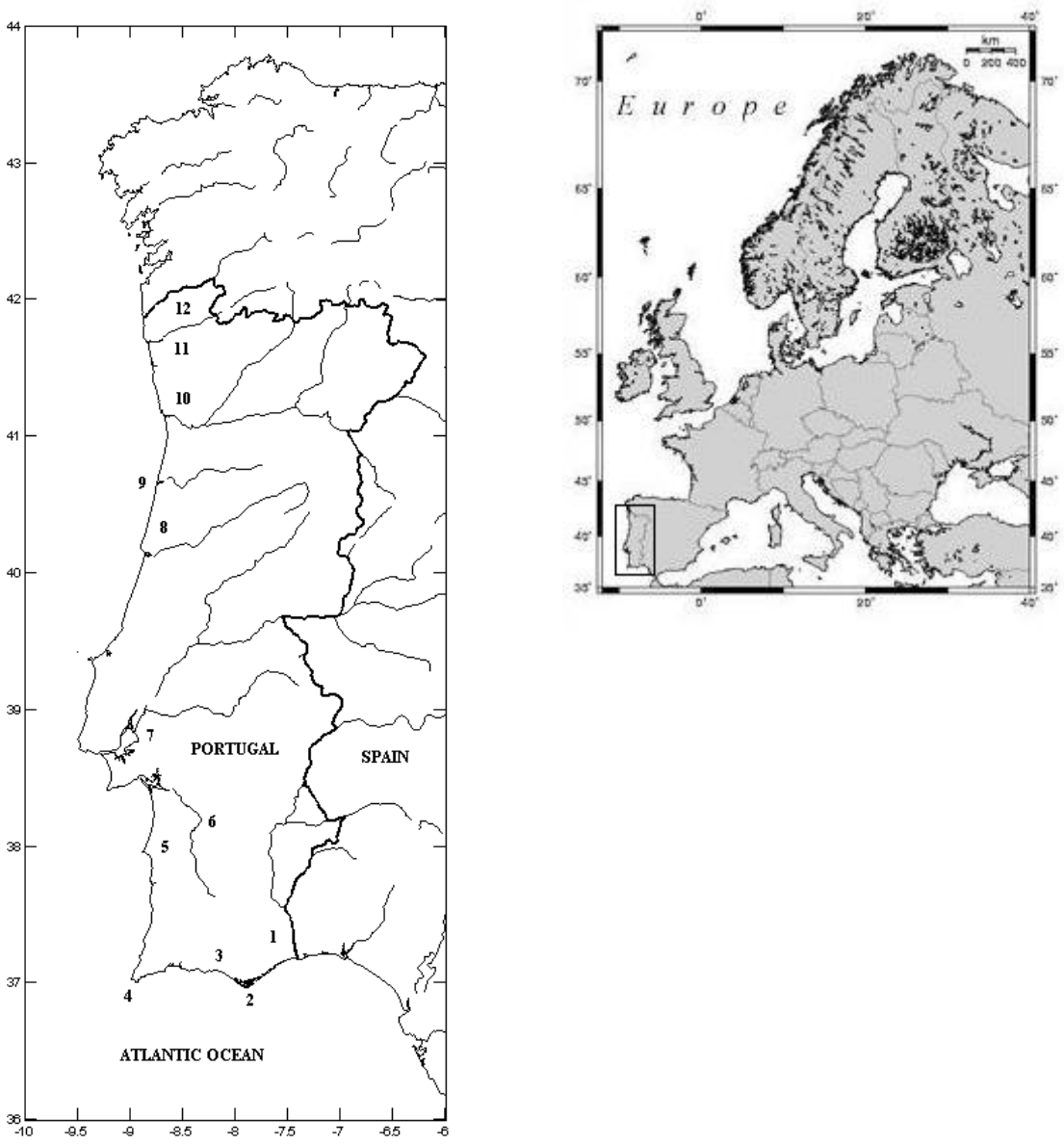

Figure 2.1 – Location of some surface water bodies in Portugal. 1 – Guadiana River; 2 – Ria Formosa coastal lagoon; 3 – Foz de Almargem coastal lagoon; 4 – Sagres; 5 – Santo André coastal lagoon; 6 - Sado River; 7 – Tagus River; 8 – Mondego River; 9 – Ria de Aveiro; 10 – Douro River; 11 – Lima River; 12 – Minho River.

Table 2.I – Data presented, methods employed and sampling strategy in published articles on phytoplankton monitoring in Portuguese waters (inv = inverted microscopy; epifl = epifluorescence microscopy).

System	Phytoplankton composition	Phytoplankton abundance	Phytoplankton biomass (biovolume)	Chlorophyll a	Sampling frequency	Number of sampling stations	References
Guadiana estuary	Inv + epifl (groups)	Inv + epifl	-	+	Monthly (Oct 1996 – Mar 1998)	4	Rocha et al. (2002)
Guadiana estuary	Inv + epifl. (groups)	Inv + epifl	Inv + epifl	+	Fortnightly (Abr – Oct 2001)	1	Domingues et al. (2005)
Guadiana estuary	Inv (groups)	Inv	-	+	Every 2-3 months (Dec 1999 –Nov 2001)	6	Chícharo et al. (2006)
Guadiana estuary	Inv + epifl (groups)	Inv + epifl	Inv + epifl	+	Monthly/fortnightly (Mar 2002 – Oct 2003)	3	Domingues et al. (2007)
Guadiana estuary	Inv + epi (groups)	Inv + epi	-	+	Monthly/fortnightly (Mar 2004 – Oct 2005)	3	Domingues & Galvão (2007)
Ria Formosa coastal lagoon	Inv (spp.)	Inv	-	+	Every 3 months (Jun 2001 – July 2002)	3	Loureiro et al. (2006)
Quinta do Lago lake	Inv + epifl (groups)	Inv + epifl	-	+	Fortnightly (Feb – Sep 1998)	3	Morais et al. (2003)
Foz de Almargem coastal lagoon	Inv (spp.)	Inv	-	+	Every 45 days (Jun 2001 – Jul 2002)	3	Coelho et al. (2007)
Sagres	Inv (spp.)	Inv	Inv	+	Weekly (May-Sep 2001)	1	Loureiro et al. (2005a)
Santo André lagoon	Inv (spp.)	Inv	-	+	Monthly (Jan 1998 – Jan 1999)	1	Macedo et al. (2001)
Santo André lagoon	Inv (groups)	Inv	-	+	Monthly (Jan 1998 – Jan 1999)	1	Duarte et al. (2006)
Tagus estuary	Inv (spp.)	Inv	-	+	Monthly (Mar 1999 – Mar 2000)	4	Gameiro et al. (2004)
Tagus estuary	HPLC (groups)	-	-	+	Monthly (Mar 1999 – Nov 2005)	4	Gameiro et al. (2007)
Tagus estuary	Inv (spp.)	Inv	-	-	3 sampling dates	19	Brogueira et al. (2007)
Lisbon Bay	Inv (spp.)	Inv	-	+	Weekly (Apr 2004-May 2005)	1	Silva et al. (2008)
Vela lake	Light (groups)	Inv	-	+	Fortnightly (1 year)	1	Abrantes et al. (2006)
Vela lake	Light (groups)	Inv	-	+	Fortnightly (Nov 2000 – Nov 2001)	1	de Figueiredo et al. (2006)

Still, it is worthy to mention that paleoecological approaches can also be used to go back to the past, where historical phytoplankton data are absent, to characterize non-impaired conditions. In fact, fossil pigment assemblages (Riedinger-Whitmore et al., 2005; Bunting et al., 2007) and historical cyst record (Dale, 2001; Chmura et al., 2004) can serve as reliable bioindicators of past and present phytoplankton community structure and environmental trends.

2.2.2 Sampling strategy

The Water Framework Directive establishes that each surface water system should be divided into homogeneous water bodies further used for monitoring and management purposes (EC, 2000). For Portuguese waters, Ferreira et al. (2005, 2006) developed a multi-criteria semi-quantitative approach to divide transitional and inshore coastal waters, integrating both natural characteristics and human dimension. However, phytoplankton data available for Portuguese surface waters do not provide information for all proposed water bodies. In this context, only the Tagus estuary (Brogueira et al., 2007), with four water bodies, the Guadiana estuary (Rocha et al., 2002, Chícharo et al., 2006), with three water bodies, and Santo André coastal lagoon (Macedo et al., 2001; Duarte et al., 2006), with one water body, were wholly sampled. Thus, the spatial coverage of most Portuguese surface waters is incomplete.
The sampling frequency proposed by the WFD for surveillance monitoring of phytoplankton composition, abundance and biomass in lakes, rivers, transitional and coastal waters is every six months (EC, 2000). A "Monitoring Plan for Portuguese Coastal Waters" (Ferreira et al., 2005) was recently commissioned by the Portuguese government to prepare a scheme for compliance with the Water Framework Directive monitoring (Ferreira et al., 2007). The proposed sampling frequencies for Portuguese surface waters are: (1) seasonal sampling for phytoplankton abundance, biomass and composition in open coastal water bodies; (2) monthly sampling for phytoplankton abundance and biomass and every six months for phytoplankton composition in transitional and inshore coastal waters. Additionally, Ferreira et al. (2007) appropriately recommended the evaluation of tidal variability, at least at high and low tides, in estuaries and coastal lagoons. However, these monitoring frequencies do not seem suitable to measure all the required variables. In fact, the required monitoring efforts to ensure a precise classification of ecological status are considerably higher than predicted by the WFD, so the proposed sampling frequencies will not provide sufficient precision (Carstensen, 2007). These sampling frequencies may even preclude the detection/observation of important phytoplankton bloom events. Dubelaar et al. (2004) referred a minimum sampling

frequency of 5 to 6 days per week to follow some algal blooms, since many species may reach blooming conditions and start disappearing again within one week.

Indeed, algal blooms can be prolonged recurrent seasonal phenomena, rare events associated to exceptional conditions or short-term episodic events (see Cloern, 1996). The high *in situ* doubling times displayed by phytoplankton cells (e.g., 6.5 hours for eukaryotic picophytoplankton, 8.1 hours for diatoms, 16.5 hours for the cyanobacterium *Synechococcus* and 17.3 hours for nanoflagellates, in the Ria Formosa coastal lagoon: Barbosa, 2006) clearly demonstrate that a bloom can be triggered on a very small time-scale. However, as rapidly as it initiates, a bloom can terminate within a short period of time, particularly due to herbivory by phagotrophic protists, the most efficient controllers of phytoplankton biomass, due to their high specific growth rates (see Irigoien et al., 2005). Nutrient and/or light limitation can lead to a decrease in phytoplankton *in situ* growth rate, also contributing to overall bloom termination. The interplay between phytoplankton growth rate and mortality rate will control the duration of phytoplankton blooms. For instance, in the Guadiana estuary and the Ria Formosa coastal lagoon, cyanobacteria, eukaryotic picophytoplankton and diatom blooms can last less than 2 weeks (Domingues et al., 2005; Barbosa, 2006; Domingues and Galvão, 2007). Additionally, a toxic dinoflagellate bloom advected from off-shore fronts lasted less than 1 week in the Ria Formosa (Barbosa, 2006). Furthermore, the frequency of phytoplankton blooms is increasing worldwide (e.g., Hallegraeff, 1993; Carstensen et al., 2007), so their detection is extremely important, especially if the bloom-forming species are toxin-producers, such as many dinoflagellates and cyanobacteria.

We recognize that the Water Framework Directive foresees more frequent sampling programs associated to operational and investigative monitoring. However, these types of monitoring implicitly assume situations of non-compliance with quality status or risk of failing to meet the environmental objectives. Thus, the general detection of a risk situation is dependent on its previous observation under the surveillance monitoring program. Still, the proposed sampling frequencies may unable the detection of risk situations that should be further monitored.

A phytoplankton surveillance monitoring program should, therefore, consider the time-scale of variability of photoautotrophic processes; sampling for determination

of biomass, abundance and composition should be as frequent as possible. Whilst weekly sampling is most likely unaffordable, monthly sampling is usually feasible and should be considered in phytoplankton monitoring programs. This is especially important in temperate climate regions, such as Portugal, where phytoplankton composition displays a high seasonal variability (e.g., Domingues et al., 2005; Gameiro et al., 2007).

2.2.3 What and how to measure

Chlorophyll *a* concentration has been widely used in aquatic studies as an indicator of phytoplankton biomass (e.g., Gameiro et al., 2004; Kromkamp and Peene, 2005), but it has also been recommended as a proxy of phytoplankton abundance (e.g., Bettencourt et al., 2003; Devlin et al. 2007). However, chlorophyll *a* concentration, biomass and abundance are three different variables.

Abundance represents the number of cells per volume of water. Phytoplankton biomass, usually represented in carbon units, corresponds to the amount of organic carbon present in the phytoplankton cells per volume of water. Chlorophyll *a*, the key photosynthetic pigment, is indeed present in all phytoplankton cells, but it only represents a fraction of the whole phytoplankton biomass. It seems obvious that abundance, biomass and chlorophyll *a* are three different phytoplankton metrics. Chlorophyll *a* can be analysed using a set of different techniques (spectrophotometry, fluorimetry, HPLC, remote sensing); the most widespread methods, spectrophotometry and fluorimetry, are time and cost-effective, highly reproducible and allow the comparison and integration of different sets of data. Therefore chlorophyll *a* concentration is extensively used to estimate phytoplankton biomass, usually through the application of a carbon/chlorophyll *a* ratio typically between 30 and 50 (e.g., Legendre et al., 1999). However, the relationship between carbon biomass and chlorophyll *a* (C:Chl) is highly variable on both intra- and inter-specific levels. In fact, C:Chl ratio depends on the physiological state of the cell and it usually increases with increasing nutrient stress, and decreases with decreasing light (e.g., Zonneveld, 1998; Kruskopf and Flynn, 2005). In respect to inter-specific variability, diatoms usually present a low C:Chl ratio (33-35 mg C mg Chl^{-1}), dinoflagellates

exhibit a high C:Chl ratio (90-120 mg C mg Chl^{-1}) (Chan, 1980) and small cells typically have high C:Chl ratios (see Putland and Iverson, 2007). Thus, phytoplankton community in specific systems can exhibit a wide temporal and spatial variability in C:Chl values (e.g. 5-345 mg C mg Chl^{-1}: see Putland and Iverson, 2007), which will complicate the estimation of phytoplankton biomass using chlorophyll *a* values. For instance, in case of phytoplankton communities dominated by dinoflagellates, the use of average C:Chl ratios can lead to severe underestimates of phytoplankton biomass. This was obvious in the Ria Formosa coastal lagoon, when a bloom of a toxic gymnodinoid dinoflagellate (527 x 10^3 cells L^{-1}) that induced the prohibition of bivalve harvesting was clearly detected on biomass (carbon) analyses but depicted no obvious signal on chlorophyll *a* concentration (Barbosa, 2006). In addition, chlorophyll *a* is a poor indicator of total phytoplankton biomass in poor light environments (Buchanan et al., 2005). Available data for Portuguese systems are scarce, and indicate that some systems exhibit a significant and positive correlation between total phytoplankton biomass and chlorophyll *a* concentration (Ria Formosa coastal lagoon: Barbosa, 2006), whilst others present non significant correlations between those variables (shallow coastal waters off southeast Portuguese coast: Barbosa, 2006; Guadiana upper estuary: Domingues et al., unpublished data).

In addition, chlorophyll *a* should be used cautiously as an alternative for phytoplankton abundance and biomass, especially when pico- and nanophytoplankton (<2 μm and 2-20 μm, respectively, *sensu* Sieburth, 1979) are important components of the community. The relative contribution of picophytoplankton biomass to total biomass decreases with increasing chlorophyll *a* concentration, thus coastal and estuarine waters present low relative contributions of picophytoplankton, usually ranging between 10 and 20% (Bell and Kalff, 2001). However, picophytoplankton contribution to total phytoplankton abundance is largely higher than its contribution to phytoplankton biomass. For instance, in the Ria Formosa coastal lagoon, picophytoplankton accounts on average for 13% of total phytoplankton biomass and 82% of total phytoplankton abundance (Barbosa, 2006). In addition, important blooms of picocyanobacteria (Phlips et al., 1999) and eukaryotic picophytoplankton (Vaquer et al., 1996) may occur in coastal and estuarine systems but, due to their small size, these events may not be detected using

chlorophyll *a* as a phytoplankton metric. In some cases picophytoplankton bloom events may present deleterious ecological impacts, thus impairing overall environmental quality status. Hence, blooms of potentially toxic picocyanobacteria (Sorokin et al., 2004) and pico-eukaryotic species such as the pelagophyte *Aureococcus anophagefferens* (Gobler et al., 2002; Nuzzi and Waters, 2004) that may result in fish and shellfish mortality, may not be detected. For instance, in the Guadiana estuary, the cyanobacteria summer bloom, mainly composed of picocyanobacteria and the potentially toxic *Microcystis*, is recognizable in the abundance plot, but clearly undetectable in the biomass curve (Fig. 2.2). Additionally, the evaluation of picophytoplankton abundance should be considered in water quality monitoring programs, given that this phytoplankton metric may be used as an indicator of least-impaired and oligotrophic conditions in coastal environments (Lacouture et al., 2006).

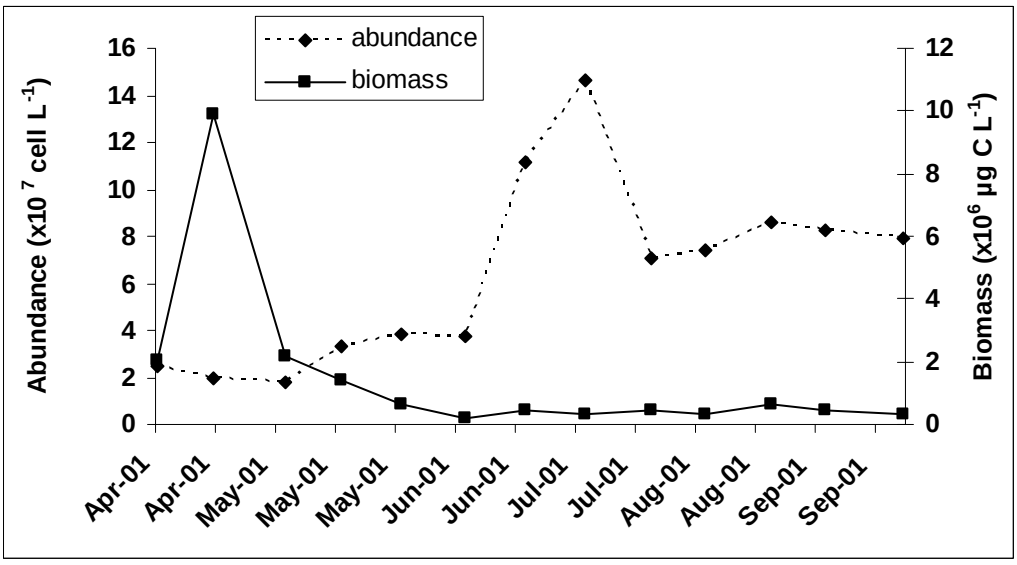

Figure 2.2 – Total phytoplankton abundance (x10^7 cell L^{-1}) and biomass (x10^6 µg C L^{-1}) in the Guadiana estuary from April through September 2001 (see Domingues et al., 2005).

Overall, the lack of information on smaller-sized phytoplankton groups is related to the deeply rooted use of the inverted microscope technique (Utermöhl, 1958). Whilst this method is effective for microphytoplankton identification and counting, it underestimates nano-sized cells and does not allow the observation of pico-sized forms. Therefore, most phytoplankton studies in Portuguese waters only account for

microphytoplanktonic species and some nano-sized genera (e.g., Gameiro et al., 2004; Loureiro et al., 2005a; Chícharo et al., 2006; Loureiro et al., 2006; Brogueira et al., 2007; Coelho et al., 2007). A growing number of studies indicate that nano- (Sin et al., 2000; Ansotegui et al., 2003; Ornolfsdottir et al., 2004; Thomas et al., 2005) and picophytoplankton (Iriarte and Purdie, 2004; Beg et al., 2005) are important constituents of the community in different aquatic ecosystems, such as lakes, rivers, estuaries and the pelagic ocean (see Carrick and Schelske, 1997), but their contribution is frequently overlooked with the inverted microscope.

The quantitative evaluation of pico- and nanophytoplankton should be based on epifluorescence microscopy (Haas, 1982) or flow cytometry. Although fluorescence techniques have been extensively used in Portugal for heterotrophic bacterioplankton enumeration (e.g., Barbosa, 1991; Cunha et al., 2000; Almeida et al., 2005), it is seldom used in phytoplankton studies. Thus, the quantitative analysis of the whole phytoplankton community abundance, biomass and composition should be undertaken using both inverted and epifluorescence microscopy. Using both techniques, phytoplankton biomass can be calculated using species-specific measurements of biovolume (Hillebrand et al., 1999) and specific carbon to volume relationships (e.g., Domingues et al., 2005). We acknowledge that this estimate of phytoplankton biomass has inherent weaknesses (see Lacouture et al., 2006 and references therein) but we consider this approach more accurate than the simple multiplication of chlorophyll *a* concentration by an average C:Chl ratio.

In Portuguese waters, the combination of both inverted and epifluorescence microscopy was applied only in the Guadiana estuary (Rocha et al., 2002; Domingues et al., 2005, 2007; Domingues and Galvão, 2007) and the Ria Formosa coastal lagoon (Morais et al., 2003; Barbosa, 2006). This approach showed that pico- and nanophytoplankton are important contributors for total phytoplankton biomass and abundance in these systems. For instance, in the Ria Formosa coastal lagoon, picophytoplankton (<2 μm) accounts on average for 82% of the total abundance and approximately 13% of the community's biomass, whilst nanophytoplankton (2-20 μm) contributes to 15% and 41-51% of the community's abundance and biomass, respectively (Barbosa, 2006). In the Guadiana estuary, the cyanobacteria summer bloom is mainly composed by picocyanobacteria and the nanoplanktonic genus

Microcystis (Domingues et al., 2005). In the saline lake of Quinta do Lago (Ria Formosa) picocyanobacteria and nanoflagellates are the dominant groups (Morais et al., 2003).

2.3 Conclusions and future prospects

In this viewpoint, we tried to show that the use of phytoplankton as a biological quality element in Portuguese waters will certainly pose several constrains, especially because existing data on phytoplankton in Portuguese aquatic ecosystems are scarce and the methodologies used do not allow the achievement of the Water Framework Directive aims for phytoplankton. A proper evaluation of phytoplankton community structure in Portugal is urgent, providing that phytoplankton is one of the biological quality elements upon which reference conditions of water quality should be based.

Therefore, a solid phytoplankton monitoring program should be implemented for Portuguese water bodies using an adequate sampling strategy and microscopy techniques that allow the evaluation of composition, abundance and biomass of the whole phytoplankton community. However, microscopy techniques are time-consuming and may not be cost-effective, so their incorporation in environmental monitoring programs may be somewhat unrealistic. Other techniques should then be considered as supplements for the evaluation of phytoplankton community. For instance, flow cytometry can be used for more frequent sampling (e.g., Dubelaar et al., 2004), whilst chemotaxonomic analysis (e.g., Ansotegui et al., 2003) of specific photosynthetic pigments using HPLC provide a good estimate of group-specific phytoplankton biomass. In addition, in costal zones remote sensing can also be an important aid to achieve different phytoplankton ecological indicators, providing highly resolved data both in time and space (see Platt and Sathyendranath, 2008). *In situ* instrumentation with moorings for fluorescence measurements can also provide early warnings for the occurrence of phytoplankton blooms. This type of *in situ* instrumentation is already available in the Guadiana, Tagus and Mondego estuaries (http://webserver.mohid.com/simpatico/). Moreover, although not referred by the WFD, a functional-based approach could also be used in monitoring programs. For

example, determination of phytoplankton primary production (phytoplankton growth) can be used as an indicator of increased growth, or eutrophication (Andersen et al., 2006). Although chlorophyll *a* concentration is frequently used as a key factor to predict the production of phytoplankton biomass, this approach is only applicable to aquatic systems that exhibit a significant relationship between phytoplankton biomass and production or growth rate. This type of relationship is usually considered an indicator of bottom-up control of phytoplankton growth (see Sin et al., 1999). However, when top-down control (e.g., grazing, viral lysis, advection) is prevalent, phytoplankton biomass and phytoplankton production or growth can be clearly uncoupled (e.g., Malone et al., 1988; Tillmann et al., 2000).

Furthermore, the evaluation of biotic integrity/quality status should be accomplished using suitable phytoplankton-based multimetric indexes. The ability of these metrics to discriminate between impaired and least-impaired systems should be tested, in order to find the most suitable to specific aquatic systems (e.g., Buchanan et al., 2005; Lacouture et al., 2006; Devlin et al., 2007), and the inclusion of harmful phytoplankton species in these metrics should be considered. Since harmful algal blooms (HABs) in Portuguese waters can be clearly associated to natural phenomena, such as upwelling/downwelling events (e.g., GEOHAB, 2005 and references therein; Moita et al., 2006), the inclusion of HAB species in these metrics should be considered if there is evidence that these species are related to anthropogenic impact. Ultimately, this strategy will allow the definition of different target communities of phytoplankton, both on spatial and temporal levels that can act as specific impairment indicators.

Chapter 3

Tidal variability of phytoplankton and environmental drivers in the freshwater reaches of the Guadiana estuary

Tidal variability of phytoplankton and environmental drivers in the freshwater reaches of the Guadiana estuary (SW Iberia)

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Abstract

The effects of different phases of semidiurnal and spring-neap tidal cycles on phytoplankton and environmental drivers were evaluated in a tidal, freshwater location of a mesotidal estuary (Guadiana estuary, SW Iberia). An Eulerian approach was used and sampling covered different seasons during 2008. Samples were collected during spring and neap tides, at high tide, mid-ebb, low tide and mid-flood. Several physical-chemical variables were measured, as well as phytoplankton abundance and biomass.

Salinity was higher at high-tide and suspended particulate matter was higher during spring tides and flood, due to higher vertical mixing and resuspension of bottom sediments. Chlorophyll *a* concentration during winter and summer neap tides was higher than during spring tides, whilst the abundance of pennate diatoms was higher during winter and Spring spring tides than during neap tides, probably reflecting differences in river discharge. Overall, tidally-induced differences detected in the freshwater tidal reaches of the Guadiana estuary were not as considerable as those observed in the lower estuary. However, the occurrence of tidally-induced variability in some seasons reflects that thorough sampling programs to study estuarine tidal dynamics should be conducted throughout the year. Occasional sampling will not reflect the typical variability of these highly dynamic systems.

Keywords: semidiurnal tides, spring-neap tides, Portugal, Spain

3.1. Introduction

Phytoplankton distribution patterns reflect the interplay between phytoplankton growth rates, commonly regulated by light, inorganic nutrients, temperature, and turbulence, and their loss rates, controlled by grazing, viral lyses, sinking, and advection (Cloern and Dufford, 2005). In estuaries, tidal flushing constitutes a relevant phytoplankton driving force, since it induces substantial horizontal and vertical mixing of the water column, as well as upstream and downstream displacement of water masses along the main longitudinal estuarine axis. While tidally-induced horizontal mixing and advection have more mechanical than physiological effects on phytoplankton (Legendre and Demers, 1984), vertical mixing can seriously affect phytoplankton physiology and growth due to its strong impact on the availability of key phytoplankton resources, nutrients and light (Demers et al., 1979, 1986). Tidally-induced vertical mixing may also modulate phytoplankton loss rates either because it affects the resuspension of benthic microalgae into the water column (see MacIntyre and Cullen, 1996) or because it effectively controls the grazing impact of benthic filter feeders (Lucas and Cloern, 2002).

Overall, tidal forcing is therefore responsible for short-term changes in phytoplankton biomass, composition, growth and production, occurring at daily and fortnightly time scales (Sinclair et al., 1981; Demers et al., 1986; Wetz et al., 2006). These tidally-induced phytoplankton alterations are particularly significant in shallow tidally-driven estuarine systems (Cloern, 1991; Wetz et al., 2006), and have been extensively addressed in mid- and lower estuarine reaches (e.g., Therriault and Lacroix, 1976; Duedall et al., 1977; Fortier et al., 1978; Demers et al., 1979; Fortier and Legendre, 1979; Lafleur et al., 1979; Riaux and Douvillé, 1980; Demers and Legendre, 1981; Haas et al., 1981; Riaux, 1981; Litaker et al., 1987, 1993; Cloern et al., 1989; Dustan and Pinckney, 1989; Powell et al., 1989; Gianesella et al., 2000; Jouenne et al., 2005; Helbling et al., 2010) characterized by marked longitudinal gradients in salinity and water column stratification (Sinclair et al., 1981; Cloern, 1991). On the contrary, only a limited number of studies have addressed phytoplankton tidal dynamics in the upper estuarine zones (Madariaga 1995, Trigueros and Orive, 1995; Lehman, 2000; Lucas et al., 2006; see Table 3.I). Although these upper estuarine reaches

present smoother environmental gradients, they are usually relevant sources of nutrients and phytoplankton biomass to downriver estuarine reaches and adjacent coastal areas (Rocha et al., 2002; Domingues and Galvão, 2007), and clearly deserve further investigation. Freshwater tidal regions are extreme environments to phytoplankton, namely in turbid estuaries, since despite high nutrient concentrations, high turbidity usually leads to strong light limitation and low phytoplankton growth rates (Cole et al., 1992). Furthermore, phytoplankton losses due to freshwater discharges and downriver displacement are constant (Muylaert et al., 2000) and occasional saltwater intrusion may cause salinity stress and mortality (Morris et al., 1978). Overall, the effects of tidal forcing on estuarine phytoplankton differ over time and space, and are not always easy to predict (see Roden, 1994 and references therein). Furthermore, the low-frequency sampling that is usually employed in monitoring programs may incorrectly characterize maxima, minima, mean values and long-term trends (Lucas et al., 2006), given that many physical, chemical and biological processes occur on intradaily time-scales.

The upper, permanently mixed, freshwater section of the Guadiana estuary (SE Portugal-SW Spain) is subjected to a strong tidal influence, but the extent of tidally-induced variability in abiotic and biotic variables has never been examined. Published studies on phytoplankton dynamics in the Guadiana upper estuary (Rocha et al., 2002; Sobrino et al., 2004; Domingues et al., 2005, 2007; Domingues and Galvão, 2007) have tried to systematically sample the same tidal phase, to avoid the interference of semidiurnal and fortnightly tidally-induced variability. Indeed, tidally-induced variability, usually neglected in routine sampling strategies in estuaries worldwide (see Li and Smayda, 2001), may potentially affect the analysis and interpretation of long-term trends in estuarine phytoplankton and related environmental variables.

Table 3.I – Comparison of published studies on tidally-induced variability of phytoplankton in upper/low salinity estuarine zones.

Reference	Estuary	Zones sampled	Objectives	Tidal cycles evaluated	Tidal differences
Madariaga (1995)	Urdaibai, Spain	From mouth to upper limit of saltwater intrusion	Analyse short-term environmental patterns in relation to physiological properties of phytoplankton	Spring-neap	Higher phytoplankton biomass during neap tides
Lehman (2000)	San Francisco Bay, USA	Low salinity zone (LSZ, salinity 0.6-4.0)	Characterize spatial and temporal variation of Chl*a*, cell diameter, species composition in the LSZ during spring	Spring-neap	Maximum phytoplankton biomass during strong spring tide and strong neap tide
Trigueros and Orive (2000)	Urdaibai, Spain	From mouth to upper limit of saltwater intrusion	Assess longitudinal distribution of blooming phytoplankton species through the estuary during ebb	Spring-neap and semidiurnal	Advective seaward losses of bloom-forming diatoms during ebb were compensated by intense growth, allowing development of stable populations in the estuary
Lucas et al. (2006)	Sacramento-San Joaquin River Delta, USA	Freshwater tidal zone	Investigate intradaily variability of specific conductance, water temperature and Chl*a*	Semidiurnal	
present study	Guadiana, Portugal/ Spain	Freshwater tidal zone	Analyse tidally-induced variability of phytoplankton and environmental drivers along semidiurnal and spring-neap tidal cycles	Spring-neap and semidiurnal	Higher phytoplankton biomass during neap tides

The reduced number of published studies on freshwater/low salinity estuarine zones have addressed specific questions (see Table 3.I), but a global overview of tidally-induced variability of phytoplankton and their environmental drivers in freshwater tidal systems is still needed. In addition, we are not aware of studies describing tidally-induced differences over spring-neap and semidiurnal tidal cycles in freshwater tidal zones of Mediterranean estuaries. Therefore, the main goal of this article is to analyse tidal variability of phytoplankton and their environmental drivers (salinity, temperature, nutrients, light), along semidiurnal and fortnightly time scales, in the freshwater tidal reaches (upper estuary) of the mesotidal Guadiana estuary. Since phytoplankton in this estuarine region usually reveals a relative horizontal homogeneity (Rocha et al., 2002; Domingues and Galvão, 2007), and persistent light limitation coupled to episodic nutrient limitation (Domingues et al., 2005; Barbosa et al., 2010), we hypothesise that: (1) tidally-induced phytoplankton changes in the Guadiana upper estuary will be mostly driven by the spring-neap tidal cycles; and (2) phytoplankton will depict high biomass values during neap tides due to decreased vertical mixing and turbidity.

3.2. Material and Methods

3.2.1 Study site and sampling strategy

The Guadiana River's (drainage area 67,039 km^2, length 810 km) estuary forms the border between SE Portugal and SW Spain. Located in a Mediterranean climate area, it is a mesotidal estuary with semidiurnal tides, partially stratified in its lower and middle sections (but depending on river flow, tidal phase and tidal amplitude, Oliveira et al., 2006) and always well mixed in the upper section. In respect to tidal amplitude, the estuary can be considered synchronous, given that tidal amplitude is constant at least up to 50 km from the mouth (Morales, 1995). Freshwater inputs to the estuarine zone used to vary sharply between dry and humid months (1995-2000: 333.0 ± 1095.9 m^3 s^{-1}, http://snirh.pt), but in the last years the recently built Alqueva dam has promoted a more regular but reduced freshwater flow (2008: 14.2 ± 9.1 m^3 s^{-1}). The upper estuary, or freshwater tidal zone, is usually located between Álamo (25

km from the river mouth) and extends upriver from Mértola (approx. 70 km from the river mouth) (Fig. 3.1).

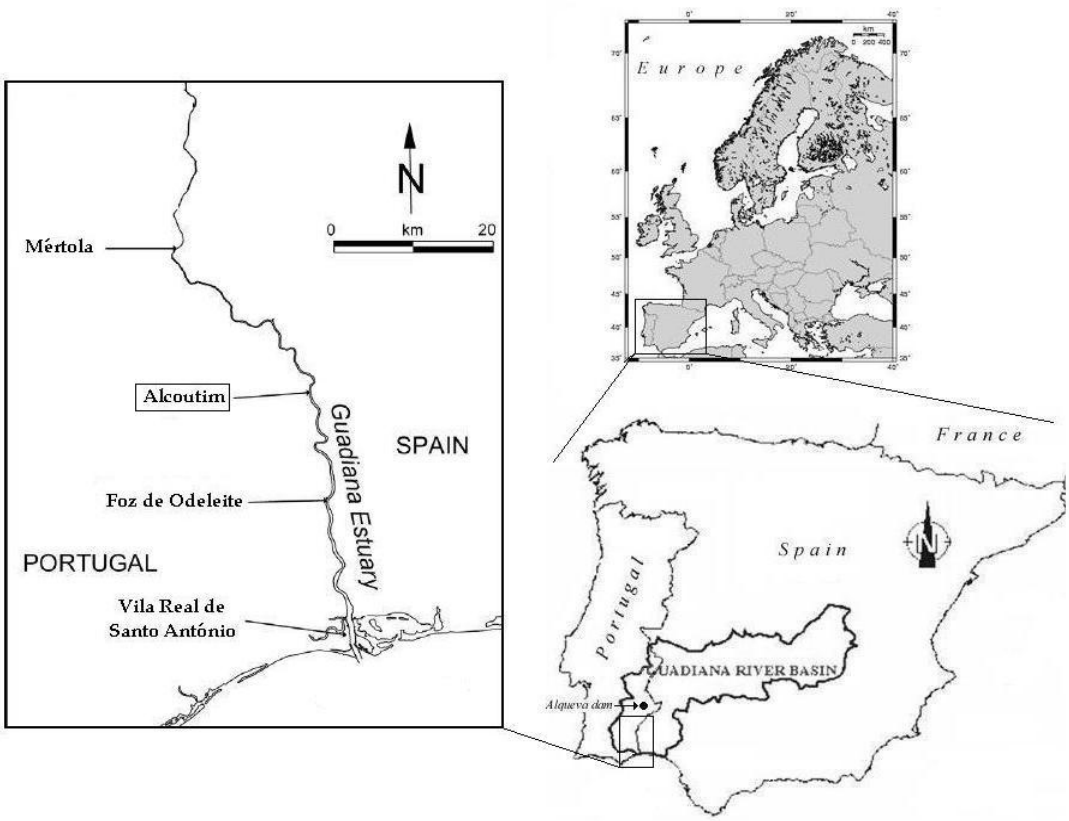

Figure 3.1 – Location of the sampling site, Alcoutim, in the Guadiana estuary (south-western Iberian Peninsula).

Sampling for the evaluation of tidally-induced variability was conducted at station Alcoutim, located at approx. 38 km from the river's mouth (Fig. 3.1). Sampling was undertaken throughout 2008, in the winter (February), spring (April), summer (August) and autumn (October); both spring (tidal amplitudes between 2.45 and 2.90 m) and neap tides (tidal amplitudes between 0.86 and 2.08 m) were sampled for each season. For each sampling date, samples were collected approximately every three hours, at high tide (slack water), mid-ebb, low tide (slack water), and mid-flood. This sampling was part of a broader monitoring program held in several locations covering the upper (Mértola and Alcoutim), middle (Foz de Odeleite) and lower (VRSA) estuaries throughout 2008 (see Fig. 3.1). This information on longitudinal variability was considered only as an aid to interpret tidal variability.

3.2.2 Environmental variables

Profiles of water temperature and salinity (measured as conductivity) were determined in situ using a YSI 556 MPS probe. Vertical profiles of photosynthetically active radiation (PAR) intensity were determined using a LI-COR radiometer and light extinction coefficient (k_e, m^{-1}) was calculated using an exponential function, $I_z = I_0\ e^{-Ke.Z}$, where I_z is the light intensity at depth level Z (m) and I_0 is the light intensity at the surface. Mean light intensity in the mixed layer (I_m, µmol photons $m^{-2}\ s^{-1}$) was calculated according to $I_m = I_o\ (1\text{-}e^{(-Ke.Zm)})(K_e.Z_m)^{-1}$, where I_o is the light intensity at the surface, k_e (m^{-1}) the light extinction coefficient and Z_m (m) the depth of the mixed layer (Jumars, 1993). The mixed layer corresponded to the whole water column, since there was neither haline nor thermal stratification (see section 3.3 Results). Subsurface (approx. 0.5 m) water samples were collected for subsequent laboratorial analyses of dissolved inorganic macronutrients, suspended particulate matter, chlorophyll *a* concentration and phytoplankton composition, abundance and biomass. Samples for nutrient determination were collected and immediately filtered through cellulose acetate filters (Whatman, nominal pore diameter = 0.2 µm). Ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_4^{4-}) were determined immediately after sample collection, whilst samples for nitrate (NO_3^-) and nitrite (NO_2^-) where frozen (-20ºC) 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, phosphate and silicate, and an autoanalyzer Skalar for nitrate and nitrite. Dissolved inorganic nitrogen (DIN) was calculated as the sum of nitrate, nitrite and ammonium. Concentration of suspended particulate matter (SPM) was determined gravimetrically. For each sample, the analysis was made in duplicate. 250 mL were filtered onto pre-combusted (4 hours at 450ºC) glass fibre filters (Whatman GF/F, nominal pore diameter = 0.7 µm), dried in a Memmert incubator at 50ºC for 24 hours and then weighed after cooling down to room temperature. Data on the Guadiana mean daily river flow, measured at Pulo do Lobo hydrometric station (85 km from the river's mouth), was obtained from the Portuguese National Water Institute (http://snirh.pt).

3.2.3 Phytoplankton variables

Chlorophyll *a* concentration was determined spectrophotometrically using glass fibre filters (Whatman GF/F, nominal pore diameter = 0.7 μm) (Parsons et al., 1984b). Chlorophyll *a* was extracted overnight at 4ºC with 90% acetone; after centrifugation, absorbance of the supernatant was measured in the spectrophotometer 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 pico- (<2 μm) and nanophytoplankton (2 - 20 μm) were preserved with glutardialdehyde (final concentration 2%) immediately after collection, stained with proflavine and filtered (1-5 mL, depending on the amount of suspended matter) onto black polycarbonate membrane filters (Whatman, nominal pore diameter = 0.45 μm). Preparations were made using glass slides and non-fluorescent immersion oil (Cargille type A), within 24 h of sampling, and then frozen (-20ºC) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5x magnification using an epifluorescence microscope (Leica DM LB). Samples for enumeration of microphytoplankton (>20 μm) were preserved with acid Lugol's solution (final concentration approx. 0.003%) immediately after collection, settled in sedimentation chambers (2-10 mL, depending on the amount of suspended matter; sedimentation time = 24 hours) and observed at 400x magnification with an inverted microscope (Zeiss Axiovert S100). Phytoplankton cells were identified, whenever possible, to species level. 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).

3.2.4 Data analyses

Horizontal profiles of salinity, SPM, light extinction coefficient, DIN concentration and chlorophyll *a* concentration along the main estuarine axis were created using Surfer 8.01 software (Golden Software Inc.), using kriging (linear variogram model)

as the gridding method. Basic statistics (mean, median, standard deviation), statistical tests, and correlation coefficients were performed using STATISTICA 6.0® software package. The strength of associations between variables was assessed using Spearman rank correlation coefficients (r). Differences across different tidal stages, both at semidiurnal and fortnightly time scales, were evaluated using non-parametric tests. Differences between the four semidiurnal tidal stages were tested using a non-parametric Kruskall-Wallis analysis of variance on ranks, and a Tukey's post-hoc test. In the case of fortnightly tidal variability, differences in median values between neap tides and spring tides were assessed using a Mann-Whitney rank sum test. All statistical analyses were considered at a significance level of 0.05.

3.3. Results

3.3.1 Longitudinal distribution of phytoplankton and environmental drivers

Mean daily river flow at Pulo do Lobo hydrometric station throughout 2008 ranged between 1.8 $m^3 s^{-1}$ and 125.6 $m^3 s^{-1}$, and averaged 13.1 ± 9.1 $m^3 s^{-1}$ (Fig. 3.2). Salinity was always detectable in the lower and middle estuarine zones, but the upper estuary remained freshwater throughout the year (Fig. 3.3A). Suspended particulate matter (SPM) increased downriver, with lower values in the upper estuary (Mértola: 12.9 ± 9.1 mg L^{-1}; Alcoutim: 44.0 ± 15.7 mg L^{-1}) and higher close to the river mouth (VRSA: 114.9 ± 28.4 mg L^{-1}) (Fig. 3.3B). Light extinction coefficient in the upper and middle estuaries (2.98 ± 1.69 m^{-1}) was higher than in the lower estuary (0.81 ± 0.32 m^{-1}) (data not shown). Consequently, mean light intensity in the mixed layer (I_m) presented higher values in the lower estuary (176.0 ± 101.6 μmol photons $m^{-2} s^{-1}$) than in the middle and upper estuaries (63.5 ± 81.0 μmol photons $m^{-2} s^{-1}$) (Fig. 3.3C). The concentration of dissolved inorganic nitrogen (DIN) did not show a significant spatial variability within the upper and middle estuaries (Mértola: 29.1 ± 18.3 μM; Alcoutim: 30.4 ± 14.5 μM; Foz de Odeleite: 31.5 ± 10.1 μM) throughout 2008, but lower values were observed in the lower estuary (VRSA: 17.9 ± 12.9 μM) (Fig. 3.3D). Occasionally, DIN maxima were observed in Mértola (max 67.4 μM, Fig. 3.3D).

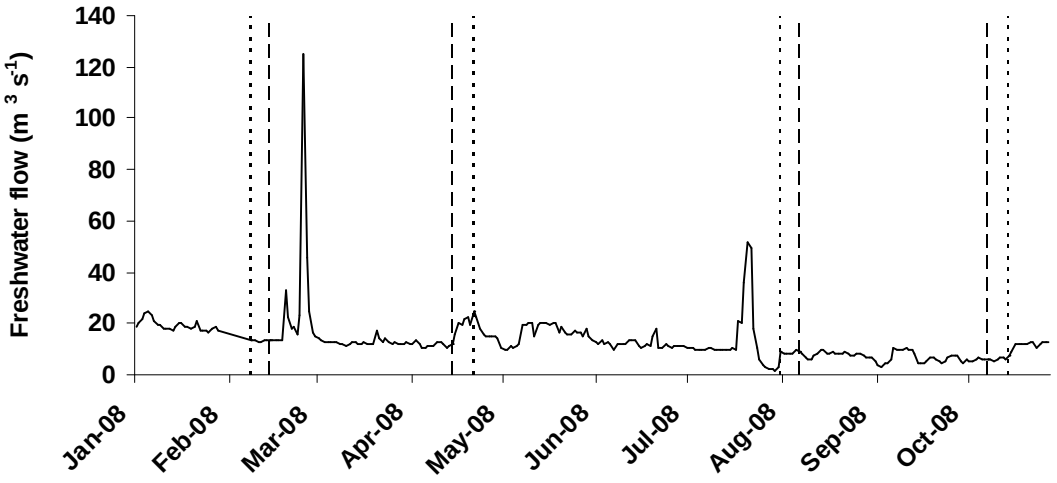

Figure 3.2 – Freshwater flow (m^3 s^{-1}) measured at Pulo do Lobo throughout 2008. Dotted (…..) vertical lines represent sampling on spring tides and dashed (- - -) vertical lines represent sampling dates during neap tides.

Dissolved reactive silicate (DSi) and dissolved reactive phosphate (DRP) exhibited a similar spatial distribution along the main longitudinal estuarine axis (data not shown), with higher concentrations in the upper (Mértola: 46.4 ± 26.4 µM DSi and 2.3 ± 0.6 µM DRP; Alcoutim: 56.4 ± 32.6 µM DSi) and middle (Foz de Odeleite: 47.4 ± 30.8 µM DSi) estuarine regions and slightly lower concentrations in the lower estuary (VRSA: 17.5 ± 12.0 µM DSi, and 1.7 ± 1.4 µM DRP). Chlorophyll *a* concentration (Chl*a*), a proxy for phytoplankton biomass, ranged between undetectable values and 16.0 µg L^{-1} and was higher in the upper estuary (Mértola: 5.2 ± 2.9 µg L^{-1}; Alcoutim: 8.3 ± 3.3 µg L^{-1}), decreasing downriver (VRSA: 1.8 ± 1.5 µg L^{-1}) (Fig. 3.3E). Chl*a* exhibited a clear seasonality with the highest values during summer, namely in Alcoutim.

3.3.2 Tidal variability of environmental drivers

Vertical profiles of water temperature in Alcoutim showed significant diel differences, with lower temperatures early in the morning and higher in the afternoon (Table 3.II). These differences were related to changes in air temperature rather than to semidiurnal tidal cycles. Salinity did not show significant differences between neap and spring tides. However, significant ($p < 0.001$) semidiurnal differences were evident throughout the year, except in the Spring, with higher salinity values during high tide (Fig. 3.4A, Table 3.II). Daily salinity ranges in Alcoutim attained a maximum during the autumn spring tide (1.19 to 6.74, for low-tide and high-tide, respectively). Vertical profiles of temperature and salinity evidenced the absence of water column stratification in Alcoutim, with similar T (maximum differences between surface and bottom <0.5^{o}C) and S (maximum differences between surface and bottom <0.5) values at the surface and bottom.

Light extinction coefficient (k_e) did not vary significantly between the different tidal phases of the semidiurnal and fortnightly cycles (Fig. 3.4B). Suspended particulate matter (SPM) was significantly higher during spring tides than during neap tides in the Spring and Autumn ($p < 0.05$), whilst no significant differences were observed in the Winter and Summer (Fig. 3.4C, Table 3.II). In all the spring tides, SPM was higher during flood, whilst during neap tides SPM showed minimum values at low tide. Maximum daily SPM range in Alcoutim was measured during the autumn spring tide (50.2 mg L^{-1} and 129.2 mg L^{-1} for low tide and flood, respectively. SPM was positively correlated to light extinction coefficient ($r = 0.656$, $p > 0.0001$, $n = 32$). Nitrate and phosphate concentrations did not show any significant tidal differences along the spring-neap and semidiurnal tidal cycles (Table 3.II). Silicate concentration showed significant differences only in the Spring fortnightly cycle, with higher Si during the neap tide ($p < 0.0001$).

Fig. 3.3

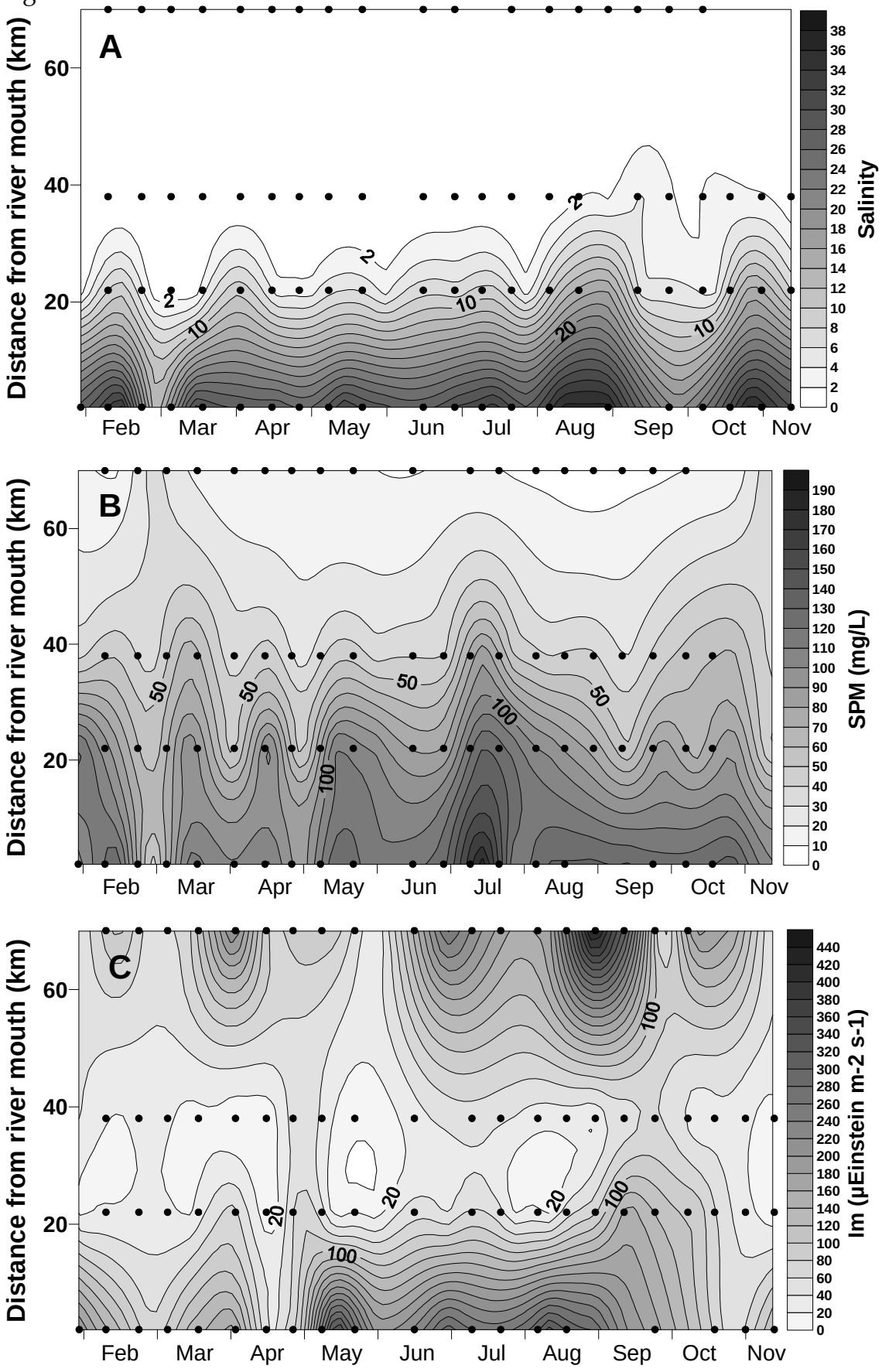
A
Distance from river mouth (km)
60
40
20
2
10
20
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Salinity
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
B
Distance from river mouth (km)
50
100
SPM (mg/L)
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
C
Distance from river mouth (km)
Im (µEinstein m-2 s-1)
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0

Figure 3.3 (cont.)

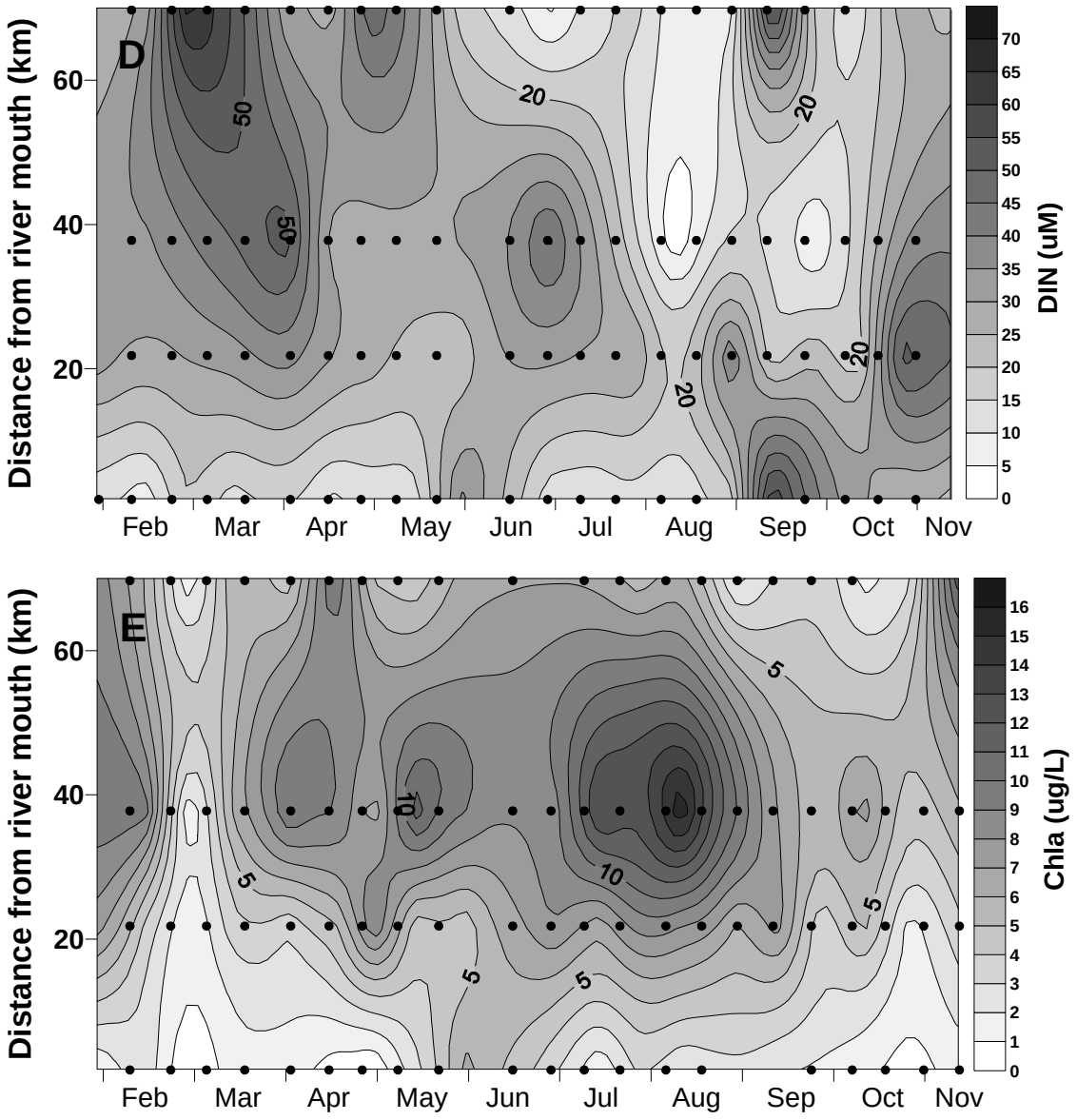

Figure 3.3 – Horizontal profiles of (A) salinity, (B) suspended particulate matter (SPM, mg L^{-1}), (C) mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}), (D) concentration of dissolved inorganic nitrogen (DIN, µM), and (E) chlorophyll *a* concentration (Chl*a*, µg L^{-1}), throughout 2008. Profiles were generated used kriging as the gridding method. Station Alcoutim is located approximately 38 km from the river mouth.

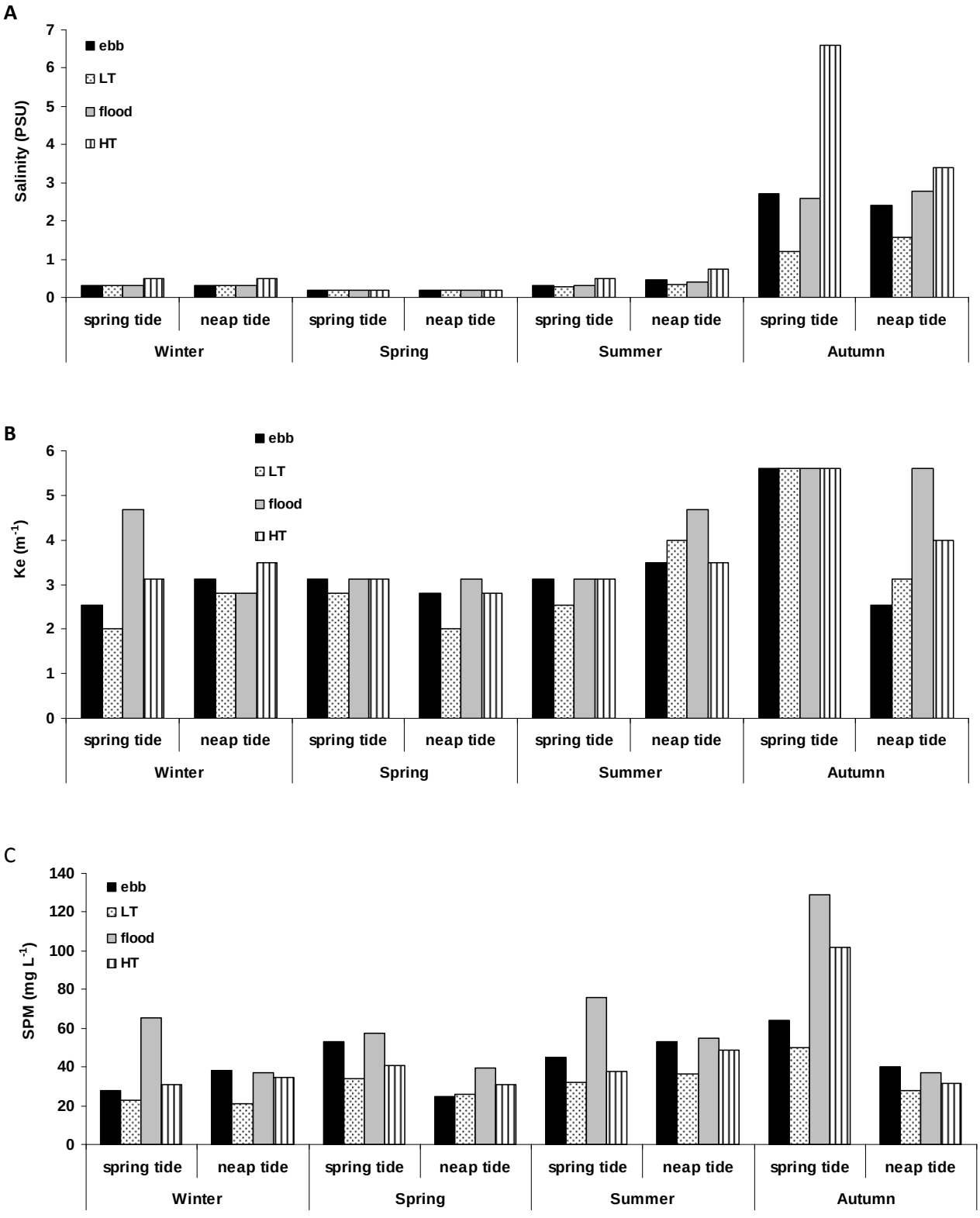

Figure 3.4 – (A) salinity (PSU), (B) light extinction coefficient (k_e, m^{-1}) and (C) concentration of suspended particulate matter (SPM, mg L^{-1}) in Alcoutim along the spring-neap and semidiurnal tidal cycles.

Table 3.II – Surface (surf.) and bottom (bott.) values for water temperature (T, ºC) and salinity (S), subsurface suspended particulate matter concentration (SPM, mg L^{-1}), light extinction coefficient (k_e, m^{-1}), and subsurface nutrient concentration (N – dissolved inorganic nitrogen; P – dissolved reactive phosphate; Si – dissolved reactive silicate; µM), measured in each phase of the different tidal cycles in Alcoutim, throughout 2008.

Season	Spring neap	Semi-diurnal	T		S		SPM	k_e	N	P	Si
			surf.	bott.	surf.	bott.					
winter	spring 7Feb	ebb	13.0	12.8	<0.3	<0.3	27.8	2.5	34.1	2.0	48.6
		LT	13.0	12.9	<0.3	<0.3	22.6	2.0	32.0	2.0	44.9
		flood	13.3	13.1	<0.3	<0.3	65.2	4.7	34.0	2.2	48.1
		HT	13.3	13.1	0.5	0.5	30.6	3.1	35.4	1.9	51.0
	neap 14Feb	ebb	13.8	13.5	<0.3	<0.3	38.4	3.1	30.6	2.2	45.4
		LT	14.0	13.7	<0.3	<0.3	21.2	2.8	29.1	2.7	39.3
		flood	13.9	13.7	<0.3	<0.3	37.0	2.8	29.9	2.2	37.3
		HT	13.6	13.3	0.5	0.5	34.4	3.5	31.5	2.6	42.2
spring	spring 21Apr	ebb	18.0	18.0	<0.3	<0.3	53.2	3.1	29.9	2.4	73.2
		LT	18.1	18.0	<0.3	<0.3	34	2.8	25.0	1.6	73.7
		flood	18.5	18.3	<0.3	<0.3	57.6	3.1	29.0	2.4	72.3
		HT	18.7	18.3	<0.3	<0.3	40.6	3.1	29.8	2.3	76.1
	neap 14Apr	ebb	17.6	17.5	<0.3	<0.3	24.6	2.8	24.2	1.9	104.6
		LT	17.1	17.1	<0.3	<0.3	26.2	2.0	27.3	2.3	101.8
		flood	17.1	17.1	<0.3	<0.3	39.2	3.1	24.6	2.3	99.2
		HT	17.4	17.3	<0.3	<0.3	30.6	2.8	24.4	2.0	103.4
summer	spring 31Jul	ebb	25.7	25.7	<0.3	<0.3	44.8	3.1	8.8	2.7	52.2
		LT	25.9	25.9	<0.3	<0.3	32.2	2.5	10.1	0.6	48.4
		flood	26.1	26.0	<0.3	<0.3	75.8	3.1	11.8	2.9	52.5
		HT	26.1	26.1	0.5	0.5	37.6	3.1	14.4	2.9	54.9
	neap 6Aug	ebb	26.9	26.9	0.5	0.5	52.8	3.5	11.8	2.8	57.0
		LT	27.2	27.2	<0.3	<0.3	36.2	4.0	9.1	2.7	59.3
		flood	27.3	27.3	0.4	0.4	54.6	4.7	8.3	2.8	50.8
		HT	26.6	26.6	0.7	0.8	48.6	3.5	8.4	2.9	57.8
autumn	spring 14Oct	ebb	20.8	20.8	2.8	2.7	64.0	5.6	20.3	3.5	48.5
		LT	20.9	20.9	1.2	1.2	50.2	5.6	19.7	3.4	57.6
		flood	21.0	21.0	2.5	2.7	129.2	5.6	21.5	3.3	45.2
		HT	21.2	21.2	6.5	6.7	101.8	5.6	16.0	3.2	36.1
	neap 7Oct	ebb	22.2	22.2	2.4	2.4	40.2	2.5	22.3	3.8	47.8
		LT	22.3	22.2	1.5	1.6	28.0	3.1	23.5	3.6	45.5
		flood	22.0	22.0	2.7	2.9	37.0	5.6	23.7	3.5	43.9
		HT	22.1	22.0	3.2	3.7	31.6	4.0	23.5	3.1	41.9

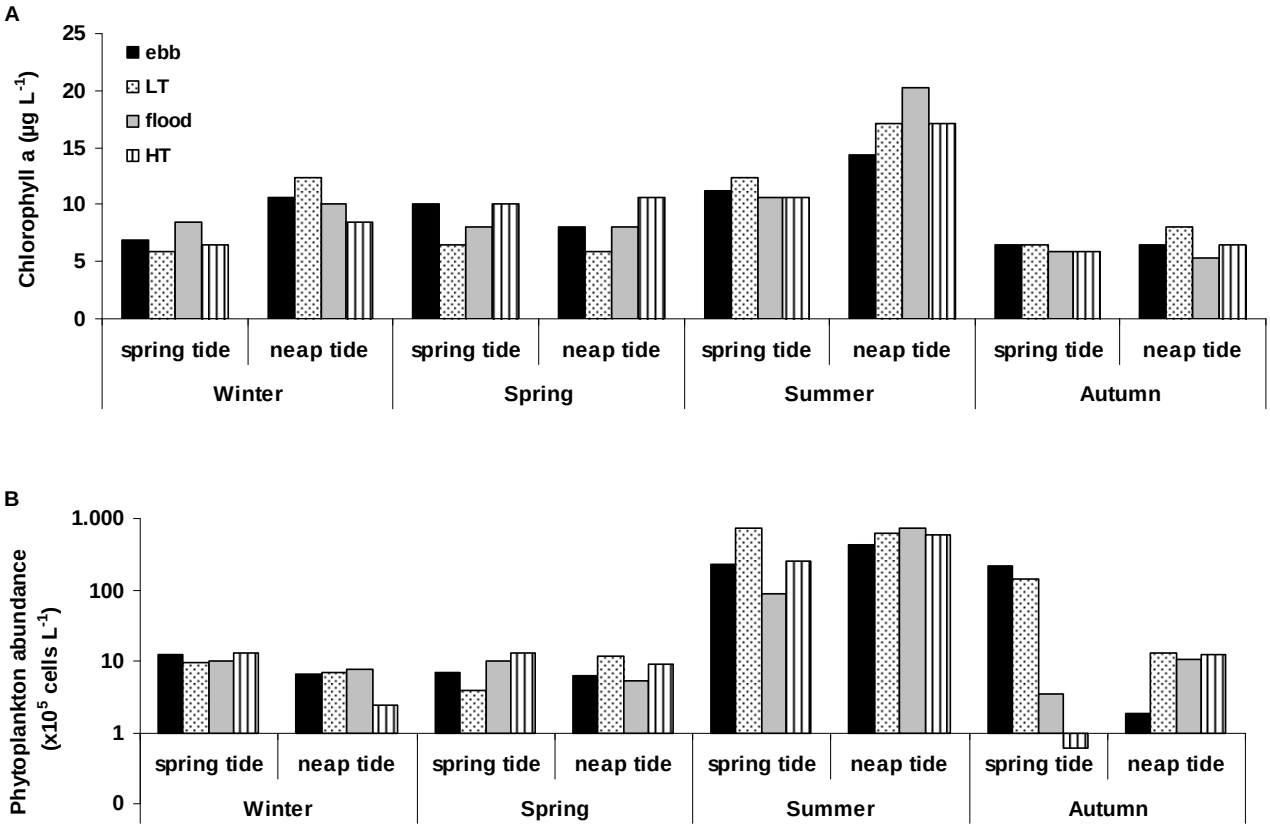

Figure 3.5 – (A) Chlorophyll *a* concentration (µg L^{-1}) and (B) phytoplankton total abundance (x10^5 cells L^{-1}) in Alcoutim along the spring-neap and semidiurnal tidal cycles.

3.3.3 Tidal variability of phytoplankton

Chlorophyll *a* concentration (Chl*a*) varied between 5.3 and 20.2 µg L^{-1}, and no significant semidiurnal tidal differences were detected (Fig. 3.5A, Table 3.III). Fortnightly differences were found in the winter and summer ($p < 0.05$), with higher Chl*a* values during neap tides. Total abundance of phytoplankton varied between 6.2 x 10^4 and 7.4 x 10^7 cells L^{-1}, with clear summer maxima, and did not exhibit significant tidal differences along the spring-neap and semidiurnal tidal cycles (Fig. 3.5B, Table 3.III). Phytoplankton community was mainly composed by diatoms (solitary and chain-forming centric genera, and pennate benthic genera), green algae (mostly *Scenedesmus*, *Monoraphidium* and *Pediastrum*), and coccoid cyanobacteria. Furthermore, dinoflagellates (*Kryptoperidinium foliaceum*) and nano-cryptophytes were more abundant during summer and winter, respectively (data not shown). The

abundance of each phytoplanktonic group did not show any significant tidal differences along the spring-neap and semidiurnal tidal phases (Table 3.III). However, during winter and spring, the abundance of pennate diatoms was significantly higher during spring tides in relation to neap tides ($p < 0.05$; Fig. 3.6A). It is worth mentioning that during these seasons, mean river flow over an 8-day period preceding sampling dates was significantly higher during spring tides than during neap tides ($p < 0.001$; see Fig. 3.6B).

Table 3.III – Chlorophyll *a* concentration (Chl*a*, µg L^{-1}) and abundance of total and specific phytoplankton groups (x 10^5 cells L^{-1}) measured in subsurface samples at each phase of the different tidal cycles at station Alcoutim, throughout 2008. TOT – total phytoplankton; DIA – diatoms; GA – green algae; CYA – cyanobacteria; nd – not detected.

Season	Spring neap	Semi-diurnal	Chl*a*	TOT	DIA	GA	CYA
winter	spring 7Feb	ebb	6.9	12.29	4.01	3.29	nd
		LT	5.9	9.63	2.95	2.73	0.01
		flood	8.5	9.98	4.02	3.11	nd
		HT	6.4	13.14	1.12	3.48	nd
	neap 14Feb	ebb	10.7	6.64	2.73	0.95	0.02
		LT	12.3	7.06	1.39	1.17	nd
		flood	10.1	7.84	2.64	3.58	nd
		HT	8.5	2.44	1.28	1.16	nd
spring	spring 21Apr	ebb	10.1	7.08	6.24	0.73	nd
		LT	6.4	3.90	3.18	0.67	nd
		flood	8.0	10.21	6.97	0.33	nd
		HT	10.1	13.14	7.97	0.89	0.03
	neap 14Apr	ebb	8.0	6.24	5.35	0.78	nd
		LT	5.9	11.53	4.07	0.22	0.03
		flood	8.0	5.41	5.17	0.24	nd
		HT	10.7	8.87	7.20	1.48	nd
summer	spring 31Jul	ebb	11.2	221.93	2.75	0.29	2.14
		LT	12.3	735.00	6.27	nd	7.06
		flood	10.7	85.61	5.36	nd	0.66
		HT	10.7	251.47	5.13	0.89	2.35
	neap 6Aug	ebb	14.4	440.18	2.75	0.19	4.28
		LT	17.1	608.40	5.79	0.10	5.87
		flood	20.2	737.64	4.02	nd	7.30
		HT	17.1	591.73	7.19	nd	5.84
autumn	spring 14Oct	ebb	6.4	213.66	0.19	0.74	2.04
		LT	6.4	141.57	0.14	nd	1.40
		flood	5.9	3.46	2.44	0.81	nd
		HT	5.9	0.62	0.62	nd	nd
	neap 7Oct	ebb	6.4	1.88	0.75	1.13	nd
		LT	8.0	13.01	1.29	nd	0.07
		flood	5.3	10.35	0.37	nd	0.09
		HT	6.4	12.57	1.41	1.18	0.07

3.4. Discussion

3.4.1 Tidal variability of environmental drivers of phytoplankton

Tidal variability of phytoplankton is modulated by tidally-induced changes in the availability of phytoplankton resources, dissolved inorganic nutrients and light, as well as changes associated to phytoplankton loss rates. Thus, the analysis of tidal dynamics of environmental drivers is relevant to understand and predict phytoplankton tidal behaviour.

River flow has been recognized as a major influence on several physical-chemical variables such as nutrient loading, light availability and water column stratification, therefore affecting phytoplankton dynamics (Cloern et al., 1983; Mallin et al., 1993 and references therein). In the Guadiana estuary, river flow exhibited low values throughout 2008 (13.1 ± 9.1 m^3 s^{-1}), comparable to those observed in extremely dry years (e.g., 2005: 18.6 ± 10.5 m^3 s^{-1}). Therefore, the influence of tidal cycles was probably more important than river flow in the regulation of estuarine dynamics throughout 2008.

Short-term changes in salinity may occur in response to both tidal and river runoff variations, whilst strong salinity gradients may occur when river discharge increases after rain pulses (Madariaga, 1995). In the Guadiana upper estuary, salinity did not vary significantly within the spring-neap tidal cycles, but significant semidiurnal variability was observed. Salinity was usually higher at high tide, reflecting the intrusion of saltwater upriver. As the sampling station (Alcoutim) is located only a few km upstream from the transition zone between the middle (brackish) and upper (freshwater) estuarine zones, salinity may be detectable occasionally, depending on the tidal phase and river flow. For instance, salinity values surpassing 3 (max 5) were observed in Alcoutim during 1999 and during the Alqueva dam filling period (2002–2003), indicating a pronounced saltwater intrusion under minimum river discharge conditions (<3 m^3 s^{-1}; Domingues et al., 2007; Barbosa et al., 2010). Throughout 2008, salinity in the upper limit of the estuary (Mértola) was always lower than 0.3, whereas in Alcoutim salinity reached a maximum value of 7, with mean salinity values <1. Furthermore, neither haline nor thermal stratification was observed at the

sampling station (see Table 3.II) thus reflecting a well mixed water column, independently of tidal stage or river flow. The absence of periodic stratification-destratification was already observed in the Guadiana upper estuary (Rocha et al., 2002; Domingues and Galvão, 2007). Conversely, haline stratification is commonly found in the lower estuary, with salinity differences between surface and bottom up to 10 (Domingues, unpublished data).

Tidally-induced resuspension is considered a primary mechanism governing the variability of suspended particulate matter (SPM) and light in estuaries (Monbet, 1992), at both semidiurnal and fortnightly scales. The combination of strong tidal currents and a shallow water column results in a particularly high sediment transport capacity (Lionard et al., 2008) in the freshwater tidal zone. Therefore, turbidity is typically high and consequently light, rather than nutrients, usually controls phytoplankton growth in these regions (Sin et al., 1999). In fact, it has been shown for phytoplankton primary production models that the most significant errors occur when the temporal pattern of light penetration, linked to the tidal cycle of solids settling and resuspension, is neglected (Desmit et al., 2005). Microcosm experiments recently carried out in the Guadiana upper estuary confirmed that phytoplankton is co-limited by nutrients (mainly nitrogen) during the productive period and by light throughout the year (see chapters 4 and 6). The positive correlation between SPM and light extinction coefficient (k_e) indicated that, as in many other shallow water systems (e.g., May et al., 2003), light attenuation in Alcoutim was mainly controlled by SPM. However, k_e did not always follow the same tidal variability as SPM, probably because light attenuation depends not only on the SPM concentration, but also on SPM composition. For instance, if SPM is mainly dominated by quartz, its effect on light attenuation will be lower than SPM dominated by clays, which play an important role in light absorption. In the lower Guadiana estuary, the highest SPM values were observed (114.9 ± 28.4 mg L^{-1}), but the lowest light attenuation was determined (0.81 ± 0.32 m^{-1}), given that suspended sediments in this estuarine region are mainly composed by quartz (Machado et al., 2007).

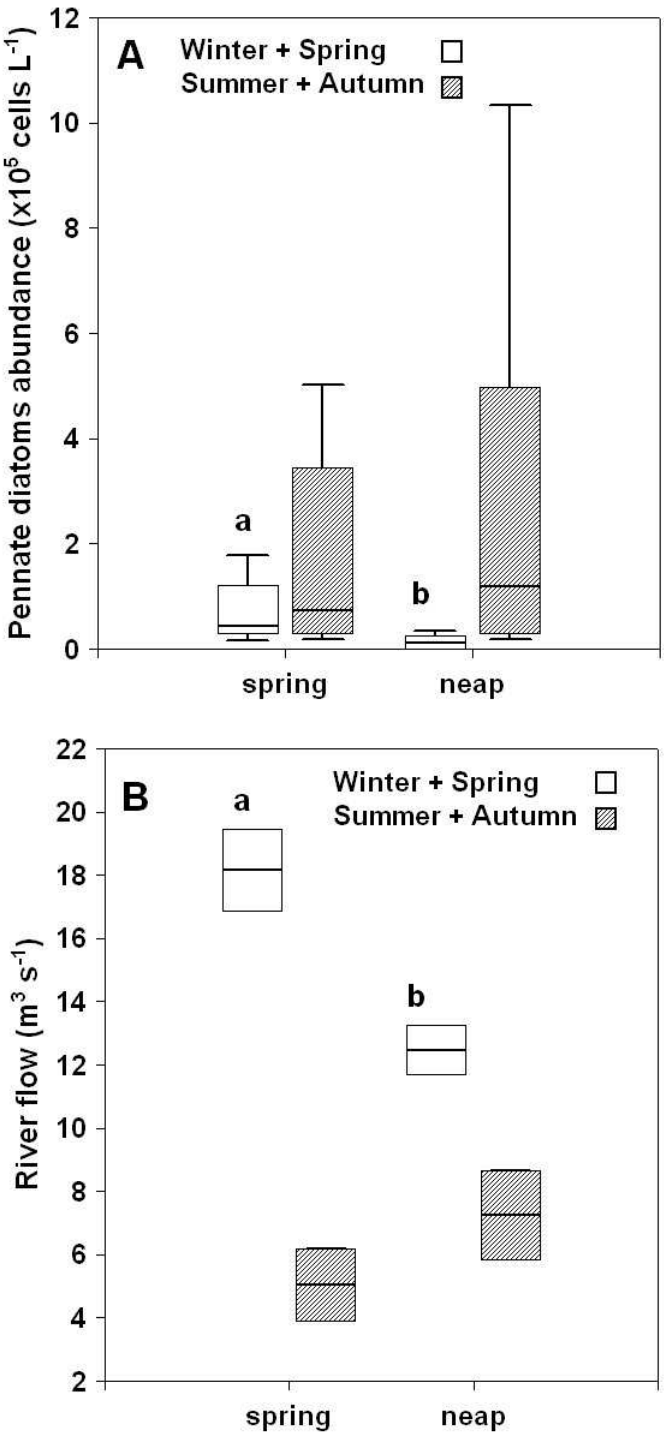

Figure 3.6 - Box and whisker plots showing A) distribution of pennate diatoms and B) river flow (average of the 8 days preceding sampling) binned in different seasons (winter + spring, summer + autumn) along spring-neap tidal cycles. Median value is represented by the horizontal line within the box, 25th to 75th percentiles are denoted by box edges and 5th to 90th percentiles are depicted by the error bars. Different letters (a, b) above the bars represent significant differences.

Furthermore, significant differences in SPM were observed at a fortnightly scale, with higher SPM values during spring tides and lower during neap tides. This is a common observation in many coastal ecosystems (e.g., Koh et al., 2006; Bartholomä et al., 2009) and is primarily related to stronger tidal currents during spring tides and, consequently, higher resuspension of bottom sediments. In respect to the semidiurnal tidal cycle, flood and ebb tidal currents are usually associated to semidiurnal peaks of sediment erosion and transport (Monbet, 1992), thus promoting higher SPM concentrations. In the Guadiana estuary, flood currents are associated to peaks in the concentration of suspended sediments, due to the resuspension of fine sediments deposited during the preceding relatively long low tide slack (Garel et al., 2009). Indeed, SPM was higher during flood than during low tide, and slightly higher during flood than during the other tidal phases.

Considering the other environmental drivers of phytoplankton growth, no significant tidally-induced differences were found, neither on fortnightly nor on semidiurnal time scales. These results reflect the homogeneity of the water masses around the sampling station. In fact, tidal excursion in the Alcoutim area is only ca. 6 km, considering an average velocity of 0.3 m s^{-1} (E. Garel, pers. comm.). Overall, tidal differences in phytoplankton and physical-chemical variables in the upper, freshwater estuary were trivial when compared to those observed in lower estuarine zones (e.g., Trigueros and Orive, 2000; Morais et al., 2009a) subjected to a stronger sea influence, sharper environmental gradients and higher tidal currents velocities (Garel et al., 2009).

3.4.2 Tidal variability of phytoplankton

The distribution of phytoplankton in specific semidiurnal tidal phases depends on the biological properties of the water masses that recurrently oscillate up- and downriver from the sampling site. Therefore, both the longitudinal distribution of phytoplankton along the main estuarine axis, namely in the vicinity of sampling stations, and the tidal excursion of the water mass along the semidiurnal tidal cycle will control phytoplankton distribution in specific estuarine locations. In general, higher phytoplankton biomass is usually associated to low tide, due to the advection

of phytoplankton-rich water masses from the upper estuary (Trigueros and Orive, 2000; Wetz et al., 2006). However, maxima may also occur during any phase of the semidiurnal tidal cycle (e.g. Gianesella et al., 2000; Lehman, 2000; Aubry and Acri, 2004). In the Guadiana upper estuary, the relative homogeneity of water masses upriver and downriver from Alcoutim, sustained by a maximum tidal excursion of 6 km (E. Garel, pers. comm.), is probably responsible for the absence of significant differences in chlorophyll *a* concentration or phytoplankton abundance along the semidiurnal cycle. In fact, marked horizontal differences between Alcoutim and the locations upstream (Mértola) and downstream (Foz de Odeleite) were observed only episodically. The longitudinal gradients in the vicinity of the middle and lower estuarine regions, much stronger than those observed in the upper estuary (see Fig. 3.3), will probably reverberate into a more marked tidal variability in the lower and middle estuaries.

Considering the spring-neap tidal cycle, the occurrence of higher phytoplankton biomass during neap tides is usually reported for light-limited systems (e.g. Madariaga, 1995; Bustos-Serrano et al., 1996), due to the decrease in turbidity and the shallowing of the mixed layer that consequently lead to an increase in mean light intensity in the mixed layer (Cloern, 1996). In addition, neap tides are also associated to a reduced vertical turbulence and higher water levels during low-tide, therefore reducing the effect of benthic grazing upon phytoplankton (Lucas et al., 1999). Benthic grazing is in fact an important sink for phytoplankton, especially in shallow, well mixed water columns (Lucas and Cloern, 2002).

In the Guadiana upper estuary, significant differences were found in chlorophyll *a* concentration between neap and spring tides only during winter and summer, with higher Chl*a* values occurring at neap tides, as initially hypothesized. This pattern was not significantly related to any growth regulatory variable or to the typical shallowing of the mixed layer, given that the Guadiana upper estuary is always well mixed. The differences in Chl*a* between spring and neap tides in each season can be associated to different factors. In the winter, mean (8 days before sampling) river flow was significantly higher in the spring tide than in the neap tide ($p < 0.05$); higher river flow can be responsible for higher turbulence, lower light, and thus, lower Chl*a*; additionally, higher vertical mixing may have increased the effect of

benthic grazing upon phytoplankton (Lucas et al., 1999). In the summer, higher Chl*a* values during neap tides may be related to a reduced effect of benthic grazing due a higher water level at low-tide. However, the effect of benthic grazing as a sink for phytoplankton has never been evaluated in the Guadiana estuary. Massive occurrence of the invasive Asian clam *Corbicula fluminea* and other freshwater bivalves have been reported for the upper estuary (Morais et al., 2009b), but their effect as a sink for phytoplankton biomass has never been addressed in this system. Indeed, studies on grazing and other mortality processes are crucial to understand the interactive effects of the environmental drivers and their tidally-induced alterations on phytoplankton dynamics.

The abundances of the main phytoplankton groups did not show any clear patterns or significant differences between different phases of spring-neap and semidiurnal tidal cycles. Nevertheless, significant differences ($p < 0.05$) were found in pennate diatoms abundance in the winter and spring, with higher values during spring tides and lower during neap tides. This may probably be related to a higher vertical mixing induced by higher river flow during spring tides, leading to resuspension of microphytobenthic diatoms (Gianesella et al., 2000; Brunet and Lizon, 2003).

3.5. Conclusions

1. Overall, the water column within the Guadiana upper estuary was vertically and horizontally homogeneous, showing no evidence of haline or thermal water column stratification.

2. No significant tidally-induced differences were found for most physical-chemical variables in the upper estuary, either on semidiurnal and fortnightly time scales, reflecting the homogeneity of the water column up- and downriver from the sampling station.

3. Tidally-induced differences in suspended particulate matter, with higher values during spring tides and flood, were related, respectively, to stronger tidal currents and resuspension of fine sediments deposited during the preceding long low tide slack.

4. Tidally-associated differences in chlorophyll *a* were observed seasonally (winter and summer), most likely due to short-term alterations in river flow and benthic grazing.

5. Tidally-induced differences on SPM and Chl*a* in the Guadiana upper estuary should be considered in the design of sampling programs, and integrated when comparing data collected at different tidal stages.

6. Furthermore, these seasonal differences reflect that in order to study estuarine tidal dynamics, sampling must be conducted throughout the year. Occasional sampling will not reflect the typical variability of these highly dynamic systems.

Chapter 4

Nutrient limitation of phytoplankton growth in the freshwater tidal zone of a turbid, Mediterranean estuary

Nutrient limitation of phytoplankton growth in the freshwater tidal zone of a turbid, Mediterranean estuary

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Estuarine, Coastal and Shelf Science (in press)

Abstract

Identification of the limiting nutrient(s) is a requirement for the rational management of eutrophication. Here, we present the first experimental analysis of nutrient limitation of phytoplankton growth and its seasonal variation in the Guadiana estuary (SE Portugal-SW Spain). Ten microcosm experiments were performed during 2005 and 2008, using water samples collected in the freshwater tidal zone of the Guadiana estuary. Nitrate, phosphate and silicate were added in a single pulse, alone and in combinations. Experimental treatments were incubated for 4 days under controlled laboratory conditions. Phytoplankton response to nutrient enrichment was evaluated through changes in biomass (Chl*a*), and abundance of specific phytoplankton groups.

Overall, phytoplankton growth seemed to be nitrogen-limited throughout the productive period, especially green algae in 2005 and diatoms in 2008. In the summer 2008, cyanobacteria and the harmful dinoflagellate *Kryptoperidinium foliaceum* responded to N enrichment in the absence of Si. Indeed, the presence of *K. foliaceum* was observed for the first time in the freshwater tidal reaches of the Guadiana estuary, where dinoflagellates were usually absent or rare. The significant increase on dinoflagellates and cyanobacteria growth in response to N enrichment in the absence of Si is alarming, because anthropogenic nutrient enrichments usually increase N and P, but not Si. Furthermore, relatively high N concentrations, up to 22 μM, were found to be limiting to phytoplankton growth. These results should therefore be used as a management tool when establishing nutrient criteria and nutrient loading budgets to estuarine waters.

Keywords: Water Framework Directive, phytoplankton, biomass, abundance, chlorophyll a, Portuguese waters

4.1 Introduction

The development of human population centres in coastal areas and particularly in the catchment of estuaries has led to widespread eutrophication with its associated problems, such as harmful algal blooms and deterioration of water quality. In fact, eutrophication of estuaries has been pointed out as one of the most pressing problems of the 21st century (Turner and Rabalais, 2003). Nutrient availability is frequently referred as key factor regulating phytoplankton growth, biomass and species composition (Roelke et al., 1999 and references therein). Therefore, the role of nutrients, especially nitrogen and phosphorus, as limiting factors of phytoplankton is an important aspect for eutrophication mitigation and management (e.g., Conley, 2000; Conley et al., 2009; Paerl, 2009). Knowledge of the limiting nutrient enables managers to draw up appropriate nutrient loading budgets for estuarine catchment areas and to respond to possible perturbations on an informed basis (Beardall et al., 2001). A comprehensive understanding of how nutrients affect phytoplankton growth, diversity, and production, is therefore needed to properly assess the impact of nutrient enrichment and the efficiency of subsequent nutrient reduction strategies (Gobler et al., 2006).

Generally, nitrogen (N) is considered limiting in marine systems (Ryther and Dunstan, 1971) and phosphorus (P) in freshwaters (Schindler, 1977), but these two deeply rooted dogmas have been questioned (e.g., Sterner, 2008). In estuaries, there is evidence of temporal and spatial changes in the limiting nutrient (D'Elia et al., 1986; Domingues et al., 2005; Fisher et al., 2006). A switch from P limitation in spring to N limitation during summer is often observed in estuarine systems (D'Elia et al., 1986; Pennock and Sharp, 1994; Fisher et al., 1999); dissolved silica (Si) may also be limiting to diatom growth (Gobler et al., 2006).

Common approaches to determine the limiting nutrient include bioassays with test organisms, enrichment experiments with natural assemblages, elemental ratios and macromolecular composition, nutrient uptake kinetics, and several biochemical and molecular approaches (see Beardall et al., 2001). Nutrient enrichment experiments using natural phytoplankton as inoculum have been used (1) to identify the limiting nutrient by higher phytoplankton growth following enrichment with a particular

nutrient in relation to the control, and (2) to extrapolate the outcomes to natural ecosystems, i.e., to identify changes in phytoplankton composition, growth and succession following specific nutrient enrichment scenarios.

However, extrapolation of bioassays results to natural systems is not straightforward. Enclosing phytoplankton in small volumes may isolate the cells from physical, chemical and biological factors experienced in situ and may also magnify their contact with others (Venrick et al., 1977). For instance, sedimentation, grazing and advection may be excluded, whilst nutrient cycling may be reduced (Loureiro et al., 2005b and references therein). Nevertheless, nutrient enrichment experiments provide valuable insights into nutrient and phytoplankton dynamics, and may accurately describe processes in natural phytoplankton communities (Loureiro et al., 2005b and references therein). Additionally, data obtained using nutrient enrichment experiments constitute an interesting management tool, as they provide quantitative measures of the phytoplankton response to altered nutrient regimes and potential shifts in community structure (Örnólfsdóttir et al., 2004).

Freshwater tidal estuarine zones represent extreme environments to phytoplankton, characterized by salinity conditions similar to the river (<0.5), but subjected to a strong tidal influence. Tidal forcing may induce the resuspension of bottom sediments, which will result in increased turbidity, leading to strong light limitation and low phytoplankton growth rates, despite high nutrient concentrations (Cole et al., 1992; Muylaert et al., 1997). Furthermore, phytoplankton losses due to freshwater discharges and downriver displacement are constant (Muylaert et al., 2000), and occasional saltwater intrusion may cause salinity stress and mortality (Morris et al., 1978). Nevertheless, freshwater tidal estuarine zones often support dense phytoplankton communities, with higher chlorophyll *a* concentrations than those found downstream (Muylaert et al., 2000 and references therein).

In contrast to the high number of studies addressing nutrient limitation of phytoplankton growth in marine and brackish estuarine zones (e.g., D'Elia et al., 1986; Harrison et al., 1990; Rudek et al., 1991; Pennock and Sharp, 1994; Roelke et al., 1997; Richardson et al., 2001; Twomey and Thompson, 2001; Yin et al., 2001, 2004; Örnólfsdóttir et al., 2004; Wawrik et al., 2004; Gobler et al., 2006), only a reduced

number of studies deals with nutrient limitation in freshwater tidal estuarine zones (e.g., O'Donohue and Dennison, 1997; Thompson, 1998; Mallin et al. 1999; Tomasky et al., 1999; Ault et al., 2000; Smith and Kemp, 2003). Furthermore, 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. Considering that Mediterranean estuaries are located in an extremely vulnerable region to climate change (IPCC, 2001), eutrophication has been increasing in many hydrographic basins, and freshwater estuarine regions are important sources of nutrients and phytoplankton biomass to downriver estuarine reaches and adjacent coastal areas (Rocha et al., 2002; Domingues and Galvão, 2007), it is crucial to fully understand the effects of nutrients on phytoplankton growth and the consequences of nutrient enrichment in these sensitive and nutrient-rich regions.

The freshwater tidal zone of the Guadiana estuary, or upper estuary, represents the largest estuarine region in length, extending from Álamo (25 km from the river's mouth) up to the tidal limit (>70 km from the river's mouth) (Morales, 1995) (Fig. 4.1). Based on field observations of nutrient concentrations and ratios and species composition, the Guadiana upper estuary is usually considered co-limited by light and nutrients (Domingues et al., 2005, 2007), but the effect of either nutrients or light on phytoplankton growth was never tested. Furthermore, in the last years, due to water and sediment retention in the recently built Alqueva dam, phytoplankton biomass has been decreasing with decreasing turbidity, which may point to a shift from a light-limited environment towards a more nutrient-limited one (Barbosa et al., 2010). The upper estuary has also been subjected to increasing human influences, including urban and agricultural runoffs, and, consequently, nutrient enrichment, that can affect phytoplankton community structure. Therefore, we performed nutrient enrichment bioassays containing natural phytoplankton populations from the freshwater tidal reaches of the Guadiana estuary to determine the limiting nutrient for phytoplankton growth and its seasonal variation, and to understand the effects of potential anthropogenic nutrient enrichments on phytoplankton community structure.

4.2. Material and Methods

4.2.1 Study site

The Guadiana River's (drainage area 67,039 km^2, length 810 km) estuary forms the southern border between Portugal and Spain. Located in a Mediterranean climate area, it is a mesotidal estuary, partially stratified in the lower and middle sections, but well mixed in the upper section. The upper estuary, or freshwater tidal zone, usually extends from Álamo (25 km from the river mouth) to Mértola (approx. 70 km from the river mouth), but depending on tidal stage and river flow (Fig. 4.1). Freshwater inputs to the estuarine zone used to vary sharply between dry and wet months (1995 - 2000: 333.0 ± 1095.9 m^3 s^{-1}, http://snirh.pt), but in the last years the recently built Alqueva dam has promoted a more regular and reduced freshwater flow (2008: 14.2 ± 9.1 m^3 s^{-1}). The estuary also receives reduced freshwater inputs from some tributaries, whilst other inputs include sewage, mainly near the mouth.

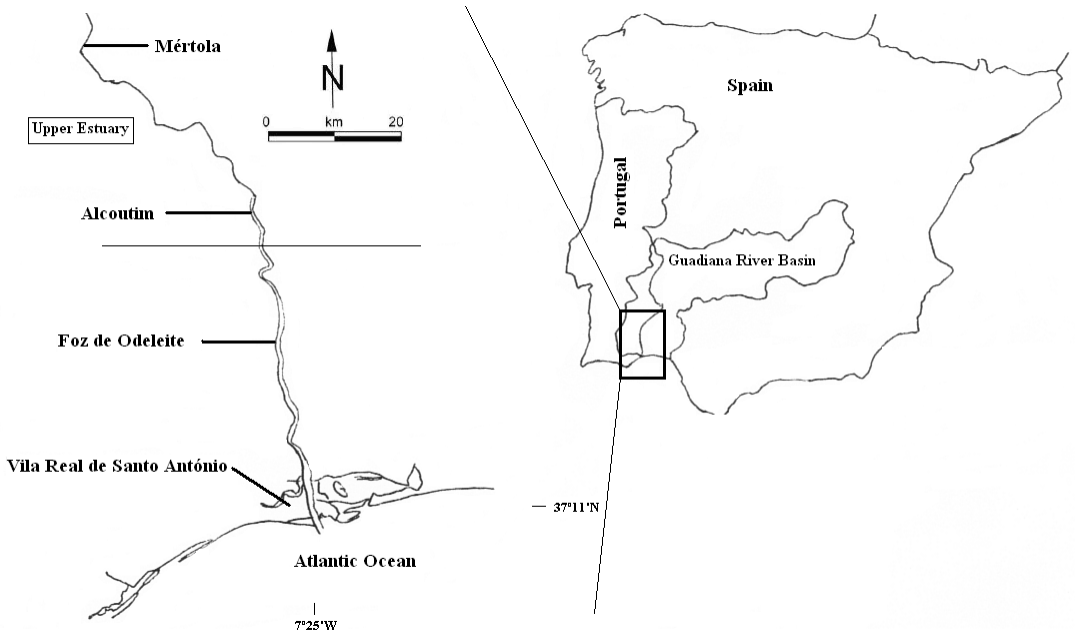

Figure 4.1 – Map of the Guadiana estuary and sampling station (Alcoutim).

4.2.2 Sampling strategy

Nutrient addition experiments were undertaken using water samples collected in the freshwater tidal reaches (upper estuary) of the Guadiana estuary (see Fig. 4.1). Samples for nutrient enrichment experiments were collected immediately after high tide, during neap tides. Samples were collected near the surface (approx. 0.5 m depth), assuming that the whole water column was well mixed (Domingues and Galvão, 2007; Morais et al., 2009a). Acid-cleaned polycarbonate bottles were used for sample collection and samples were kept in cold and dark conditions between collection and experiment set-up (approx. 2 hours). Vertical profiles of water temperature and photosynthetically active radiation (PAR) intensity were determined in situ using a YSI 556 MPS probe and a LI-COR radiometer, respectively. Light extinction coefficient (k_e, m^{-1}) was calculated using an exponential function, $I_z = I_0\ e^{-Ke.Z}$, where I_z is the light intensity at depth level Z (m) and I_0 is the light intensity at the surface. Mean light intensity in the mixed layer (I_m, µmol photons $m^{-2}\ s^{-1}$) was calculated as $I_m = I_o\ (1\text{-}e^{(-Ke.Zm)})(K_e.Z_m)^{-1}$, where I_o is the light intensity at the surface, k_e (m^{-1}) the light extinction coefficient and Z_m (m) the depth of the mixed layer (Jumars, 1993). The mixed layer was taken as the whole water column, due to the absence of haline and thermal stratification.

4.2.3 Nutrient addition experiments

Two different sets of experiments were performed during 2005 and 2008. The 2005 experiments served as a preliminary study to test and improve the methods. Ten experiments in total were performed, two per each representative season for phytoplankton growth: winter (February), spring (May), spring-summer transition (June), summer (August) and autumn (October). For each experiment, several treatments were prepared in duplicate. Nutrients were added, alone and in combinations, at day 0 in a single pulse, according to Table 4.I. Nitrogen was added as potassium nitrate (KNO_3), phosphorus as potassium dihydrogen phosphate (KH_2PO_4) and silicium as sodium hexafluorosilicate (Na_2SiF_6). In 2005, the experimental treatments were incubated in 2 L polycarbonate bottles in an outdoor

tank filled with tap water to avoid extreme variations in temperature and covered with several layers of screen to simulate the light intensity in the mixed layer at time of sampling. In 2008, 1 L polycarbonate bottles were incubated inside a growth chamber under in situ temperature and in situ light-dark cycle at approx. 110 µmol photons m^{-2} s^{-1}. This light intensity was higher than I_m at the time of sampling, but since the daily variability of I_m is significant, depending on atmospheric factors and tidal phase, and samples were collected in the morning, when irradiance is lower, the light intensity chosen for the incubations can be observed *in situ*, so the cells were not exposed to light intensities higher than those that they usually experience in their natural environment.

Table 4.I – Experimental treatments with indication of concentrations (µM) of nutrients added during the 2005 and 2008 experiments.

	Winter 2005	**Others 2005**	**2008**
Control	no additions	no additions	no additions
N	+150	+200	+150
P	+8.5	+3.1	+15
Si	+175	+150	+150
NP	+16.5 N +1.5 P	+200 N +3.1 P	+150 N +15 P
SiN	+37.5 Si +25 N	+150 Si +200 N	+150 N +150 Si
SiP	-	+150 Si +3.1 P	+150 Si +15 P
NPSi	-	+200 N +150 Si +3.1 P	+150 N +15 P +150 Si

The bottles were opened daily and gently shaken twice a day. Sub-samples for nutrient determination were collected from each bottle at days 0, 1, 2, 4 (and day 6 in 2005). Chlorophyll *a* and phytoplankton composition and abundance were evaluated at days 0, 1, 2, 4 and 6 in 2005. During the 2005 experiments, phytoplankton growth was exponential until day 4, and in many experiments, until day 6. Therefore, in

2008, due to logistic and financial reasons, chlorophyll and phytoplankton were evaluated only at days 0 and 4. In the winter and spring 2008 experiments, daily measurements of in vivo Chl*a* fluorescence confirmed the exponential growth of phytoplankton until day 4 (determination coefficients of regression lines time vs. ln(Chl*a*) ranging between 0.90 and 0.99).

4.2.4 Laboratory analyses

Samples for nutrient determination were collected and immediately filtered through cellulose acetate filters (Whatman, nominal pore diameter = 0.2 μm). Ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_4^{4-}) were determined immediately after sample collection, whilst samples for nitrate (NO_3^-) and nitrite (NO_2^-) where frozen (-20ºC) 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, phosphate and silicate, and an autoanalyzer Skalar for nitrate and nitrite.

Chlorophyll *a* concentration was determined according to Parsons et al. (1984b), using glass fibre filters (Whatman GF/F, pore diameter = 0.7 μm). Chlorophyll *a* was extracted overnight at 4ºC 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 pico- (<2 μm) and nanophytoplankton (2 - 20 μm) were preserved with glutardialdehyde (final concentration 2%) immediately after collection, stained with proflavine and filtered (1-5 mL, depending on the amount of suspended matter) onto black polycarbonate membrane filters (Whatman, nominal pore diameter = 0.45 μm). Preparations were made with glass slides and non-fluorescent immersion oil (Cargille type A), within 24 h of sampling, and then frozen (-20ºC) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5x magnification using a Leica DM LB epifluorescence microscope. Samples for enumeration of microphytoplankton

(>20 μm) were preserved with acid Lugol's solution (final concentration approx. 0.003%) immediately after collection, settled in sedimentation chambers (2 - 10 mL, depending on the amount of suspended matter; sedimentation time = 24 hours) and observed at 400x magnification with a Zeiss Axiovert S100 inverted microscope. Phytoplankton cells were identified, whenever possible, to species level. 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).

4.2.5 Data analyses

Data analyses were performed using GraphPad Prism 5 software. For each experimental treatment, nutrient concentrations, chlorophyll *a* and phytoplankton abundances were statistically compared within replicates of the same treatment 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, all values were combined for the subsequent data analysis. Nutrient net consumption rates for each treatment were estimated as the slope of a linear or exponential function adjusted to the data points. It is important to stress that what we determine in fact were nutrient disappearance rates, that integrate not only uptake rates (inward transport through the cell membrane), but also excretion and nutrient regeneration. Community net growth rate and specific net growth rate of different phytoplankton groups (μ, d^{-1}) were estimated as the slope of ln N(t) versus time (4 days), where N(t) is chlorophyll *a* concentration or phytoplankton abundance at day t, respectively, assuming exponential growth (confirmed by in vivo Chla fluorescence). Slopes and standard errors of the estimated regression lines were then compared to assess significant differences between consumption/growth rates of the controls and the treatments.

4.3. Results

4.3.1 Initial conditions

In the 2005 and 2008 experiments, nitrogen was the potential limiting nutrient in the beginning of all the experiments (Table 4.II). N:P ratio was always <16, and Si:N was always >1. Initial concentration of dissolved inorganic nitrogen (DIN) was higher in the 2008 experiments, but always <40 μM. In 2005, initial N did not surpassed 24 μM. Mean light intensity in the mixed layer (I_m) at the time of sampling was higher during 2005 (74 - 183 μmol photons m^{-2} s^{-1}) than 2008 (9 - 105 μmol photons m^{-2} s^{-1}) (Table 4.II).

Phytoplankton community was mainly composed of diatoms, green algae and cyanobacteria in the 2005 experiments, whilst in 2008 cyanobacteria were only detected in the summer and dinoflagellates were frequently observed. Phytoplankton abundance and chlorophyll *a* in the beginning of the experiments were higher in 2005 than in 2008. Initial abundances and chlorophyll *a* concentration are presented in Table 4.III.

Table 4.II – Initial nutrient concentrations (μM) and molar ratios, potential limiting nutrient according to the Redfield ratio (Redfield et al., 1963) and mean light intensity in the mixed layer (I_m, μmol photons m^{-2} s^{-1}) at the time of sampling during 2005 and 2008 (SS trans. – spring-summer transition).

	DIN	**Si**	**P**	**N:P**	**Si:N**	**Potential Limitation**	**I_m**
2005							
Winter	23.9	67.7	1.9	12.6	2.8	N	93
Spring	2.0	4.7	1.6	1.3	2.3	N	74
SS trans.	19.8	27.0	2.0	9.9	1.4	N	183
Summer	5.6	13.5	1.1	5.1	2.4	N	165
Autumn	13.0	51.1	3.0	4.3	3.9	N	92
2008							
Winter	39.5	125.6	2.6	15.2	3.2	N	32
Spring	21.6	63.9	2.7	8.0	3.0	N	63
SS trans.	20.0	42.8	2.2	9.1	2.1	N	87
Summer	3.8	59.7	2.5	1.5	15.7	N	105
Autumn	23.4	48.2	3.0	7.8	2.1	N	9

Table 4.III – Phytoplankton abundance (x10^5 cells L^{-1}) and chlorophyll *a* concentration (µg L^{-1}) at the time of sampling in 2005 and 2008. DI – diatoms; GA – green algae; DINO – dinoflagellates; CYA – cyanobacteria; Chl*a* – chlorophyll *a* concentration; nd – not detected.

	DI	GA	DINO	CYA	Chl*a*
2005					
Winter	68	13	nd	nd	4.3
Spring	320	690	nd	240	14.9
SS trans.	17	140	nd	740	9.1
Summer	8.9	160	nd	720	41.6
Autumn	11	60	nd	590	2.1
2008					
Winter	0.63	0.19	nd	nd	1.1
Spring	49	0.19	2.8	nd	19.7
SS trans.	3.1	0.47	0.47	nd	13.9
Summer	2.5	1.8	0.29	960	6.9
Autumn	1.3	0.19	0.74	nd	8.0

4.3.2 Nutrient uptake rates

Significant nutrient consumption occurred in all the experiments, except in the autumn 2008 experiment, when no significant nutrient consumptions in the nutrient-enriched treatments in relation to the control were observed. Nitrate net consumption rates in all N-enriched treatments (N, NP, SiN, NPSi) were significantly higher than in the controls in the winter, spring, spring-summer transition and summer experiments. Nitrate consumption rates in the N-enriched treatments varied between 6.3 and 20.5 µM d^{-1}, whilst rates in the controls ranged between 1.1 and 4.8 µM d^{-1} (Figs. 4.2A, 4.3A, 4.4A, 4.5A). The highest nitrate net consumption rates were observed in the summer experiment (9.7 - 19.6 µM d^{-1}; control 1.2 µM d^{-1}), and the lowest in the winter experiment (7.2 - 10.3 µM d^{-1}; control 1.1 µM d^{-1}).

Silicate net consumption was observed in the winter, spring and summer 2008 experiments. Si consumption in the Si-enriched treatments varied between 29.3 and 42.7 µM d^{-1}, whilst consumption in the controls varied between 9.4 and 14.1 µM d^{-1} (Figs. 4.2A, 4.3A, 4.5A). In the spring-summer transition and autumn experiments, no significant consumptions in relation to the controls were observed.

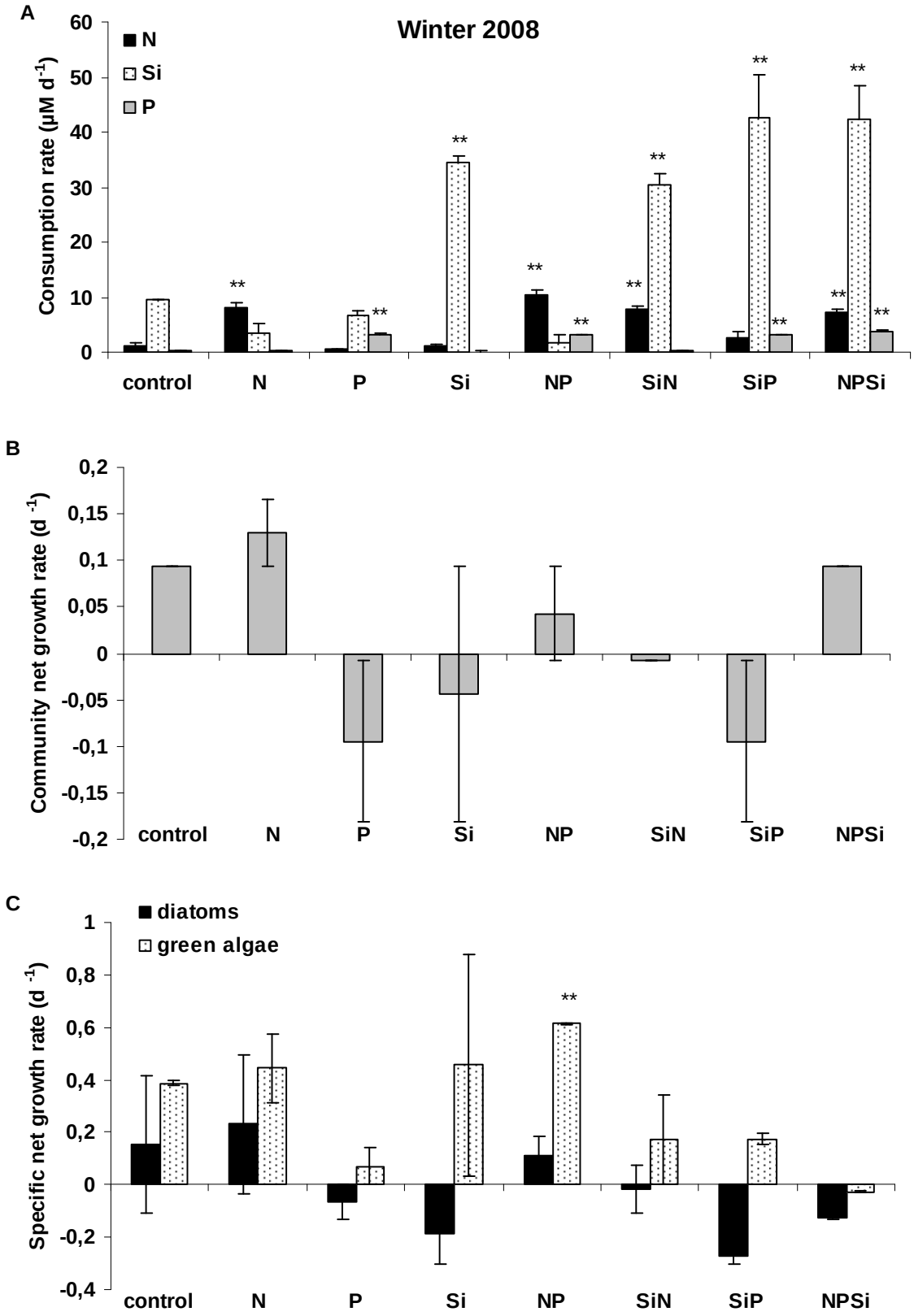

Figure 4.2 - A) Nitrate (N), silicate (Si) and phosphate (P) net consumption rates (µM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms and green algae based on abundance during the 2008 winter experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

Significant phosphate net consumptions were observed only in the winter (3.3 - 3.9 μM d^{-1}; control 0.2 μM d^{-1}: Fig. 4.2A) and spring (0.8 - 1.6 μM d^{-1}; control 0.5 μM d^{-1}: Fig. 4.3A) 2008 experiments. In the spring-summer transition, summer and autumn experiments, phosphate net consumption rates in the P-enriched treatments were not significantly different from the controls (Figs. 4.4A, 4.5A, 4.6A).

4.3.3 Phytoplankton growth rates

The response of phytoplankton to nutrient enrichment was evaluated by means of changes in chlorophyll *a* concentration, used as a proxy for community biomass, and changes in the abundance of specific phytoplankton groups. Different responses of the phytoplankton community were observed throughout the 2008 experiments. In the winter, no trends could be deduced due to high variability within experimental treatments (Fig. 4.2B) and in the spring-summer transition no significant differences between the experimental treatments and the controls were observed (Fig. 4.4B). In the spring, summer and autumn 2008 experiments, significant responses of the phytoplankton community to nitrate additions were observed. In the spring (0.22 - 0.46 d^{-1}, Fig. 4.3B) and summer (0.39 - 0.69 d^{-1}, Fig. 4.5B), community net growth rates were significantly higher than in the controls (0.09 d^{-1} and -0.01 d^{-1}, respectively). In the autumn, community net growth rates were also significantly higher (0.39-0.47 d^{-1}) than in the control (0.29 d^{-1}) in the N-enriched treatments except in treatment SiN (Fig. 4.6B).

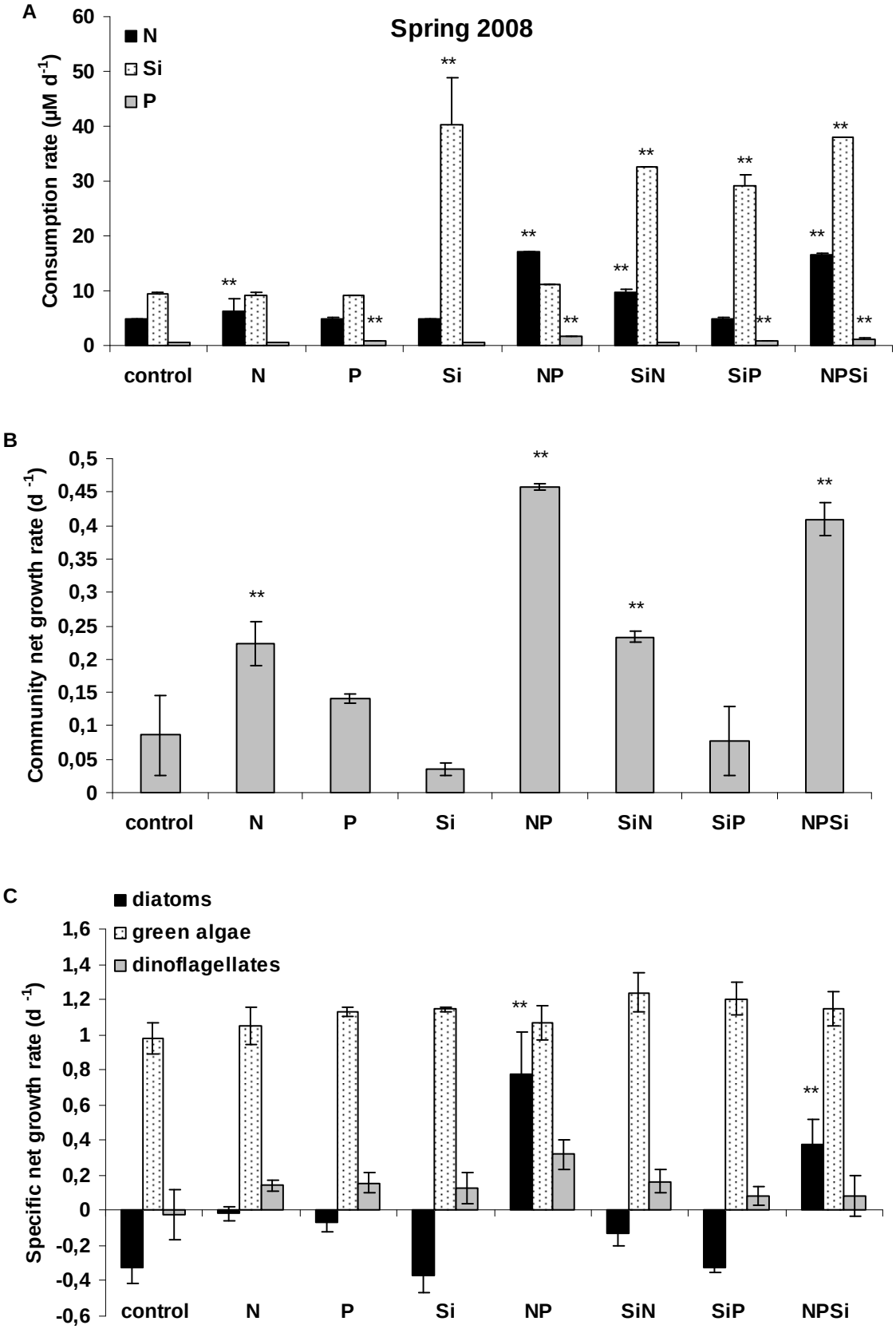

Figure 4.3 - A) Nitrate (N), silicate (Si) and phosphate (P) net consumption rates (µM d^{-1}, n = 8 for each bar), B) phytoplankton community net growth rate (d^{-1}) based on chlorophyll *a* concentrations, and C)

specific net growth rates (d^{-1}) of diatoms, green algae and dinoflagellates based on abundance during the 2008 spring experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

Considering each functional group separately and the two sets of experiments, 2005 and 2008, green algae (Class Chlorophyceae) and diatoms (Class Bacillariophyceae) were present in basically all the experiments. Green algae responded positively to nitrate enrichment in the spring (Fig. 4.7B), spring-summer transition (Fig. 4.7C) and summer (Fig. 4.7D) 2005 experiments, and also in the summer 2008 experiment (Fig. 4.5B). The most common genera in these experiments were *Scenedesmus*, *Pediastrum* and *Monoraphidium*. Net growth rates of green algae in the N-enriched treatments varied between 0.25 and 1.23 d^{-1}, whilst rates in the control ranged between 0.10 and 0.43 d^{-1}. In the winter 2008 experiment, green algae, composed mainly by *Scenedesmus*, was favoured in the treatment NP.

The responses of diatoms to nutrient enrichment were not consistent throughout the 2005 and 2008 experiments. During 2005, net growth rates of diatoms were significantly higher than the controls in the spring, spring-summer transition and summer experiments. No responses were observed in the winter and autumn. In the spring 2005 experiment, diatoms showed negative growth rates in the treatments, except in treatment NPSi, where growth rate (0.07 d^{-1}) was significantly higher than the control (-0.11 d^{-1}) (Fig. 4.7B). In the summer 2005, positive responses were also observed only in treatment NPSi (0.52 d^{-1}) in relation to the control (0.26 d^{-1}) (Fig. 4.7D). In the spring-summer transition 2005, growth rates of diatoms in treatments with simultaneous addition of N and P (NP, NPSi) were significantly higher (0.41 and 0.14 d^{-1}) than the control (0.05 d^{-1}) (Fig. 4.7C). During 2008, positive responses of diatoms were observed in the spring, summer and winter experiments.

In the spring, specific net growth rates of micro- (>20 µm) and nano-sized (2 - 20 µm) centric diatoms in treatments NP and NPSi (0.38 - 0.77 d^{-1}) were significantly higher than in the control (-0.33 d^{-1}). In the summer, diatoms, mainly represented by nano-sized centric diatoms and pennate diatoms belonging to the family Naviculaceae, responded significantly in all N-enriched treatments (0.92 - 1.11 d^{-1}) in relation to the control (0.74 d^{-1}) (Fig. 4.5 C). In the autumn, however, net growth rate of diatoms was

significantly higher than the control (1.15 d^{-1}) only in treatment NPSi (1.32 d^{-1}) (Fig. 4.6C).

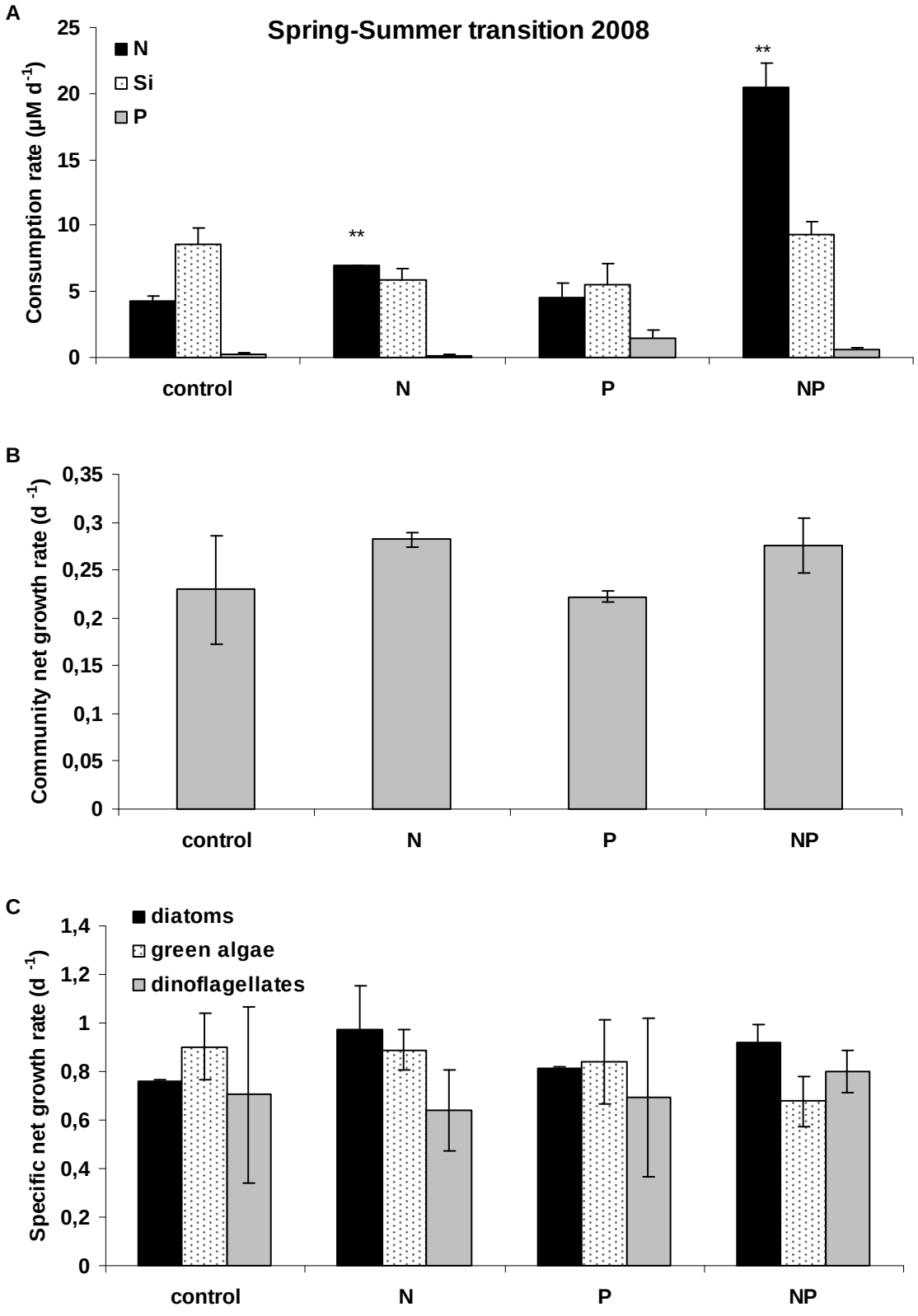

Figure 4.4 – 2008 Spring-summer transition experiment. For legend see Fig. 4.3.

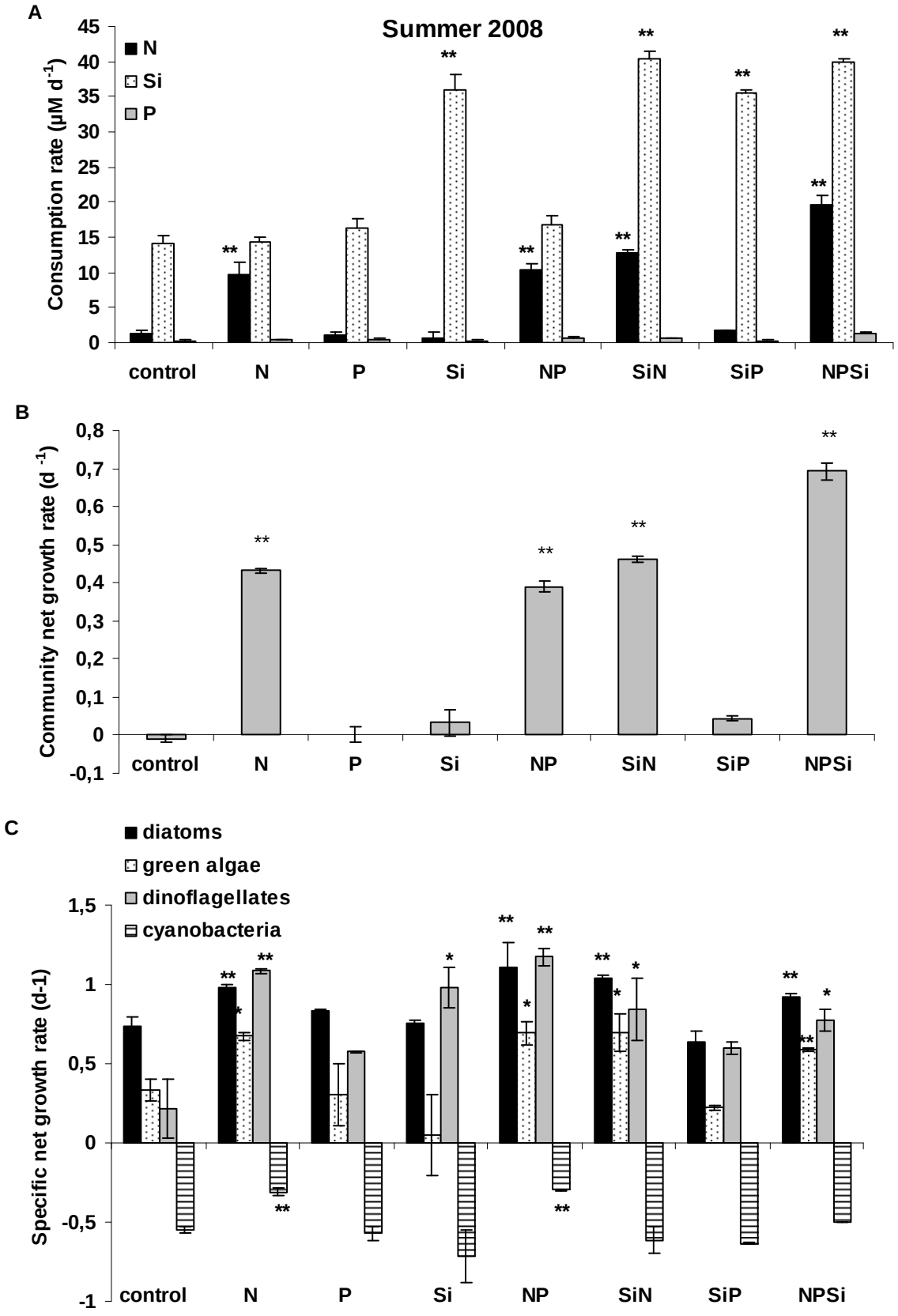

Figure 4.5 - A) Nitrate (N), silicate (Si) and phosphate (P) net consumption rates (µM d^{-1}), B) phytoplankton community net growth rate (d^{-1}) based on chlorophyll *a* concentrations, and C) specific

net growth rates (d^{-1}) of diatoms, green algae, dinoflagellates and cyanobacteria based on abundance during the 2008 summer experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

Coccoid picocyanobacteria in the summer 2008 experiment showed negative growth rates in all treatments, including the control. However, net growth rates of coccoid picocyanobacteria in treatments N and NP (-0.30 d^{-1}) were significantly higher than growth rates in the control (-0.55 d^{-1}), but SiN and NPSi additions did not induce a significant response on cyanobacteria net growth rates (Fig. 4.5C).

Dinoflagellates were present in the spring, summer and autumn 2008 experiments and were mainly represented by *Kryptoperidinium foliaceum*. In the spring, although not significantly different from the control (-0.02 d^{-1}) due to its high standard error, net growth rates of *K. foliaceum* were higher in the nutrient-enriched treatments, especially treatment NP (0.32 d^{-1}) (Fig. 4.3C). In the summer, net growth rates of the dinoflagellate *K. foliaceum* in all N-enriched treatments (0.77 – 1.17 d^{-1}) were also significantly higher than growth rates in the control (0.22 d^{-1}). Additionally, net growth rates of *K. foliaceum* in treatments enriched with N but not Si (N and NP) were significantly higher ($p < 0.05$) than those in treatments enriched with both N and Si (SiN and NPSi) (Fig. 4.5C). In the autumn experiment, dinoflagellates showed positive responses to nutrient enrichment in all treatments (0.68 – 0.78 d^{-1}) except NP and SiP, in relation to the control (0.52 d^{-1}) (Fig. 4.6C).

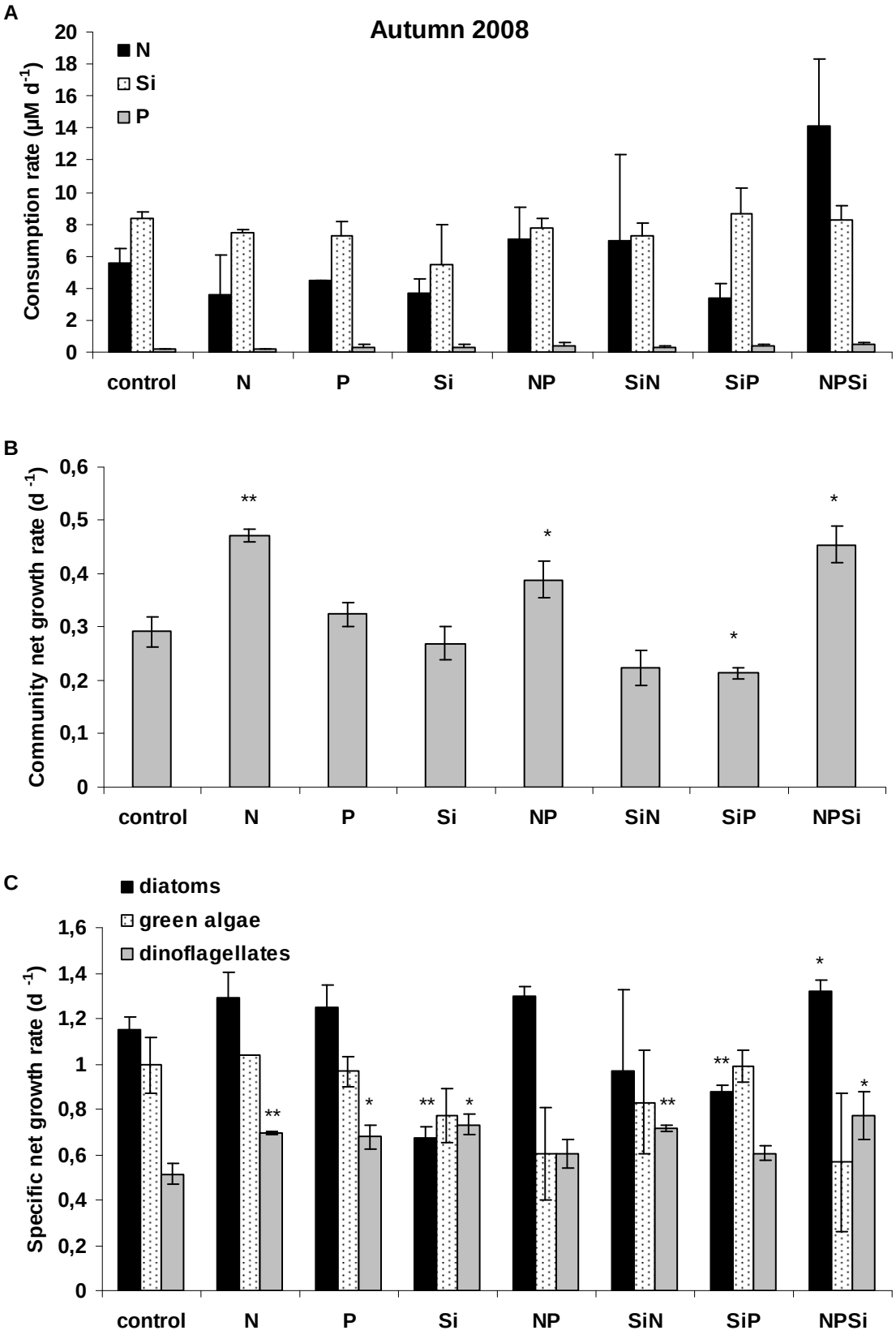

Figure 4.6 – 2008 Autumn experiment. For legend see Fig. 4.3.

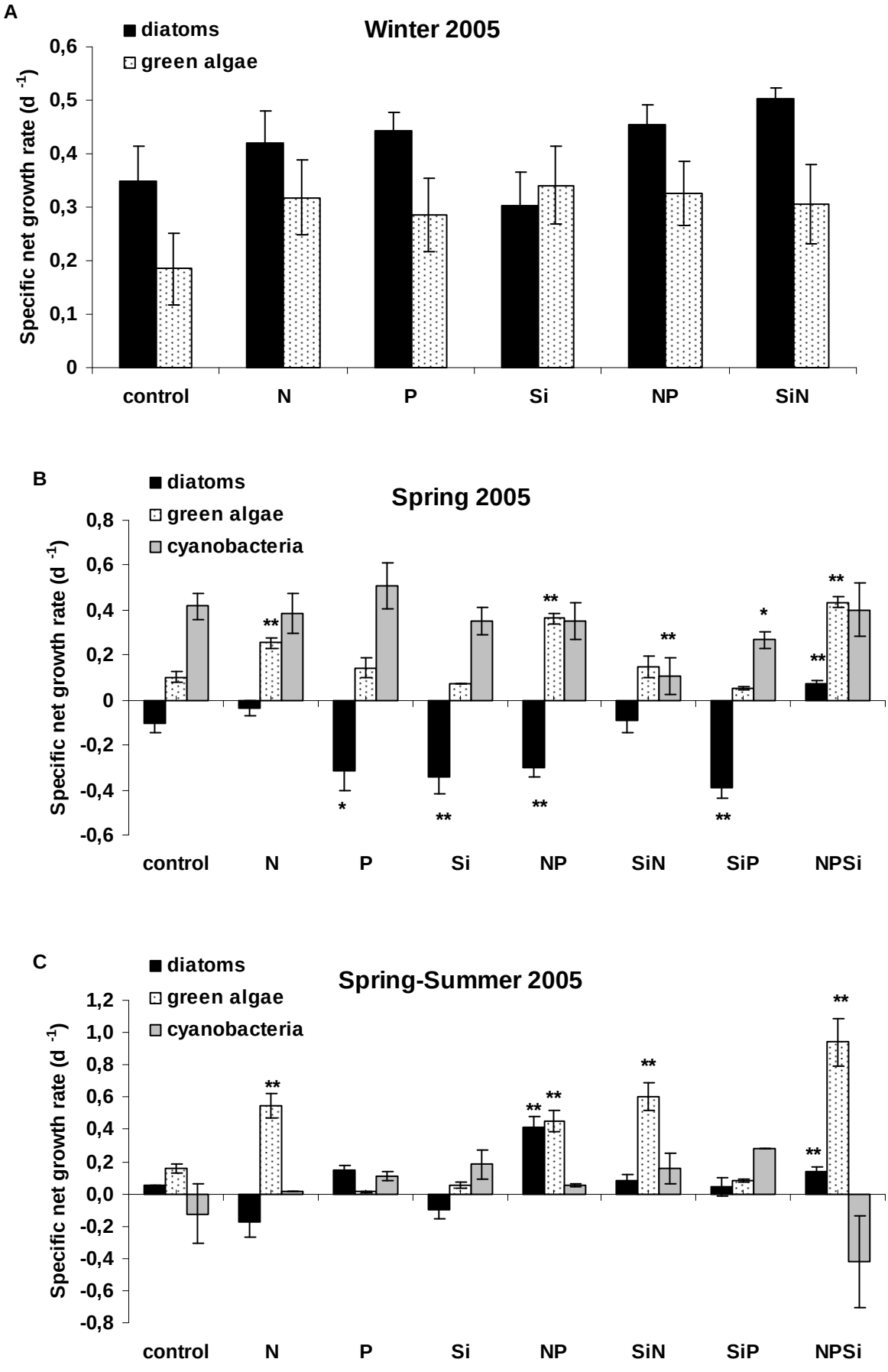
A
diatoms
green algae
Winter 2005
Specific net growth rate (d -1)
0,6
0,5
0,4
0,3
0,2
0,1
0
control
N
P
Si
NP
SiN
B
diatoms
green algae
cyanobacteria
Spring 2005
Specific net growth rate (d -1)
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
control
N
P
Si
NP
SiN
SiP
NPSi
C
diatoms
green algae
cyanobacteria
Spring-Summer 2005
Specific net growth rate (d -1)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
-0,6
-0,8
control
N
P
Si
NP
SiN
SiP
NPSi

Fig. 4.7 (cont.)

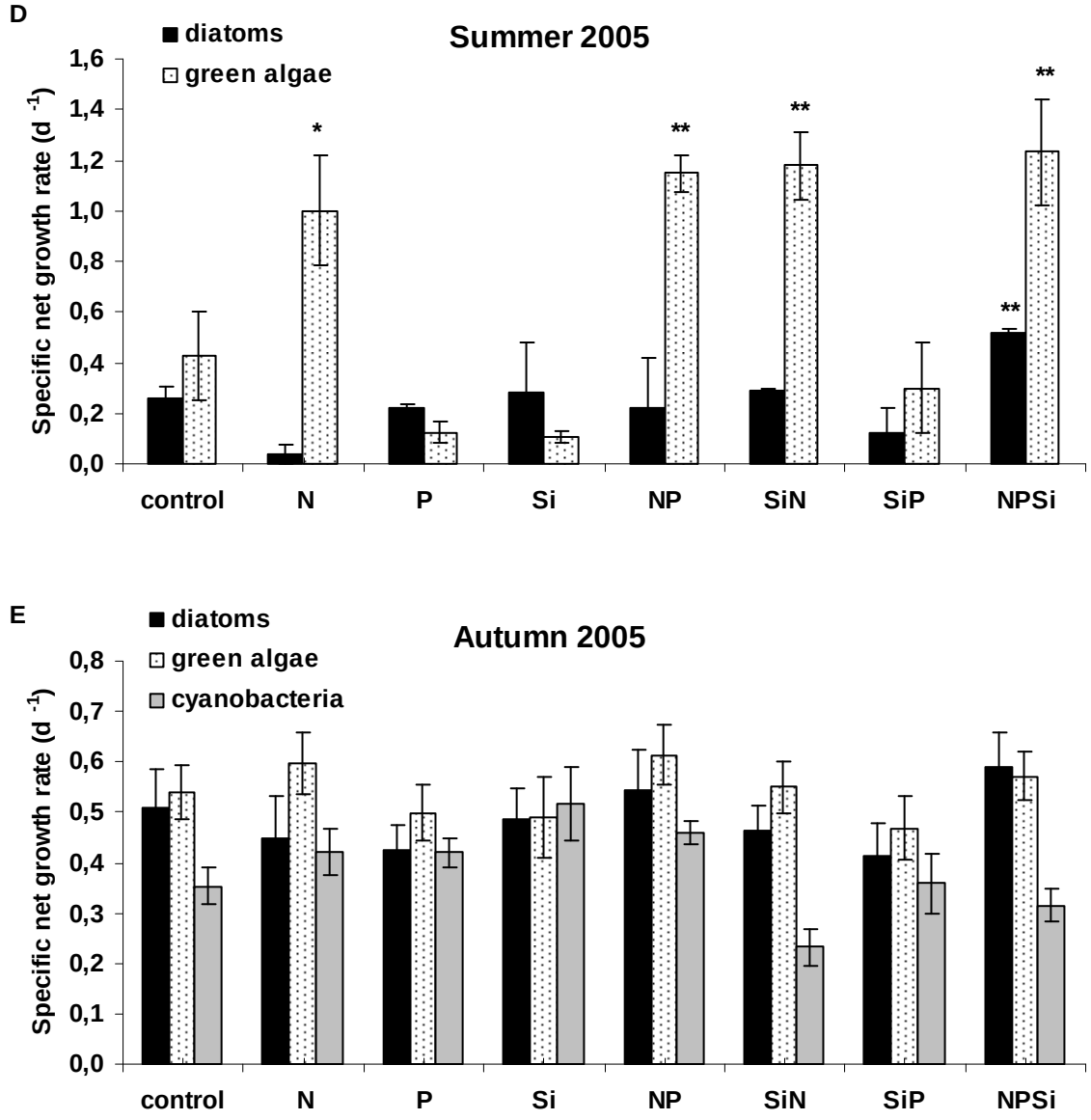

Figure 4.7 – Specific net growth rates (d^{-1}) of different phytoplankton groups during the 2005 nutrient enrichments experiments. A) winter, B) spring, C) spring-summer transition, D) summer and E) autumn. Vertical lines represent ± 1 S.D. Significant differences in experimental treatments in respect to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar. Cyanobacteria occurred during the summer experiment, but due to technical reasons, those data are not shown.

4.4. Discussion

4.4.1 Methodological concerns

Despite providing significant information on the effects of nutrient availability on phytoplankton growth and community structure, nutrient enrichment bioassays do not constitute a straightforward methodology when it comes to interpreting and extrapolating the results to natural systems. Firstly, incubating phytoplankton in bottles isolates the cells from many of the physical, chemical and biological factors they normally experience and may magnify their contact with others (Venrick et al., 1977). Natural processes such as water column mixing and nutrient inputs from autochthonous and allochthonous sources will be excluded, whilst other processes such as grazing may be enhanced. For instance, phytoplankton growth and accumulation of biomass inside nutrient enriched enclosures may not be extrapolated to a natural system where the water residence time is low and the cells are advected from the estuary before biomass can accumulate. Tomasky et al. (1999) found evidence of increased phytoplankton biomass in response to nutrient additions in experimental enclosures, but argued that such increases may not be apparent in the river itself, where phytoplankton is not enclosed and water renewal rates are high. The water residence time in the freshwater tidal zone of the Guadiana estuary varies with tidal stage and river flow, but it is long enough to allow the accumulation of phytoplankton biomass and the development of blooms, especially during spring, summer and autumn (e.g., Domingues et al., 2005). Furthermore, water masses around the sampling station are relatively homogenous when compared to the lower estuary, and tidal excursion in the Alcoutim area is only ca. 6 km (Domingues et al., 2010, see Chapter 3). Therefore, advection of phytoplankton from the upper estuary will occur only under conditions of extremely high river discharge, which are usually not observed in the Guadiana estuary, due to restrictive damming and dry climate.

The incubation conditions may also affect the outcomes of these experiments. For instance, we may eliminate variables that could affect the response of phytoplankton to nutrient enrichment (e.g., incubate light-limited cells under saturating light-

intensities, so that light limitation would be eliminated), but by doing so, we are obviously setting artificial conditions that are not found in the natural environment; the responses of phytoplankton in such conditions would have to be extrapolated with extreme caution. The Guadiana estuary is highly turbid, particularly in its middle and upper sections, and phytoplankton growth is most likely light-limited. Exposure to saturating light intensities or light intensities higher than the mean light intensity in the mixed layer would have alleviated light limitation and we could have observed the effects of nutrient enrichments without the interference of this important limiting factor. However, the results would have indicate us just the potential effects of nutrient enrichments under an artificial light environment, and not the actual effects of nutrients on a phytoplankton community already affected by low light availability. Therefore, not only PAR intensity, but also temperature and light-dark cycle were kept as close to natural conditions as possible during the 4-day incubations. Obviously, a better approach would have been to incubate the experimental treatments *in situ*, under natural light intensities, light-dark cycles, temperature and turbulence (e.g., Xu et al., 2010), but due to the distance between our lab and the sampling station (approx. 100 km), that option was disregarded.

Ault et al. (2000) argued that increases in growth rate in response to nutrient enrichment over the course of an experiment do not necessarily mean that phytoplankton growth was nutrient-limited at the time of sampling. Whilst there is a continuous supply of nutrients from different sources in the natural system, nutrient concentrations in enrichment bioassays will tend to decrease over time as a result of cellular uptake, since there is no additional nutrient inputs to the bottles. Therefore, a certain nutrient that was not limiting at the beginning of the experiment may become limiting after a few days of incubation. This problem may be overcome by following nutrient disappearance in the bottles on a daily or hourly basis. If a nutrient is not limiting at the time of sampling/beginning of the experiment, nutrient consumption in the enriched treatments after nutrient addition will not be different from consumption in the controls. Comparing nutrient disappearance to phytoplankton growth will also give a rough insight on the occurrence of other processes such as

nutrient luxury consumption or nutrient consumption by cells other than phytoplankton (e.g., heterotrophic bacteria) in the bottles.

Finally, one of the most deeply-rooted concerns of *in vitro* studies are the "bottle effects" that may be apparent over long incubation times and are thought to arise from factors such as contamination from the bottle walls or microbial growth on the walls (Marra, 2009). Several recent studies have found no evidence for substantial bottle effects (e.g., Williams et al., 2004; Hammes et al., 2010), concluding that anomalies are most likely caused by other factors (P.J. le B. Williams, pers. comm.). In our experiments, the incubation time (4 days) and the volume of sample (1 L) were similar to other enrichment bioassays where no bottle effects were described. In reality, the volume of samples and incubation times found in the literature vary tremendously, from small volumes and short incubations (e.g., 50 mL, 48 hours: Yin et al., 2001), to large volumes and long incubations (e.g., 10 L, 10 days: Balode et al., 1998), but also small volumes and long incubations (e.g., 150 mL, 2 weeks: Pollingher et al., 1988), and large volumes and short incubation times (e.g., 2 L, 24 hours: Örnólfsdóttir et al., 2004). A rough analysis of nutrient enrichment experiments found in the literature indicates that volumes of 1 - 2 L and incubation times of 2 - 5 days are the general norm (e.g., Rudek et al., 1991; Gobler et al., 2006; Xu et al., 2010).

4.4.2 Effects of nutrient enrichment on phytoplankton

Overall, net growth of phytoplankton in the Guadiana upper estuary seemed to be nitrogen limited on several occasions. The clearest case of potential N-limitation occurred during spring and summer 2008, when initial DIN concentrations were 22 and 4 μM, respectively, corresponding to N:P ratios of 8.2 and 1.6. Increased nitrogen net consumption rates in all N-enriched treatments in these experiments were associated to significant increases in community biomass (Figs. 4.3B, 4.5B) and in the abundance of specific phytoplankton groups (Figs. 4.3C, 4.5C), undoubtedly implying growth limitation by nitrogen. Nitrogen limitation of phytoplankton growth is commonly observed in other estuarine systems, especially during summer (D'Elia et al., 1985; Rudek et al., 1991; Pennock and Sharp, 1994), but limiting DIN concentrations are usually lower than those described for the Guadiana upper

estuary (e.g., 0.58 - 8.79 μM, Long Island Sound: Gobler et al., 2006; 0.32 - 2.91 μM, Galveston Bay: Örnólfsdóttir et al., 2004; 0.33 - 10 μM, Wilson Inlet: Twomey and Thompson, 2001). However, different responses have been observed in freshwater tidal estuarine areas. For instance, nitrate concentrations ranging between ~15 and >40 μM were apparently not limiting to phytoplankton growth in the freshwaters of Childs River (Tomasky et al., 1999); also, phosphorus, rather than nitrogen, was the limiting nutrient for phytoplankton growth during summer in Logan River (O'Donohue and Dennison, 1997). In the upper Port Adelaide River estuary, Si was the potential limiting nutrient to phytoplankton, whilst N and P had no effect on growth rates (Ault et al., 2000). In the freshwater reaches of the Cape Fear Estuary, no responses to nutrient enrichment were observed due to light limitation (Mallin et al., 1999). Therefore, no patterns in nutrient limitation can be inferred from these freshwater tidal estuarine zones. However, these systems are located in regions with distinct hydrographic and climatic characteristics, which is probably the cause for the different responses observed. Studies on nutrient enrichment effects on Mediterranean freshwater tidal estuaries are pressing, given their ecological importance and susceptibility to climate change.

Considering specific phytoplankton groups, diatoms (class Bacillariophyceae) and green algae (class Chlorophyceae) were the most abundant in the upper estuary, in agreement with observations in other Mediterranean climate estuaries, such as the Swan River estuary (Thompson, 1998) and the Ebro River estuary (Pérez et al., 2009). Green algae showed different responses to nutrient enrichment in 2005 and 2008. During the 2005 experiments, green algae net growth rate increased significantly in all N-enriched treatments throughout the productive period (spring, spring-summer transition and summer experiments, Figs. 4.7B, 4.7C, 4.7D), whilst in 2008, green algae responded to N-additions only in the summer experiment (Fig. 4.5C). Initial nitrate (NO_3^--N) concentrations ranged between 2.0 and 19.8 μM in these experiments, corresponding to N:P ratios from 1.3 to 9.9. These values are clearly below the optimum N:P ratio of 30 for *Scenedesmus* (Rhee, 1978), the most abundant green algae genus in the freshwater tidal reaches of the Guadiana estuary. In the other experiments, nitrate concentrations were >20 μM. Therefore, NO_3^--N <20 μM

seemed to be limiting for green algae growth. In the autumn 2005 experiment, NO_3^--N concentration was lower than 20 µM, but still green algae did not respond to N addition, probably due to light limitation and/or low water temperature. Light intensity during incubation was approx. 92 µmol photons m^{-2} s^{-1}, which is lower than typical saturating light intensities (I_k) described for green algae. In fact, green algae are usually "sun" species that achieve their maximum photosynthetic rate at higher light intensities than "shade" algae, such as dinoflagellates (e.g.,, Raven and Richardson, 1986). For instance, Senger and Fleishhacker (1978) refer a I_k for *Scenedesmus obliquus* ranging from 122 to 400 µmol photons m^{-2} s^{-1}.

Diatoms were the main component of phytoplankton community in all the experiments, although seasonal differences in specific composition were observed. Micro-sized, solitary centric diatoms were more abundant in the winter experiments, whilst the other experiments were dominated by nano-sized, solitary or chain-forming centric diatoms. Overall, diatom growth was occasionally limited by nitrogen during the productive period. Potential N-limitation of diatom growth was evident in the summer 2008 (Fig. 4.5C). Potential co-limitation by N and P also occurred in the spring-summer transition 2005 (Fig. 4.7C) and spring 2008 (Fig. 4.3C). In the spring 2008, the most abundant diatoms were unidentified centric diatoms <20 µm, which have higher maximum growth rates than larger diatoms (Sarthou et al., 2005) and are more efficient in nutrient uptake due to a higher surface to volume ratio (Eppley et al., 1969). According to Tang's (1995) allometric model, the maximum potential growth rate for diatoms of this size at *in situ* temperature (20ºC) is 1.82 d^{-1}, which is only slightly higher than net growth rates measured in treatments NP (1.74 d^{-1}) and NPSi (1.61 d^{-1}). These results show that diatoms were co-limited by N and P, and the increased nutrient uptake resulted in biomass accumulation.

Dinoflagellates were present in the spring, summer and autumn 2008 experiments, and were mainly represented by the harmful species *Kryptoperidinium foliaceum* (Stein) Lindemann 1924. *K. foliaceum* is a small, lightly armoured dinoflagellate responsible for red tides in many brackish ecosystems (Kempton et al., 2002; Figueroa et al., 2009). Blooms of *K. foliaceum* appear to be monospecific and cell density can reach 3.5 x 10^8 cells L^{-1} (Kempton et al., 2002). During this study, *K. foliaceum* was observed for the first time in the freshwater tidal reaches of the

Guadiana estuary, where dinoflagellates were usually absent or rare. Indeed, no dinoflagellates were observed in the 2005 samples. After N addition, this species' abundance increased from 2.9 x 10^4 cells L^{-1} to 3.9 x 10^6 cells L^{-1} in the summer experiment (Fig. 4.5C), corresponding to an in situ net growth rate of 1.17 d^{-1} at 25ºC, equivalent to a doubling time of 14 h. This growth rate is much higher than instantaneous growth rates observed in unialgal cultures (0.16 d^{-1} at 23ºC: Figueroa et al., 2009) and maximum potential growth rates estimated by allometric models (0.78 d^{-1} at 25ºC, Tang, 1995). Furthermore, net growth rates of *K. foliaceum* in all treatments amended with N but not Si (N, NP) were significantly higher than net growth rates in treatments enriched with N and Si simultaneously (SiN, NPSi). This response is worthy of further investigation, given that anthropogenic nutrient inputs are typically of N and P, but not Si. In another Mediterranean climate estuary, the Swan River estuary (Australia), dinoflagellate summer blooms have also been supported by nitrogen inputs (Thompson, 1998).

Cyanobacteria are usually responsible for summer to early-autumn blooms in the Guadiana estuary (Barbosa et al., 2010), due to their preference for high water temperature and low turbulence. Low N:P ratios will also give a competitive advantage to cyanobacteria (Tilman et al., 1986). Experiments recently carried out in the Guadiana estuary clearly showed that cyanobacteria growth rate increased after ammonium additions, but did not respond to nitrate-alone additions (see Chapter 5). During the 2008 experiments, cyanobacteria occurred only in the summer and their abundance decreased in all treatments after 4-day incubation (Fig. 4.5C), most likely due to a strong top-down control exerted by phagotrophic protists. Nevertheless, net growth rates of cyanobacteria in treatments N and NP were significantly higher than net growth rates in the control and in treatments enriched simultaneously with N and Si (SiN and NPSi), suggesting that they may have responded more intensively to N addition, than to combined N and Si, as observed for the dinoflagellate *K. foliaceum*. Other studies have shown increases on cyanobacteria abundance in response to all forms of N additions (Moisander et al., 2009), to P and ammonium additions (Zohary et al., 2005), and to N and P additions (Sipura et al., 2005). Additionally, the higher net growth rates of cyanobacteria in treatments N and NP

may have also been the indirect effect of increased growth of heterotrophic bacteria due to N additions and, consequently, an increase on grazing activity of heterotrophic bacteria by phagotrophic protists, alleviating the grazing pressure on cyanobacteria. The preference of planktonic protozoa for heterotrophic bacteria rather than cyanobacteria has already been reviewed (e.g., Caron et al., 1991).

Phytoplankton growth responded significantly to nutrient enrichment in most experiments. However, increased nutrient net consumption rates without simultaneous increase in phytoplankton net growth were also observed on several occasions. In the winter 2008, N, P and Si net consumption rates were significantly higher in all N-, P- and Si-enriched treatments, respectively, but no apparent stimulation of phytoplankton was observed (Fig. 4.2A). During the spring and summer 2008 experiments, the same was observed for Si, and in the spring-summer transition with N additions (Figs. 4.3A, 4.4A, 4.5A). These responses may be explained by various hypothesis, including: (a) nutrient uptake by cells other than phytoplankton (e.g. heterotrophic bacteria, algae growing on the bottle walls), (b) phytoplankton removal by grazers or other mortality sources (e.g., viral lyses), or (c) luxury consumption that will later result in delayed biomass growth (Dortch et al., 1984; Krom et al., 2005; Glover et al., 2007). Luxury consumption of N and P is a well known strategy of phytoplankton to cope with a variable nutrient regime, using transient nutrient enrichment to build-up an intracellular storage pool (Sommer, 1985, 1989) that can be used for growth after depletion of the external nutrient supply. It has also been hypothesized that diatoms can incorporate nitrate by non-nutritional mechanisms, and then release it as nitrite, ammonium or dissolved organic nitrogen (Lomas and Glibert, 1999). This N uptake would therefore not result in biomass increases. Si accumulation may also occur in diatoms, although internal pools of Si are usually small, given that Si uptake occurs only during cell wall synthesis (Martin-Jézéquel et al., 2000). Luxury uptake of Si can therefore result in thicker cell walls. Since other Si-consuming organisms (e.g., choanoflagellates, silicoflagellates) were not observed in the samples, Si luxury consumption by diatoms was most likely responsible for the significant Si uptake that occurred in all Si-enriched treatments in the winter, spring and summer 2008 experiments (Figs. 4.2C, 4.3C, 4.5C), that did not result in biomass accumulation.

4.4.3 Implications for eutrophication

Nitrogen was the potentially limiting nutrient to phytoplankton growth throughout the productive period (spring-summer), at ambient N concentrations lower than 22 μM. Nitrogen concentrations up to 20 μM (Bishop et al., 1984) and 57 μM (Xu et al., 2010) have been shown to limit phytoplankton growth. In addition, a recent review on nitrate uptake data by phytoplankton suggests that nitrate concentrations above 20 μM stimulate uptake rates in both unialgal cultures and natural phytoplankton communities (Collos et al., 2005). Many studies have been using half-saturation constants for nutrient uptake and/or growth as a threshold to evaluate nutrient limitation of phytoplankton in natural communities (e.g., Domingues et al., 2005). Therefore, had we considered half-saturation constants (K_S) for nitrate uptake described in the literature (e.g. 0.02 – 10.2 μM, Sarthou et al., 2005) and nutrient limitation criteria that use both nutrient concentrations and ratios (Fisher et al., 1988; Justic et al., 1995), we would have concluded that N was generally not limiting in the Guadiana upper estuary. These contradictory results clearly show that nutrient enrichment experiments are a solid strategy to evaluate nutrient limitation of phytoplankton growth over specific periods and ecosystems, although the outcomes of such experiments require careful analysis and interpretation. Conversely, half-saturation constants are obtained under laboratorial, steady-state conditions, and vary over time, space, inter- and intra-specifically. Therefore, application of criteria based on K_S to assess nutrient limitation of natural phytoplankton communities should be done cautiously.

The response of dinoflagellates and cyanobacteria during the summer experiment is worthy of further investigation. The harmful dinoflagellate *Kryptoperidinium foliaceum* and coccoid picocyanobacteria showed significantly higher net growth rates in response to N additions (treatments N and NP) in the absence of Si. Anthropogenic nutrient inputs are typically of high N and P, but not Si, given that the chemical weathering of silicates on land is the main process that supplies dissolved and particulate silicate to rivers (Ittekkot et al., 2000). Therefore, increased anthropogenic N supply to the Guadiana estuary may promote the development of this harmful

dinoflagellate species and cyanobacteria. Although the Guadiana estuary is not impacted by intense human pressure and it is still in a good state in respect to eutrophication, increased urban development on its margins, especially in the lower estuary, will probably be responsible for increased nutrient inputs in a near future. Furthermore, the recently reported increasing trend in light availability in the Guadiana estuary, a result of increased retention of suspended matter behind the recently constructed Alqueva dam (Barbosa et al., 2010), will most likely increase the sensitivity of this estuary to nutrient enrichment, namely during spring and summer. Our results should, therefore, be used as a management tool when establishing nutrient criteria and nutrient loading budgets. Furthermore, acclimation of phytoplankton to elevated nutrient levels have not yet received as much attention as acclimation to temperature or PAR (Collos et al., 2005), but in view of the current eutrophication trend, this subject is pertinent.

Chapter 5

Effects of ammonium and nitrate on phytoplankton growth in the freshwater tidal zone of a turbid, Mediterranean estuary

Effects of ammonium and nitrate on phytoplankton growth in the freshwater tidal zone of a turbid, Mediterranean estuary

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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 on 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 influence 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. Phytoplankton response was evaluated in terms of biomass and abundance, using inverted and epifluorescence microscopy.

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 has clearly been the main nitrogen source for phytoplankton in the study site. Overall, nitrogen 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 may result in a shift on phytoplankton community composition, towards dominance of cyanobacteria and green algae.

Keywords: Water Framework Directive, phytoplankton, biomass, abundance, chlorophyll a, Portuguese waters

5.1 Introduction

Uptake and assimilation of nitrate (NO_3^-) and ammonium (NH_4^+) by aquatic primary producers are important biochemical processes that result in the conversion of inorganic nitrogen into organic compounds within the cell. The differential utilisation of these inorganic nitrogenous compounds 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 still not been 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 manifested in a higher maximum velocity and lower half-saturation constant for ammonium uptake, in relation to nitrate (Dortch, 1990). It is also related to the lower energetic costs associated to 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 differential utilization of inorganic nitrogenous compounds by phytoplankton may have significant impacts on primary productivity on local scales. For instance, in San Francisco Bay, high ammonium concentrations resulting from agricultural drainage and sewage treatment plants can prevent the development of the spring phytoplankton bloom, due to inhibition of nitrate uptake (Dugdale et al., 2007). In this system, nitrate only becomes available to phytoplankton when ammonium concentrations are reduced to less than 4 μM, through dilution by precipitation and

runoff, enabling a rapid uptake of NO_3^- and consequent phytoplankton growth (Dugdale et al., 2007).

The interactions between nitrate and ammonium uptake have been extensively studied in cultures and marine/brackish environments (e.g., Dortch et al., 1984; Quéguiner et al., 1986; Sanders et al., 1987; Zehr et al., 1989; Glibert and Garside, 1992; Tamminen, 1995; Yin et al., 1998; 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), despite their importance as sources of nutrients and phytoplankton to downriver estuarine reaches and adjacent coastal areas (Rocha et al., 2002; Domingues and Galvão, 2007). In view of increasing human influences on estuaries and coastal zones, which include urban and agricultural runoffs and, consequently, nutrient enrichment, the analyses of nutrient interactions and uptake by phytoplankton are particularly needed in sensitive and extreme ecosystems such as freshwater tidal estuarine zones. Furthermore, knowledge on nitrate/ammonium interactions represents an important contribution towards the understanding of new versus regenerated production. Considering that new production of phytoplankton is coupled to the transfer of fixed carbon at surface waters to its vertical exportation and burial in sediments, this is a crucial topic due to the increasing concern over the implications of global warming (Dugdale and Goering, 1967; Flynn et al., 1997).

Therefore, this study aims to evaluate the effect of nitrate and ammonium on phytoplankton growth, and the effect of variable ammonium concentrations on phytoplankton growth and nitrate uptake. This is a pertinent subject given the increased urban pressure on the Guadiana margins, with associated increase of ammonium inputs and reduced nitrate inputs due to water and sediment retention behind the recently built Alqueva dam.

5.2 Materials and Methods

5.2.1 Study site

The Guadiana River's (drainage area 67,039 km^2, length 810 km) estuary 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 well mixed in the upper section. The upper, freshwater tidal section represents the largest estuarine region in length, extending approx. 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: 333.0 ± 1095.9 m^{3}s^{-1}, http://snirh.pt), but the recently built Alqueva dam 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.

5.2.2 Sampling strategy

Nutrient addition experiments were undertaken using water samples collected in the freshwater tidal reaches (upper estuary) of the Guadiana estuary (see Fig. 4.1, Chapter 4). Throughout 2008, abiotic and biotic variables were analysed fortnightly at the sampling station, Alcoutim, as part of a broader sampling program that covered the whole Guadiana estuary. 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 (Domingues and Galvão, 2007; Morais et al. 2009a), 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 experiment set-up (approx. 2 hours).

Vertical profiles of photosynthetically active radiation (PAR) intensity were determined using a LI-COR radiometer. Light extinction coefficient (k_e, m^{-1}) was calculated using an exponential function, $I_z = I_0\, e^{-Ke.Z}$, where I_z is the light intensity at

depth level Z (m) and I_0 is the light intensity at the surface. Mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}) was calculated according to $I_m = I_o (1-e^{(-Ke.Zm)})(K_e.Z_m)^{-1}$, where Z_m (m) is the depth of the mixed layer (Jumars, 1993). The mixed layer corresponded to the whole water column, since there was neither haline nor thermal stratification (Domingues and Galvão, 2007; Morais et al., 2009a). Daily freshwater flow throughout 2008, measured at Pulo do Lobo hydrometric station, 85 km from the river mouth, was obtained from the Portuguese National Water Institute public database (http://snirh.pt).

5.2.3 Nitrate and ammonium addition experiments

Two different sets of experiments were performed during 2005 and 2008. The 2005 experiments served as a preliminary study to test and improve the methods. Experiments were conducted in representative seasons for phytoplankton growth: winter (February, only in 2008), spring (May), spring-summer transition (June), summer (August) and autumn (October). For each experiment, eight experimental treatments were prepared in duplicate and ran for 4 days (6 days in 2005). Potassium nitrate (KNO_3) and ammonium chloride (NH_4Cl) were added to the experimental treatments at day 0, in a single pulse, according to Table 5.I. Ammonium was added in different concentrations (from 1 to 100 µM) to the experimental treatments whilst nitrate was added at the same concentration (100 µM). During 2005, the experimental treatments were incubated in 2 L polycarbonate bottles in an outdoor tank filled with tap water to avoid extreme variations in temperature and covered with several layers of screen to simulate the light intensity in the mixed layer at the time of sampling. During 2008, 1 L polycarbonate bottles were incubated inside a growth chamber under in situ temperature and in situ light-dark cycle at approx. 110 µmol photons m^{-2} s^{-1}, which is slightly higher than I_m at 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 is lower. The bottles were opened daily and gently shaken twice a day. Consumption of NO_3^- and NH_4^+ were determined by following their disappearance

from solution (e.g., Suttle and Harrison, 1988) at days 0, 1, 2, and 4. Chlorophyll *a* and phytoplankton composition and abundance were evaluated at days 0, 1, 2, 4 and 6 in 2005. During the 2005 experiments, phytoplankton growth was exponential until day 4, and in many experimental treatments, until day 6. Therefore, in 2008, due to logistic and financial reasons, chlorophyll and phytoplankton were evaluated only at days 0 and 4. In the winter and spring 2008 experiments, daily measurements of in vivo Chl*a* fluorescence, confirmed exponential growth of phytoplankton until day 4 (data not shown).

Table 5.I – Concentrations (μM) of nutrients added to the experimental treatments in 2005 and 2008. Nitrate was added as potassium nitrate (KNO_3) and ammonium as ammonium chloride (NH_4Cl).

	2005		**2008**	
	NO_3^-	**NH_4^+**	**NO_3^-**	**NH_4^+**
Control	-	-	-	-
NIT	200	-	100	-
AMM	-	200	-	100
1AMM + NIT	-	-	100	1
10 AMM + NIT	-	-	100	10
20 AMM + NIT	-	-	100	20
50 AMM + NIT	-	-	100	50
100 AMM + NIT	-	-	100	100

5.2.4 Laboratory analyses

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^-) where frozen (-20ºC) 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. (1984b), using glass fibre filters (Whatman GF/F, pore diameter = 0.7 μm). Chlorophyll *a* was extracted overnight at 4ºC 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 hours of sampling, using glass slides and non-fluorescent immersion oil (Cargille type A), and then frozen (-20ºC) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5x 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 400x 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).

5.2.5 Relative preference index

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.

5.2.6 Statistical analyses

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, all values were combined for the 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 to assess significant differences between nutrient consumption and phytoplankton growth rates of the controls and the treatments.

In respect to nutrient consumption, we actually 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 enrichments to the medium had occurred during the experiments.

5.3 Results

5.3.1 Ambient nitrogen and chlorophyll concentration

Mean river flow at Pulo do Lobo was 18.5 ± 15.8 m^3 s^{-1} in 2005 and 14.2 ± 9.1 m^3 s^{-1} in 2008 (Fig. 5.2). Nitrate concentration in the upper estuary was lower in 2005 than 2008, with mean values of 9.5 ± 7.1 μM and 24.2 ± 12.7 μM, respectively. Throughout 2008, nitrate concentration was always above 10 μM, except for three sampling dates in the summer. Three maxima occurred in March (52.5 μM), June (43.7 μM) and December 2008 (36.6 μM) (Fig. 5.2). Nitrate was the predominant nitrogen form during both years. During 2008, river flow was positively correlated to nitrate concentration ($r = 0.5$, $p < 0.01$, $n = 31$). Ammonium concentration during 2005 and 2008 showed similar means (2.5 ± 0.1 μM and 2.7 ± 2.2 μM, respectively) and never surpassed 9 μM (Fig. 5.2). Throughout the two years, ammonium concentration was mostly <3 μM. Ammonium represented, on average, 26.5%±23.8% and 12%±18% of the total dissolved inorganic nitrogen (ammonium+nitrate) in 2005 and 2008, respectively.

Chlorophyll *a* concentration was higher during 2005 (2.1 - 41.6 μg L^{-1}) than 2008 (1.1 - 17.1 μg L^{-1}); the highest values were observed in the summer and the lowest in the winter (Fig. 5.2). No significant relationships were found between chlorophyll *a* and nitrogenous nutrients.

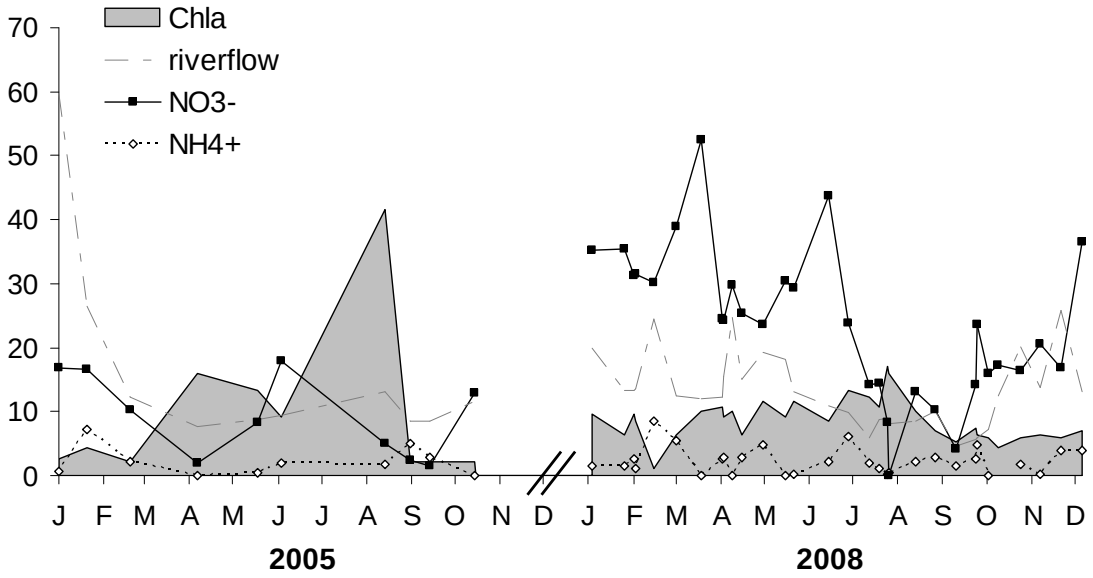

Figure 5.2 – Variation of chlorophyll *a* concentration (µg L^{-1}), river flow (m^3 s^{-1}), nitrate and ammonium concentration (µM) in Alcoutim throughout 2005 and 2008.

5.3.2 Nitrate and ammonium addition experiments

In the winter 2008 experiment, nitrate uptake was significantly lower in the nitrate-enriched treatments (0.1 - 0.8 µM d^{-1}), in relation to the control (1.6 µM d^{-1}). Ammonium net consumption rates were significantly higher than in the control (0.5 µM d^{-1}) in the ammonium-enriched treatments, and increased with increasing ammonium concentrations (0.7 - 10.8 µM d^{-1}) (Fig. 5.4A). Community net growth rates in all ammonium-enriched treatments (0.32 - 0.36 d^{-1}) were significantly higher than the control (0.19 d^{-1}) (Fig. 5.4B). Green algae showed significantly higher net growth rates in relation to the control (0.04 d^{-1}) in the ammonium-enriched treatments (0.19-0.55 d^{-1}), except treatments with the lowest ammonium concentrations (1AMM+NIT and 10AMM+NIT) (Fig. 5.4C). Nitrate-only additions had no effect on growth rates. Diatoms did not respond to nitrogen addition.

Fig. 5.3

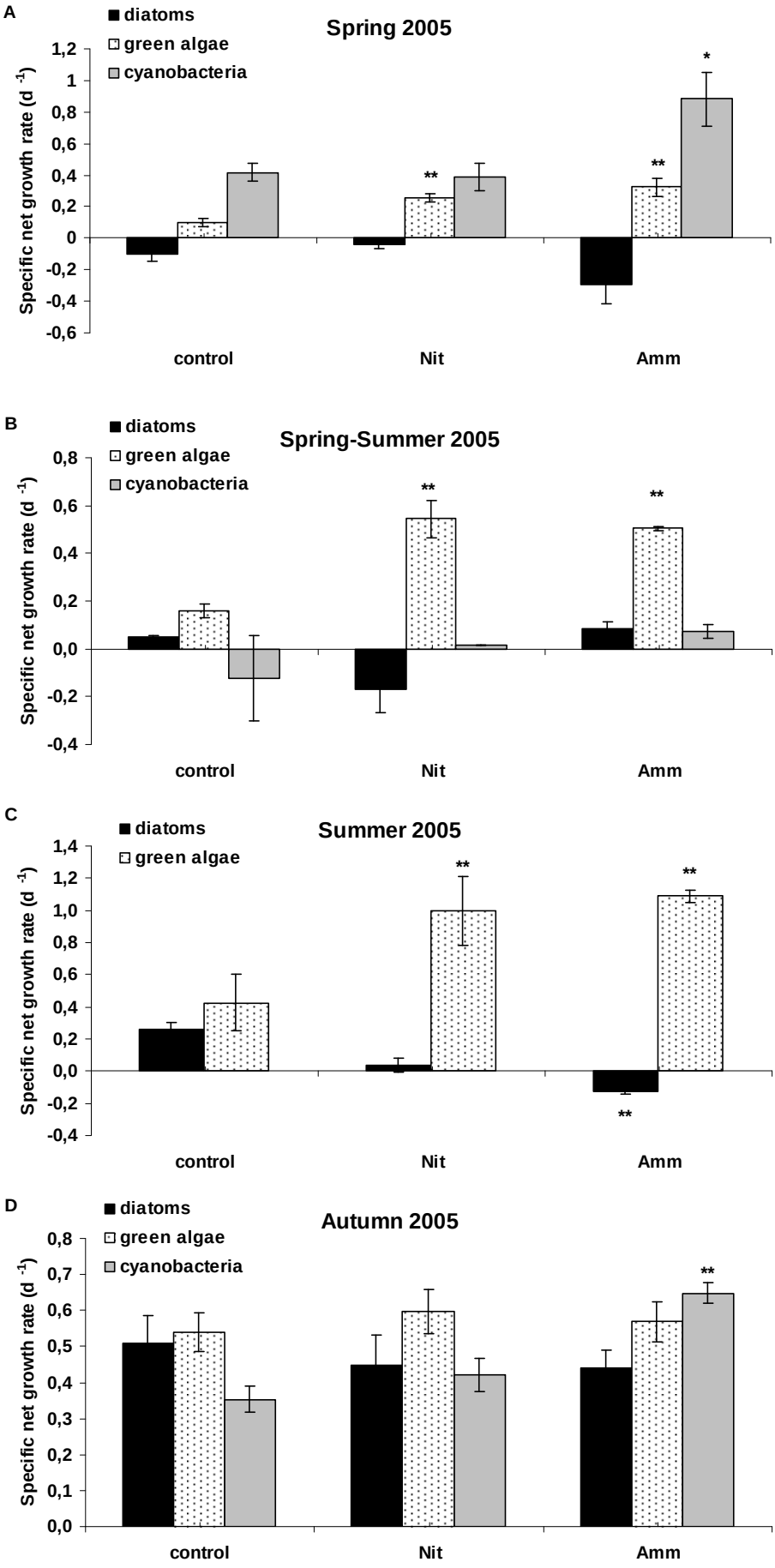
A
diatoms
green algae
cyanobacteria
Spring 2005
Specific net growth rate (d -1)
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
-0,4
-0,6
**
**
*
control
Nit
Amm
B
diatoms
green algae
cyanobacteria
Spring-Summer 2005
Specific net growth rate (d -1)
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
**
**
control
Nit
Amm
C
diatoms
green algae
Summer 2005
Specific net growth rate (d -1)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
**
**
**
control
Nit
Amm
D
diatoms
green algae
cyanobacteria
Autumn 2005
Specific net growth rate (d -1)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
**
control
Nit
Amm

Figure 5.3 (previous page) – Specific net growth rates (d^{-1}) of different phytoplankton groups during the 2005 nutrient enrichments experiments. A) spring, B) spring-summer transition, C) summer and D) autumn. Vertical lines represent ± 1 S.D. Significant differences between the treatments and the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

During spring 2005, green algae responded significantly to N (as nitrate and as ammonium) enrichment in relation to the control (0.10 d^{-1}), with growth rates ranging between 0.25 and 0.32 d^{-1} in treatments NIT and AMM, respectively (Fig. 5.3A). Diatoms, on the contrary, showed negative growth rates in all the treatments. Cyanobacteria responded significantly to ammonium addition (AMM, 0.88 d^{-1}) in relation to the control (0.42 d^{-1}) (Fig. 5.3A). During spring 2008, nitrate uptake in the only nitrate-enriched treatment (NIT, 7.7 μM d^{-1}) was not significantly different from the control (7.3 μM d^{-1}), but in the ammonium-enriched treatments, nitrate uptake decreased significantly with increasing ammonium concentrations, from 7.0 to 0.5 μM d^{-1} in treatments 1AMM+NIT and 100AMM+NIT, respectively. In contrast, ammonium uptake increased significantly in relation to the control (0.0 μM d^{-1}) with increasing ammonium concentrations, from 0.3 to 8.3 μM d^{-1} in treatments 1AMM+NIT and 100AMM+NIT, respectively (Fig. 5.5A). Community net growth rate in the spring experiment was significantly higher than the control (0.45 d^{-1}) only in treatments with intermediate concentrations of ammonium (treatments 10AMM+NIT and 20AMM+NIT, 0.57 d^{-1}), and was lower than the control in treatment AMM (0.44 d^{-1}) (Fig. 5.5B). Diatoms did not show significant differences in net growth rates in the enriched treatments in relation to the control (1.15 d^{-1}), except in treatments enriched with 100 μM ammonium (AMM and 100AMM+NIT), where their net growth rate decreased (0.99 and 0.93 d^{-1}). Green algae, on the contrary, showed significant increases in net growth rate in relation to the control (0.78 d^{-1}) in some of the ammonium enriched treatments (AMM, 20AMM+NIT and 100AMM+NIT), with net growth rates varying from 0.91 to 0.97 d^{-1}. Net growth rates of dinoflagellates in the enriched treatments were not significantly different from the control, and varied between 0.58 and 0.74 d^{-1} (Fig. 5.5C).

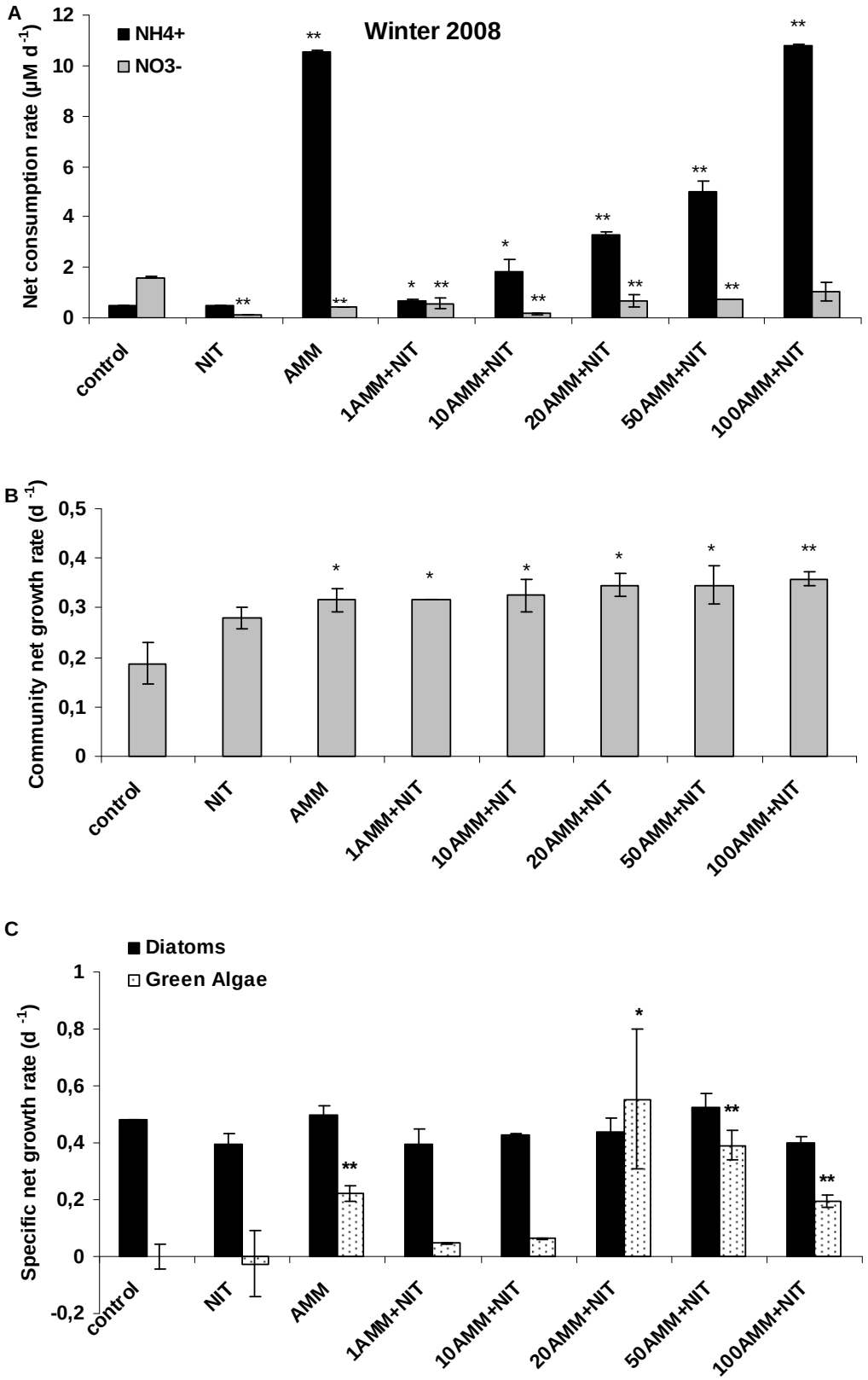

Figure 5.4 - A) Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), B) community net growth rate (d^{-1}), and C) specific net growth rates (d^{-1}) of diatoms and green algae during the 2008 winter experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

In the spring-summer transition 2005 experiment, only green algae responded significantly to both nitrate (0.54 d^{-1}) and ammonium (0.50 d^{-1}) additions in relation to the control (0.16 d^{-1}) (Fig. 5.3B). The 2008 experiment was characterized by significantly higher ammonium net consumption rates in all ammonium-enriched treatments (2.4-21.4 μM d^{-1}), in relation to the control (0.1 μM d^{-1}). As in the other experiments, ammonium net consumption rate increased with increasing ammonium additions. Nitrate net consumption rate decreased with increasing ammonium concentrations, from 7.0 μM d^{-1} in the control to 0.5 μM d^{-1} in treatment AMM. In treatments enriched only with nitrate (NIT) and with low ammonium concentrations (1AMM-NIT, 10AMM+NIT), nitrate uptake did not vary significantly in relation to the control (Fig. 5.6A). Community net growth rates of phytoplankton in the nitrogen-amended treatments (0.38-0.47 d^{-1}) basically did not vary in relation to the control (0.44 d^{-1}) (Fig. 5.6B). Net growth rates of diatoms (1.00-1.14 d^{-1}) and green algae (0.73-0.83 d^{-1}) in the nitrogen-amended treatments were not significantly different from the control (1.08 and 0.84 d^{-1}, respectively). Dinoflagellates differed from the control (0.14 d^{-1}) only in treatment AMM, where net growth rate was significantly lower and negative (-0.07 d^{-1}) (Fig. 5.6C).

During the summer 2005 experiment, green algae responded significantly to nutrient enrichment, with higher growth rates in treatments NIT (1.00 d^{-1}) and AMM (1.09 d^{-1}) than in the control (0.43 d^{-1}). Diatoms showed a significant and negative growth rate in treatment AMM (-0.13 d^{-1}) in relation to the control (0.26 d^{-1}) (Fig. 5.3C). In the summer 2008 experiment, nitrate net consumption rates were significantly higher in all nitrate-enriched treatments (11.4-35.1 μM d^{-1}), in relation to the control (3.6 μM d^{-1}), although rates decreased with increasing ammonium concentrations. In contrast, ammonium net consumption rates increased in the ammonium-enriched treatments, and were significantly higher (2.5-19.0 μM d^{-1}) than in the control (0.4 μM d^{-1}) (Fig. 5.7A). Community net growth rates in all nitrogen-enriched treatments (0.23-0.32 d^{-1}) were significantly higher than the control (0.04 d^{-1}) (Fig. 5.7B). Green algae increased in all nitrogen-enriched treatments in relation to the control (0.73 d^{-1}), with net growth rates varying from 1.06 to 1.29 d^{-1}. On the contrary, net growth rates of diatoms decreased in the treatments with higher ammonium concentrations (AMM, 50AMM+NIT and 100AMM+NIT), with net growth rates from 0.61 to 0.76 d^{-1} and

0.99 d^{-1} in the control. Cyanobacteria net growth rates increased with increasing ammonium concentrations, from 0.49 d^{-1} in treatment 1AMM+NIT to 1.29 and 1.39 d^{-1} in treatments 100AMM+NIT and AMM, respectively, whilst in the control cyanobacteria net growth rate was 0.17 d^{-1}. Dinoflagellates showed negative net growth rates in treatments enriched with 100 µM ammonium (AMM and 100AMM+NIT, -0.31 and -0.08 d^{-1}), but increased in relation to the control (0.13 d^{-1}) in treatment 50AMM+NIT (0.23 d^{-1}) (Fig. 5.7C).

In the autumn 2005, only cyanobacteria showed higher growth rates in treatment AMM (0.65 d^{-1}) in relation to the control (0.35 d^{-1}) (Fig. 5.3D). During 2008, ammonium net consumption rates in all ammonium-enriched treatments (0.5-10.3 µM d^{-1}) were significantly higher than in the control (0.1 µM d^{-1}), and increased with increasing ammonium concentrations. Nitrate net consumption rate in treatment enriched only with 100 µM ammonium (AMM, 0.06 µM d^{-1}) was significantly lower than in the control (4.9 µM d^{-1}), but in the other treatments no significant differences were found in relation to the control (Fig. 5.8A). Community net growth rates in the nitrogen-enriched treatments (0.47-0.59 d^{-1}) did not show significant differences in relation to the control (0.50 d^{-1}), except in treatment AMM, where net growth rate was significantly higher (0.62 d^{-1}) (Fig. 5.8B). Likewise, diatoms net growth rate was significantly higher in treatment AMM (1.63 d^{-1}) in relation to the control (1.42 d^{-1}) (Fig. 5.8C).

Overall, the relative preference index for nitrate in relation to ammonium concentration (Fig. 5.9) showed that nitrate was not the preferred nitrogen source in the experiments (RPI < 1) and the preference for nitrate decreased with increasing ammonium concentrations, except in the summer, when NO_3^- RPI values were always close to 1 for all ammonium concentrations.

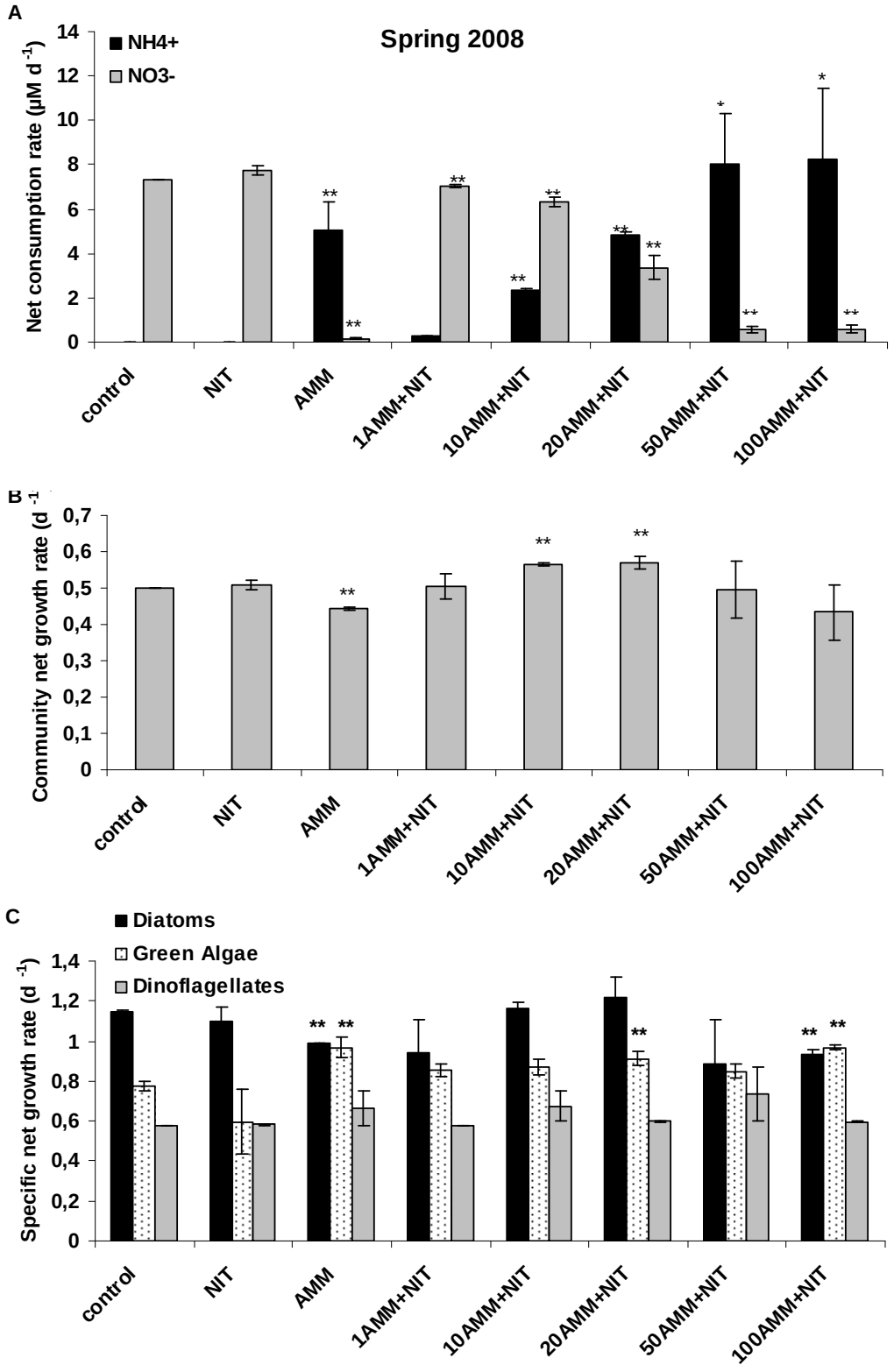

Figure 5.5 - A) Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), B) community net growth rate (d^{-1}), and C) specific net growth rates (d^{-1}) of diatoms, green algae and dinoflagellates during the 2008 spring experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

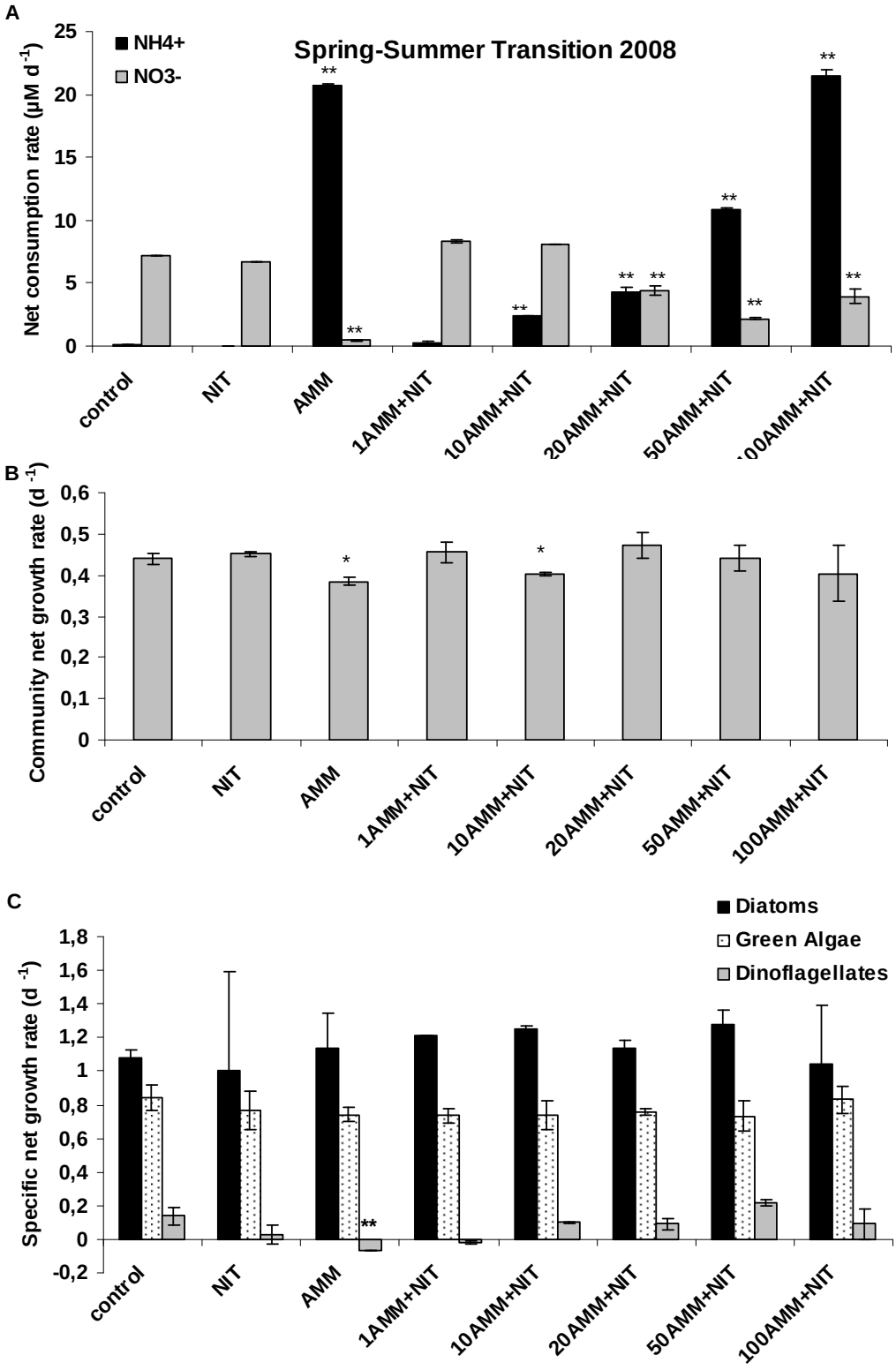

Figure 5.6 – 2008 spring-summer transition experiment. For legend see Fig. 5.5.

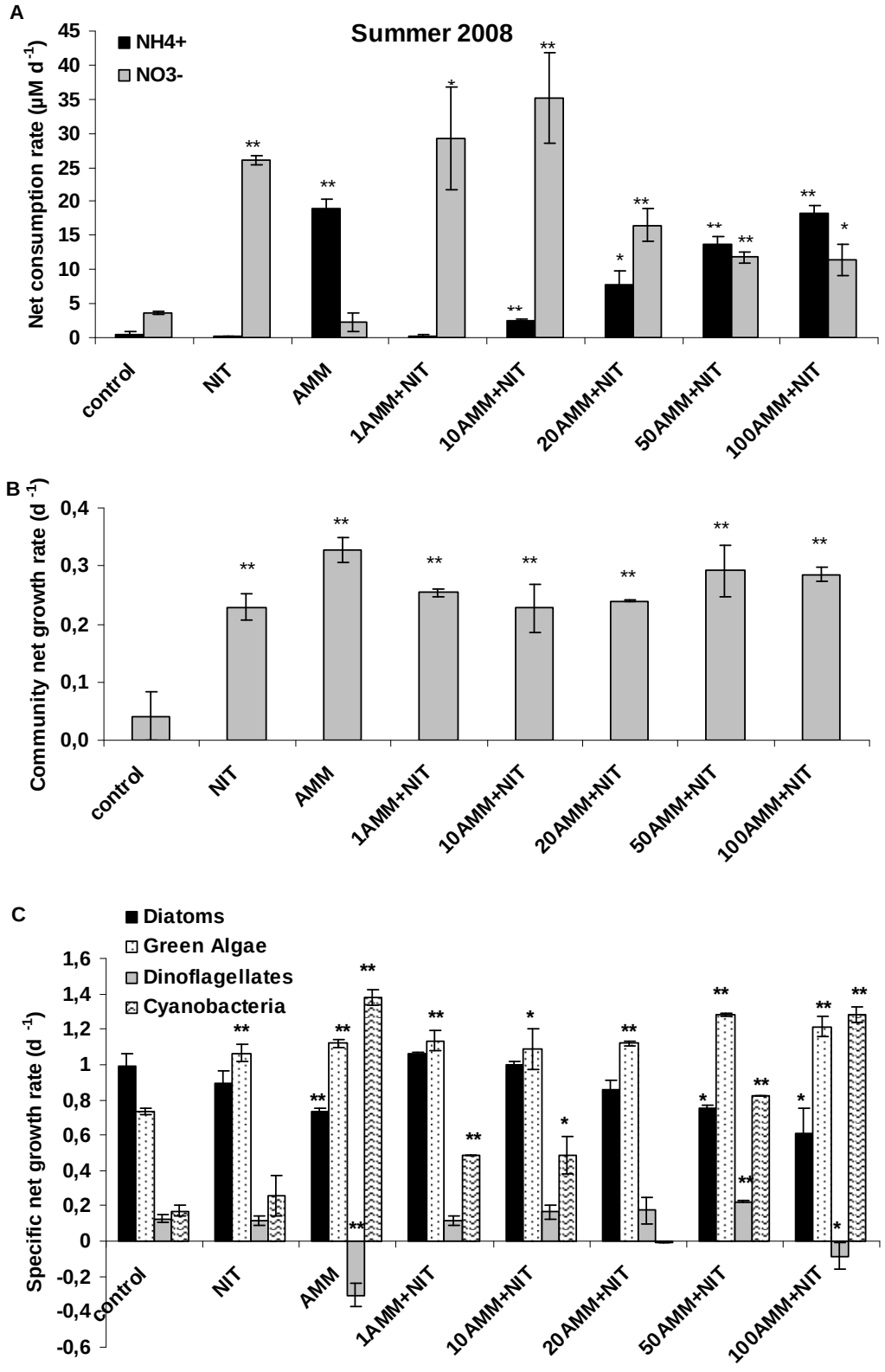

Figure 5.7 - A) Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), B) community net growth rate (d^{-1}), and C) specific net growth rates (d^{-1}) of diatoms, green algae, dinoflagellates and cyanobacteria during the 2008 summer experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

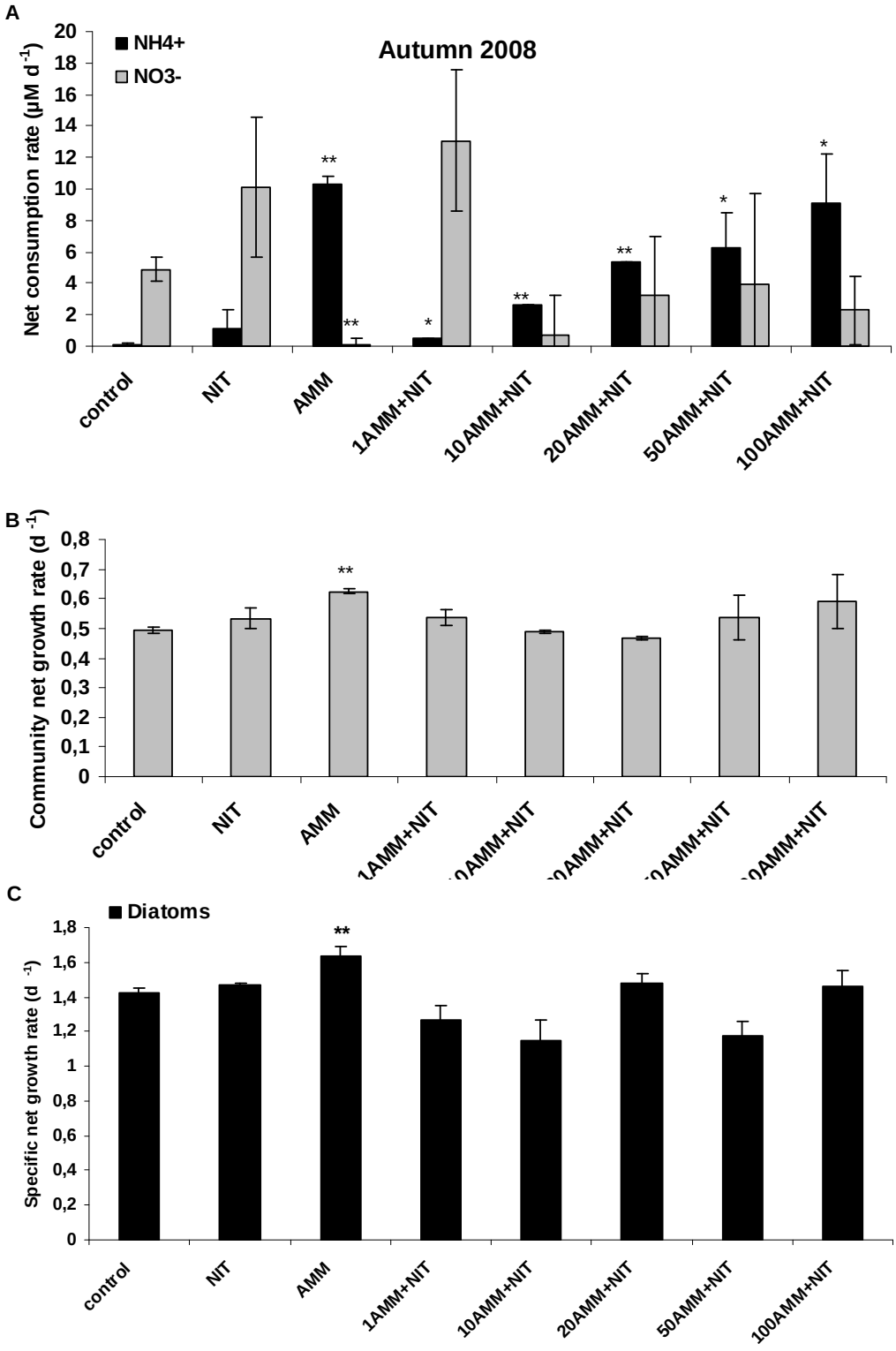

Figure 5.8 - A) Nitrate (NO_3^-) and ammonium (NH_4^+) net consumption rates (µM d^{-1}), B) community net growth rate (d^{-1}), and C) specific net growth rates (d^{-1}) of diatoms during the 2008 winter experiment. Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

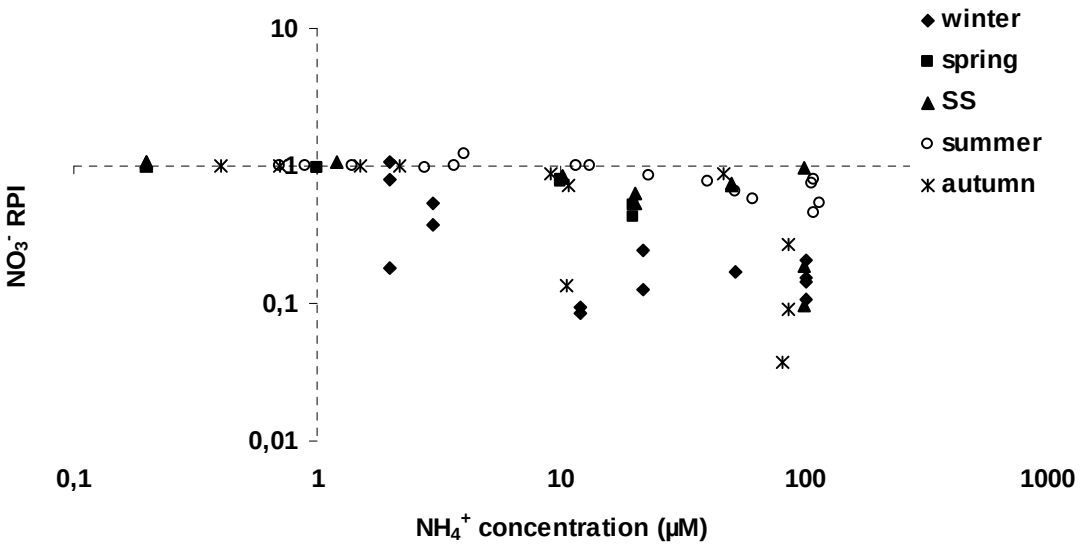

Figure 5.9 – Relative preference index (RPI) for nitrate uptake as a function of ammonium concentration, for each treatment and each experiment.

5.4 Discussion

5.4.1 Ammonium and nitrate availability in the Guadiana estuary

In the Guadiana estuary, ammonium concentrations ranged between undetectable values and 8.6 µM, but remained mostly below 3 µM throughout 2005 and 2008, which can be considered low concentrations, comparing to other estuaries (e.g., <0.2-41.5 µM, Southampton Water: Torres-Valdés & Purdie, 2006; >2 µM, Delaware Estuary: Yoshiyama & Sharp, 2006; >4 µM, San Francisco Bay: Dugdale et al., 2007). It has been extensively 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, the inhibitory effect of the low ammonium concentrations on nitrate uptake was most likely minimal in the Guadiana estuary, as was the potential toxic/inhibitory effect of ammonium on phytoplankton production. Nitrate concentrations were significantly higher than ammonium throughout the two years. It is therefore probable that phytoplankton communities in the Guadiana upper estuary are primarily 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 & Purdie, 2006). The dominance of micro- and larger nano-sized (>10 μm) phytoplankton species in the Guadiana estuary (e.g., Domingues et al., 2005, 2007) 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).

Nitrate has been the main source of nitrogen for phytoplankton in the Guadiana estuary. Significant and 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 regularisation 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; 2005 annual mean = 9.5 μM; 2008 annual mean = 23.7 μM). The decrease in the availability of nitrate and other nutrients together 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).

5.4.2 Effects of ammonium on nitrate uptake

In general, nitrate net consumption rates decreased with increasing ammonium concentrations and uptake, which could be attributed to inhibition of nitrate uptake by ammonium. Ammonium concentrations higher than 2 μM (Pennock, 1987) or 4 μM (Dugdale et al., 2007) are known to suppress nitrate uptake in estuarine systems. Although initial ammonium concentrations were low (between undetectable values and 4 μM), ammonium additions in the treatments (up to 100 μM) were high enough to exert an inhibitory effect on nitrate uptake. In the summer, however, nitrate uptake in treatment 100AMM+NIT was still significantly higher than uptake in the control, which reflects a preference for ammonium, but not a suppression/inhibition

of 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.

5.4.3 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 throughout 2008. Firstly, in the summer experiment nitrate added alone promoted a significantly higher net consumption rate than in the control, resulting in significant increases in the community biomass. Phytoplankton growth rates in all the N-enriched treatments were significantly higher than in the control, indicating that the community was indeed N-limited, when initial nitrate concentration was 15.5 μM, and that the nitrogenous nutrients were used for growth, not for storing in internal pools.
A second response type was observed in the other experiments throughout 2008, with initial nitrate concentrations ranging between 22.2 and 35.4 μM. Nitrate-alone additions had no significant effects on uptake and growth rates. Nitrate was most likely not limiting, otherwise, cells would have taken up the available nitrate. Previous nutrient enrichment experiments carried out in the Guadiana estuary have shown that nitrogen, added as nitrate, became limiting when ambient concentrations were <20-24 μM; the present experiments indicate that nitrate concentrations >20 μM were not limiting to phytoplankton growth. However, ammonium net consumption rates increased significantly in all the ammonium-enriched treatments throughout 2008. Besides the significant increases in net consumption rates only (spring-summer transition and autumn 2008), which 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 (winter 2008), 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 autumn experiments. 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 storing 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 Relative Preference Index, which is also a common observation in other estuarine systems (e.g., McCarthy et al., 1977; Carpenter & Dunham, 1985; Balode et al., 1998). Only when ammonium concentrations were undetectable, was nitrate the preferred nutrient, with RPI values slightly higher than 1.

5.4.4 Effects of ammonium and nitrate on specific phytoplankton groups

Green algae showed the most consistent responses to nitrate and ammonium additions. Throughout 2005 (except in the autumn) and in the winter, spring and summer 2008, green algae responded significantly to ammonium additions, with initial DIN concentrations ranging between 2.0 and 37.4 μM. Green algae also responded positively to nitrate additions during 2005 and in the summer 2008, when DIN concentrations were <20 μM. 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 the external nutrient concentrations, but also by the

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 green algae, namely *Scenedesmus*, *Ankistrodesmus* and *Selenastrum*, may reach similar densities growing on both nitrate and ammonium, under non-limiting conditions (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 2008 experiments was alleviated during incubation, and light intensity throughout 2005 was relatively high, green algae were most likely 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, the 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 nitrogen additions have favoured green algae throughout the experiments.

Cyanobacteria growth rates increased with increasing ammonium concentrations, indicating N-limitation. However, no response was observed to nitrate-only additions (NIT), in both set of experiments (2005 and 2008). Cyanobacteria growth rates can even decrease following nitrate additions (see Chapter 4). Although cyanobacteria usually have a preference for ammonium (Dokulil & 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 & 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 & Wangsupa, 2003), NH_4^+ in

the beginning of the experiments (<4 μM) 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 previously (see Chapter 4) for the freshwater tidal reaches of the Guadiana estuary during spring/early summer 2008. In addition, growth rates of diatoms in the spring and summer 2008 even decreased significantly in the treatments with ammonium concentration >50 μM, 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 & Sommer, 1996). However, stimulatory effects of ammonium upon 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). 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 & Taguchi, 2004). On the other hand, *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 & McLean, 2007). Overall, growth of *K. foliaceum* in the freshwater tidal zone of the Guadiana estuary seemed strongly dependent on the form and concentration of N. It reached extremely high growth rates in nitrate-enriched waters (see Chapter 4) and was inhibited by high concentrations of ammonium.

5.5 Conclusions

In the freshwater tidal reaches of the Guadiana estuary, ammonium concentrations throughout the studied years 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 Chapter and Chapter 4), 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, pointed towards an overall preference of phytoplankton for ammonium. However, preference for ammonium is group-specific. Green algae and cyanobacteria seemed to prefer ammonium, whilst nitrate was preferred by diatoms and dinoflagellates. Indeed, green algae showed the most prominent 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. Diatoms and dinoflagellates showed no positive responses to ammonium additions, using only nitrate as their nitrogen source. Lastly, future scenarios of water and sediment retention in dams leading to reduced nitrate inputs to the estuary and increases in anthropogenic-derived ammonium inputs to the Guadiana estuary, will most likely promote a shift on phytoplankton community composition 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.

Chapter 6

Light limitation and phytoplankton primary production in the freshwater tidal zone of the turbid Guadiana estuary

Light limitation and phytoplankton primary production in the freshwater tidal zone of the turbid Guadiana estuary

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Abstract

Light is usually the main driver of phytoplankton growth in turbid estuaries, but it has received far less attention than nutrients as a bottom-up factor. Here, we present the first experimental analysis of light limitation of phytoplankton growth and production and its seasonal variability in the freshwater tidal reaches of the turbid Guadiana estuary.

Natural phytoplankton communities were exposed to different photosynthetically active radiation (PAR) intensities. Short-term incubations with addition of $^{14}HCO_3^-$ were used to estimate photosynthetic parameters and long-term incubations allowed the evaluation of the effects of light on phytoplankton composition and growth.

Light limitation of phytoplankton growth occurred throughout the year in the freshwater tidal reaches of the Guadiana estuary and no photoinhibition was observed at least up to 615 μmol photons m^{-2} s^{-1}. In the summer, co-limitation by nutrients prevented a positive response of phytoplankton to light enrichment. Diatoms were the most light-limited group, whilst cyanobacteria were the only group acclimated to low light conditions. Green algae and dinoflagellates responded positively to higher PAR exposures. High saturating irradiances, high light-saturated rates of primary production and low photosynthetic efficiencies suggest that phytoplankton community was not acclimated to the low light conditions that prevail in the Guadiana estuary.

Keywords: phytoplankton, light limitation, primary production, photosynthetic parameters, Guadiana estuary

6.1. Introduction

In turbid environments, light availability plays a fundamental role as the energy source for phytoplankton growth (Alpine and Cloern, 1992; Grobbelaar, 1990; Cloern, 1996; Kocum et al., 2002), and it also affects phytoplankton community structure and algal competition (Reynolds, 1998; Litchman, 1998; Huisman et al., 1999). Phytoplankton primary productivity in estuaries can be higher in comparison with nearby coastal areas, but due to light limitation, this potential is seldom reached (Kromkamp and Peene, 1995). Indeed, in turbid, nutrient-rich estuaries, phytoplankton primary production is directly proportional to light availability (Underwood and Kromkamp, 1999 and references therein), which in turn is controlled by turbidity (Cloern, 1987). Light-limited phytoplankton growth can occur throughout the whole year (e.g., Irigoien and Castel, 1997) or seasonally (e.g., Fisher et al., 1999, Kocum et al., 2002), and it can be spatially restricted to specific estuarine areas (e.g., maximum turbidity zones, freshwater tidal estuarine zones). Furthermore, the euphotic zone in such turbid environments is usually shallow when compared to the mixing depth, so phytoplankton cells spend a small amount of time in the light; photoinhibition in these environments is thus rare (Grobbelaar, 1995).

Despite its paramount importance for phytoplankton production, light has received far less attention as a selective factor than nutrient availability (Huisman et al., 1999), which has classically been considered the most important factor regulating phytoplankton growth (e.g., Roelke et al., 1999 and references therein). The first studies on phytoplankton dynamics in the Guadiana estuary, a turbid, mesotidal Mediterranean estuarine system, identified nutrients as the main regulators of phytoplankton succession (Rocha et al., 2002; Domingues et al., 2005), but growing evidence on the importance of light has been reported (Domingues and Galvão, 2007; Domingues et al., 2007). Indeed, long-term field data indicates that phytoplankton growth in the Guadiana estuary, especially in the upper estuary, is most likely light-limited (Barbosa et al., 2010). The upper estuary, or freshwater tidal zone, is subjected to a strong tidal influence that induces the resuspension of bottom sediments, resulting in increased turbidity and strong light limitation of phytoplankton growth (Muylaert et al., 1997). Nutrient limitation in the Guadiana upper estuary occurs

mainly during the productive period (spring and summer), but co-limitation by light availability is a definite possibility (see Chapter 4). However, decreasing phytoplankton biomass coupled to decreasing turbidity and increasing light availability has been recently reported for the Guadiana estuary (Barbosa et al., 2010). Given that understanding how light (and other environmental drivers) regulates phytoplankton growth and production allows the prediction of ecosystem responses to environmental changes (see Cloern and Dufford, 2005; Smetacek and Cloern, 2008), the study presented here aims to evaluate the effects of light availability on phytoplankton abundance, composition and growth. Specifically, we intend to understand the occurrence and intensity of light limitation of phytoplankton growth throughout the seasonal cycle, and the role played by potential physiological adaptations to a low-light environment. To accomplish these goals we performed light enrichment bioassays with longer incubations times (days) to evaluate changes in phytoplankton abundance and composition, and bioassays with shorter incubation times (hours) to evaluate the effect on primary production. We hypothesized that phytoplankton growth in the freshwater tidal zone of the Guadiana estuary is light-limited throughout the year, and that phytoplankton is physiologically adapted to low-light conditions.

6.2. Materials and Methods

6.2.1 Study site and sampling strategy

The Guadiana River is one of the largest Iberian rivers, with a drainage area of 67,039 km^2, arising in Spain and draining between SE Portugal and SW Spain (see Fig. 4.1, Chapter 4). The river flows for 810 km; its last 70 km, located in a Mediterranean climate area, are influenced by semidiurnal, mesotidal tides, corresponding to the estuarine zone. The Guadiana estuary is partially stratified in its lower and middle sections and well mixed in the upper section. The upper estuary, or freshwater tidal zone, is usually located between Álamo (25 km from the river mouth) and Mértola (approx. 70 km from the river mouth), but the lower limit is subjected to changes,

depending on tidal stage and river flow (Fig. 4.1, Chapter 4). In the last years, intense damming has promoted a more regular but reduced river flow (2009: 16.0 ± 21.4 m^3 s^{-1}), contrasting with sharp variations between dry and humid months (1995 - 2000: 333.0 ± 1095.9 m^3 s^{-1}, http://snirh.pt) that used to occur before the Alqueva dam construction (140 km from the mouth).

Sampling campaigns were performed in Alcoutim, located in the upper estuary (Fig. 4.1, Chapter 4), during 2008 and 2009. Vertical profiles of photosynthetically active radiation (PAR) intensity were determined using a LI-COR radiometer and the light extinction coefficient (k_e, m^{-1}) was calculated using an exponential function (eq. 6.1):

$$I_Z = I_0 e^{-K_e Z} \qquad \text{(eq. 6.1)}$$

where I_z is the light intensity at depth level Z (m) and I_0 is the light intensity at the surface. Mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}) was calculated according to (eq. 6.2):

$$I_m = I_0 (1 - e^{(-K_e Z)})(K_e Z_m)^{-1} \qquad \text{(eq. 6.2)}$$

where I_o is the light intensity at the surface, k_e (m^{-1}) the light extinction coefficient and Z_m (m) the depth of the mixed layer (Jumars, 1993). The mixed layer in Alcoutim corresponded to the whole water column, since there was neither haline nor thermal stratification (Domingues and Galvão, 2007; Morais et al., 2009a). Vertical profiles of salinity and water temperature were determined in situ using a YSI 556 MPS probe, and were used to determine the depth of the mixed layer.

6.2.2 Short-term bioassays: photosynthesis-irradiance curves

We used the Steeman-Nielsen method (1952) to determine phytoplankton primary production in water samples collected in Alcoutim throughout 2008. Fifty mL aliquots were added to polycarbonate flasks and 100 µL (2 µCi) of ^{14}C-HCO_3^- were added to each flask. The sample flasks were incubated in triplicate under different light intensities (approx. 5, 83, 117, 302, 515 and 615 µmol photons m^{-2} s^{-1}) for 2 hours. Three dark flasks were also incubated and processed as the sample flasks. Incorporation of ^{14}C was stopped with 1 mL formaldehyde, and the samples were filtered onto nitrate cellulose filters (nominal pore diameter = 0.45 µm), which were placed inside 10 mL high-density polyethylene scintillation vials. The vials were

subsequently placed inside a fume hood with HCl, to allow the release of inorganic carbon attached to the cells. 10 ml of scintillation liquid (Universol) was added to each vial, put in 4ºC overnight, and ^{14}C activity was measured on a scintillation counter (Beckman). Primary production was calculated as (eq. 6.3):

$$PP = \frac{(Rs - Rb)xDxWxCA}{RxN} \qquad \text{(eq. 6.3)}$$

where PP is phytoplankton primary production (mg C L^{-1} h^{-1}), Rs (dpm) is the activity in the sample, Rb (dpm) is the mean activity of the dark flasks, D (=1.05) is the isotopic discrimination, W (mg C L^{-1}) is the amount of dissolved inorganic carbon in the sample (obtained through alkalinity), CA is a correction factor (total sample volume/filtered volume), R (dpm) is the total activity of the ^{14}C added to each flask, and N (hours) is the incubation time. Alkalinity was determined by titration with HCl for non-freshwater samples (S > 1 PSU) (Parsons et al., 1984b). A stepwise titration (Gran, 1950, 1952; Andersen, 2002) was used to determine alkalinity in freshwater samples (S < 1 PSU). Carbonate alkalinity was then converted to dissolved inorganic carbon and subsequently used in primary production determinations.

Primary production (PP, mg C L^{1-} h^{-1}) was converted to biomass-specific primary production (P^B = PP/Chl*a*, mg C (mg Chla)$^{-1}$ h^{-1}). The photosynthetic parameters were estimated using nonlinear regression fitting of equation 6.4 (Platt et al., 1980):

$$P^B = P^B{}_S(1 - \exp(-\alpha.E_{PAR} / P^B{}_S)) \qquad \text{(eq. 6.4)}$$

where $P^B{}_S$ is the light-saturated rate of biomass-specific primary production (mg C (mg Chla)$^{-1}$ h^{-1}), α is the initial slope of the photosynthesis-irradiance curve (mg C (mg Chla)$^{-1}$ h^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$) and E_{PAR} is the PAR irradiance during incubation (µmol photons m^{-2} s^{-1}). The saturating irradiance (E_S, µmol photons m^{-2} s^{-1}) was determined as $P^B{}_S$/ α.

It is to be noted that P-E curves are intended to reflect the physiological state of phytoplankton community at the time of sampling. During transportation to the lab (< 2 hours), light-shade adaptation could have occurred and cells could have been acclimated to lower light conditions at the beginning of the ^{14}C incubations. Considering the photosynthetic parameters obtained, in particular high saturating

irradiances, which are used to estimate the light history of the cells (Falkowski, 1983), it is unlikely that light-shade adaptation had occurred between sample collection and incubation, and that the P-E curves obtained truly reflect the physiological state of the cells at the time of collection.

6.2.3 Long-term bioassays: light-enriched microcosms

Light enrichment microcosm experiments were undertaken throughout 2009, to investigate the effects of light enrichments on representative phytoplankton communities from different seasons: winter (February), spring (April), spring-summer transition (June), summer (August) and autumn (October). Water samples were collected in Alcoutim into acid-cleaned 1 L polycarbonate bottles, during neap tides, immediately after high tide, near the water surface (approx. 0.5 m depth), assuming that the whole water column was well mixed. Samples were kept in cold and dark conditions between collection and experiment set-up (approx. 2 hours). The bottles were incubated for 4 days in a growth chamber under in situ temperature and a natural light-dark cycle (Table 6.I). Three different treatments and a control treatment were performed in duplicate as follows: control ($\approx I_m$), I_1 (70 - 90 µmol photons m^{-2} s^{-1}), I_2 (120 - 130 µmol photons m^{-2} s^{-1}) and I_3 (225 - 300 µmol photons m^{-2} s^{-1}) (Table 6.I). The bottles were opened daily and gently shaken twice a day. Sub-samples for nutrient determination were collected from each bottle at days 0, 1, 2 and 4. Chlorophyll *a* and phytoplankton composition and abundance were evaluated at the beginning and at the end of the experiments. In vivo fluorescence measurements undertaken daily in other microcosms experiments showed that phytoplankton growth during incubation was exponential until day 4 and in many cases until day 6 (see Chapter 4). Therefore, due to logistic and financial reasons, chlorophyll and phytoplankton were evaluated only at the beginning and end of these experiments. Changes in abundance and biomass (chlorophyll *a*) of phytoplankton in the treatment bottles relative to the controls were interpreted as responses to light enrichment.

Table 6.I - Incubation conditions of the long-term light enrichment treatments in different seasons (SS – spring-summer transition). L:D – duration of light:dark cycles (hours); T_L:T_D – temperature during light:temperature during dark (ºC); light intensities of treatments control, I_1, I_2 and I_3 (µmol photons m^{-2} s^{-1}); nutrient concentrations (µM) at day 0 (N – nitrate; P – phosphate; Si – silicate).

	L:D	**T_L:T_D**	**control**	**I_1**	**I_2**	**I_3**	**N**	**P**	**Si**
Winter	10h:14h	13.5:12.5	50	90	120	225	79.6	2.4	81.5
Spring	13h:11h	18.5:18.0	50	90	120	225	33.2	2.3	29.0
SS	15h:9h	25.0:24.0	50	70	130	300	17.2	2.4	11.4
Summer	14h:10h	25.5:24.5	50	70	130	300	11.0	5.9	100.8
Autumn	11h:13h	23.5:22.5	50	90	120	225	17.9	3.3	69.4

6.2.4 Laboratorial analyses

Samples for nutrient determination were filtered through cellulose acetate filters (Whatman, pore diameter = 0.2 μm). Phosphate (PO_4^{3-}) and silicate (SiO_4^{4-}) were determined immediately after sample collection, whilst samples for nitrate (NO_3^-) where frozen (-20ºC) 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 phosphate and silicate, and an autoanalyzer Skalar for nitrate. Given that ammonium and nitrite concentrations in the sampling station are usually low (Domingues et al., 2005, 2007) and nitrate is the main nitrogen source for phytoplankton (see Chapter 5), ammonium and nitrite were not analysed.

Chlorophyll *a* concentration was determined according to Parsons et al. (1984b), using glass fibre filters (Whatman GF/F, pore diameter = 0.7 μm). Chlorophyll *a* was extracted overnight at 4ºC 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%) immediately after collection, stained with proflavine and filtered (1 - 5 mL, depending on the amount of suspended matter) onto black polycarbonate membrane filters (Whatman, nominal pore diameter = 0.45 μm). Preparations were made with glass slides and non-fluorescent immersion oil (Cargille type A), within 24 h of sampling, and then frozen (-20ºC) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5x magnification using a Leica DM LB epifluorescence microscope. Samples for enumeration of diatoms, green algae and dinoflagellates (>20 μm) were preserved with acid Lugol's solution (final concentration approx. 0.003%) immediately after collection, settled in sedimentation chambers (2 - 10 mL, depending on the amount of suspended matter; sedimentation time = 24 hours) and observed at 400x magnification with a Zeiss Axiovert S100 inverted microscope. Phytoplankton cells were identified, whenever

possible, to genus level. 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).

6.2.5 Statistical analyses

Statistical analyses were performed using the GraphPad Prism 5 software. For each experimental treatment, nutrient concentration, chlorophyll *a* and phytoplankton abundance were statistically compared within duplicates of the same treatment 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, all values were combined for the subsequent data analyses. Nutrient net consumption rates (disappearance rates) for each treatment (n = 8) were estimated as the slope of a linear or exponential function adjusted to the data points. Community net growth rate and specific net growth rate of different phytoplankton groups (n = 4) (μ, d^{-1}) were estimated as the slope of ln N(t) versus time (4 days), where N(t) is chlorophyll *a* concentration or phytoplankton abundance at day t, respectively, assuming exponential growth (confirmed by in vivo Chla fluorescence). Slopes and standard errors of the estimated regression lines were then compared to assess significant differences between consumption/growth rates of the controls and the treatments.

6.3. Results

In general, exposure to higher PAR intensities increased net growth rates and primary production of phytoplankton and no photoinhibition was observed. Saturating irradiances (E_S) estimated through nonlinear fitting of equation 4 varied between 316.7 and 2252 μmol photons m^{-2} s^{-1} (mean 745.1 ± 643.1) (Fig. 6.2). Mean light-saturated rate of biomass-specific primary production (P^B_S) was 9.77 ± 4.88 mg C (mg Chl)$^{-1}$ h^{-1}, with the highest value in April (20.52 mg C (mg Chl)$^{-1}$ h^{-1}) and the lowest in December (4.53 mg C (mg Chl)$^{-1}$ h^{-1}).

Fig. 6.2

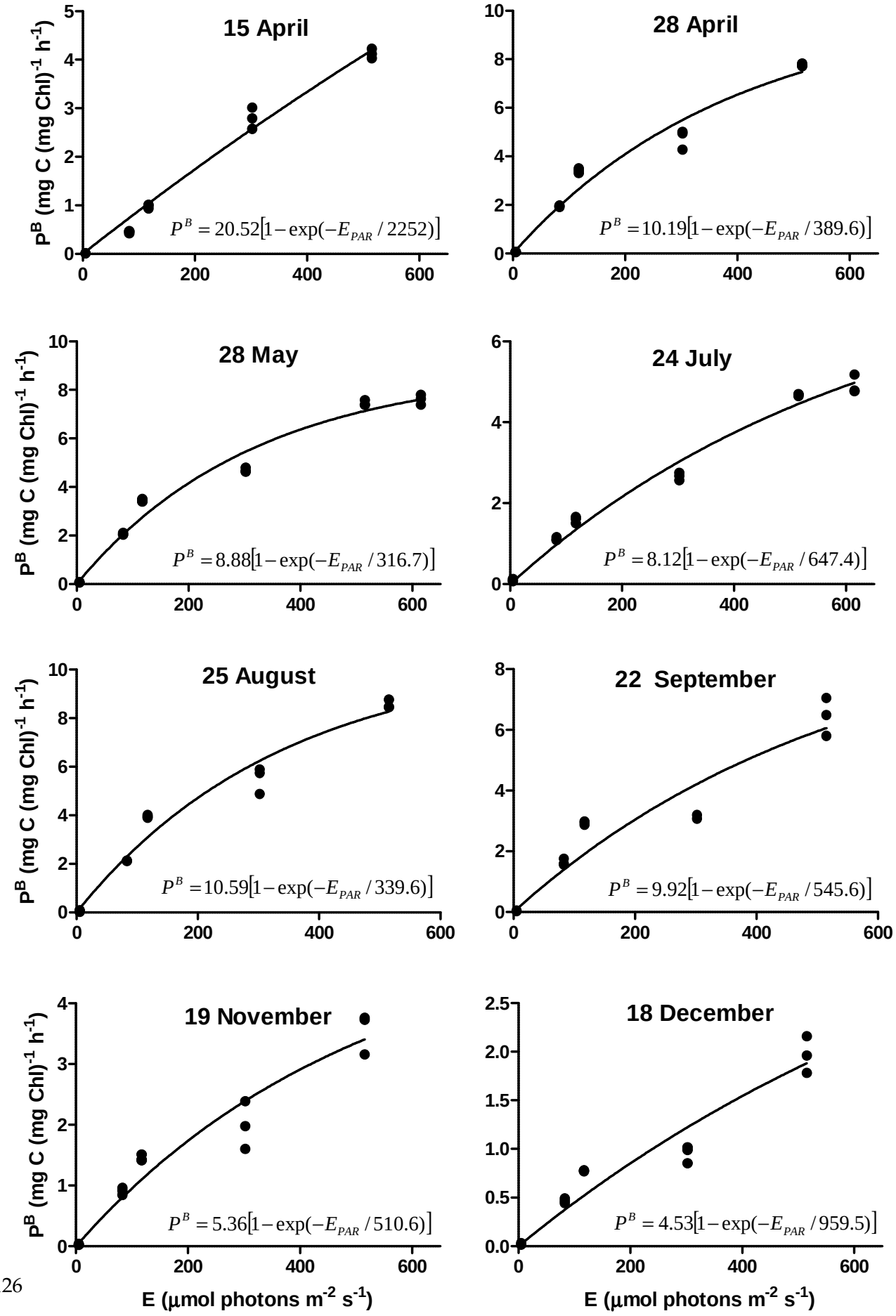

15 April
$P^B = 20.52[1-\exp(-E_{PAR}/2252)]$
28 April
$P^B = 10.19[1-\exp(-E_{PAR}/389.6)]$
28 May
$P^B = 8.88[1-\exp(-E_{PAR}/316.7)]$
24 July
$P^B = 8.12[1-\exp(-E_{PAR}/647.4)]$
25 August
$P^B = 10.59[1-\exp(-E_{PAR}/339.6)]$
22 September
$P^B = 9.92[1-\exp(-E_{PAR}/545.6)]$
19 November
$P^B = 5.36[1-\exp(-E_{PAR}/510.6)]$
18 December
$P^B = 4.53[1-\exp(-E_{PAR}/959.5)]$
P^B (mg C (mg Chl)^-1 h^-1)
E (µmol photons m^-2 s^-1)

Figure 6.2 (previous page) – Photosynthesis-irradiance (P-E) curves for ^{14}C incubations under PAR from water samples collected in Alcoutim throughout 2008. Nonlinear regressions were obtained by fitting values to equation 4. Equation represented is $P^B = P^B_S(1-\exp(-E_{PAR}/E_S))$.

No seasonal patterns of photosynthetic parameters variability were found. Significant relationships between photosynthetic parameters and water temperature, surface irradiance, mean light intensity in the mixed layer and light extinction coefficient were also not found. From 2008 through 2009, mean light intensity in the mixed layer (I_m) was 28.26 ± 16.67 µmol photons m^{-2} s^{-1} and varied between 0.99 and 63.03 µmol photons m^{-2} s^{-1}(Fig. 6.3). Light extinction coefficient (K_e) followed the same pattern of variability as I_m, with a mean value of 3.20 ± 1.37 and a ranging between 0.92 – 6.73 µmol photons m^{-2} s^{-1} (Fig. 6.3). No seasonal trends were observed for I_m and K_e throughout the sampling period.

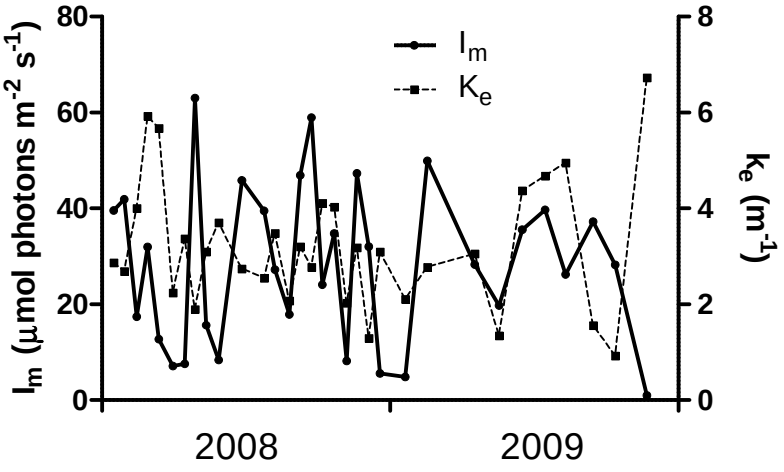


Figure 6.3 – Temporal variation of mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}) and light extinction coefficient (k_e, m^{-1}) in Alcoutim from 2008 through 2009.

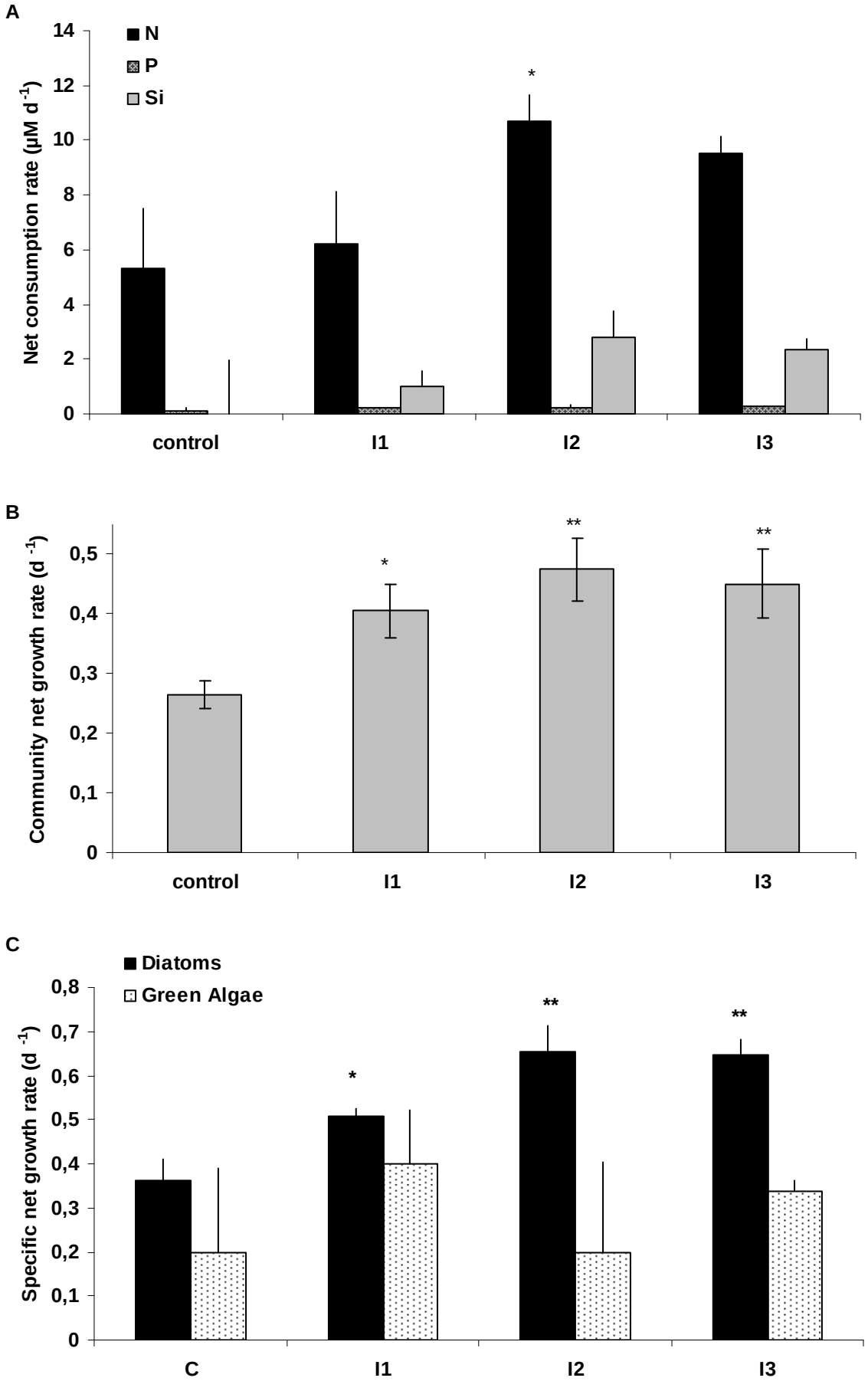
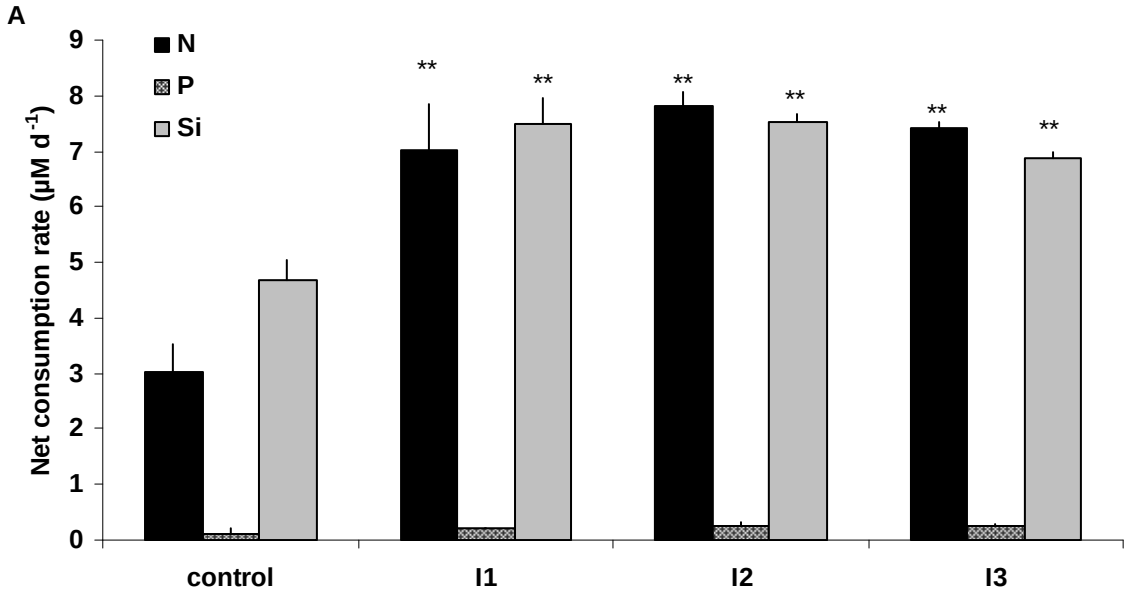

A
N
P
Si
Net consumption rate (µM d-1)
14
12
10
8
6
4
2
0
*
control
I1
I2
I3
B
Community net growth rate (d -1)
0,5
0,4
0,3
0,2
0,1
0
*
**
**
control
I1
I2
I3
C
Diatoms
Green Algae
Specific net growth rate (d -1)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
**
**
C
I1
I2
I3

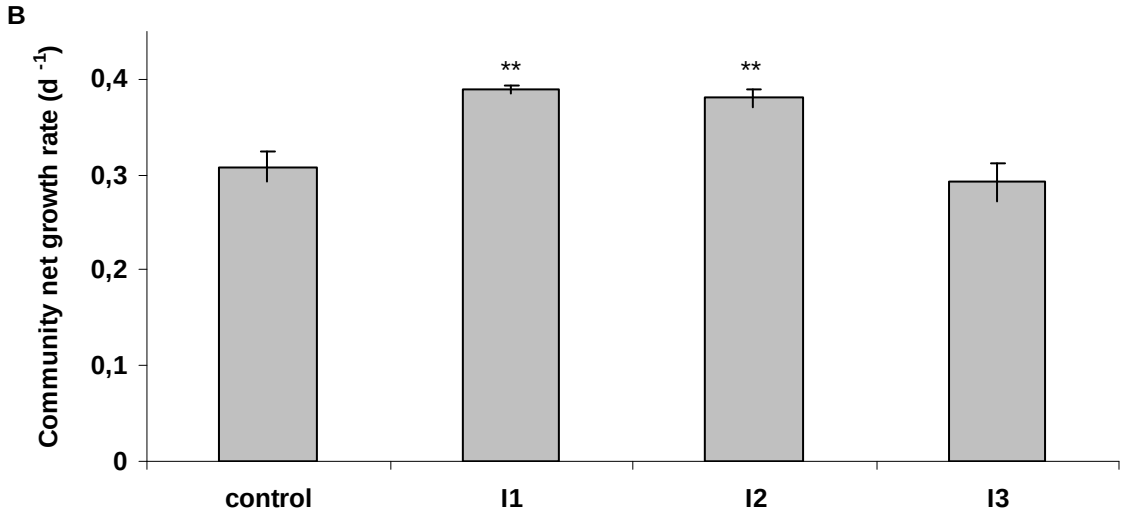
Figure 6.4 (previous page) - A) Nitrate (N), phosphate (P) and silicate (Si) net consumption rates (μM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms and green algae based on abundance during the light enrichment experiment carried out in winter 2009. Control, I_1, I_2 and I_3 correspond to PAR exposures of 50, 70, 120 and 225 μmol photons m^{-2} s^{-1}, respectively (see Table 6.I). Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

The most prominent responses to light enrichment were observed during the winter experiment. Although nutrient net consumption rates did not show, in general, significant differences in relation to the control (Fig. 6.4A), both the community net growth rate (0.40 - 0.47 d^{-1}, Fig. 6.4B) and diatoms net growth rate (0.51 - 0.65 d^{-1}, Fig. 6.4C) were significantly higher in relation to the control (0.26 d^{-1} and 0.36 d^{-1}, respectively).

In the spring experiment, both nitrate (7.0 - 7.8 μM d^{-1}) and silicate (6.9 - 7.5 μM d^{-1}) net consumption rates in all the enriched treatments were significantly higher than rates in the control (N – 3.0 μM d^{-1}; Si – 4.7 μM d^{-1}, Fig. 6.5A). Community net growth rate (Fig. 6.5B) was significantly higher in treatments I_1 (0.39 d^{-1}) and I_2 (0.38 d^{-1}), but treatment I_3 (0.29 d^{-1}) was not different from the control (0.31 d^{-1}). Diatoms net growth rate increased significantly in all light-enriched treatments (0.73 - 0.81 d^{-1}) in relation to the control (0.21 d^{-1}). Dinoflagellates also showed positive responses to light enrichment, mainly when exposed to the higher light intensity (Fig. 6.5C).

During the spring-summer transition experiment, nitrate (4.7 - 5.0 μM d^{-1}), phosphate (0.3 - 0.4 μM d^{-1}) and silicate (2.0 - 2.3 μM d^{-1}) net consumption rates were significantly higher in all the light-enriched treatments in relation to the control (N - 0.6 μM d^{-1}; P - 0.1 μM d^{-1}; Si - 0.7 μM d^{-1}; Fig. 6.6A). However, significant increases in the community net growth rate in relation to the control (0.09 d^{-1}) were only observed in treatment I_1 (0.21 d^{-1}) (Fig. 6.6B). In treatment I_3 (higher light), net growth rate decreased significantly (-0.1 d^{-1}). Considering specific phytoplankton groups (Fig. 6.6C), net growth rates in the light-enriched treatments of diatoms (0.2 - 0.4 d^{-1}) and dinoflagellates (0.3 - 0.4 d^{-1}) increased significantly in all the light-enriched treatments in relation to the control (diatoms: -0.2 d^{-1}; dinoflagellates: 0.7 d^{-1}).


A
N
P
Si
Net consumption rate (µM d-1)
9
8
7
6
5
4
3
2
1
0
**
**
**
**
**
**
control
I1
I2
I3


B
Community net growth rate (d -1)
0,4
0,3
0,2
0,1
0
**
**
control
I1
I2
I3

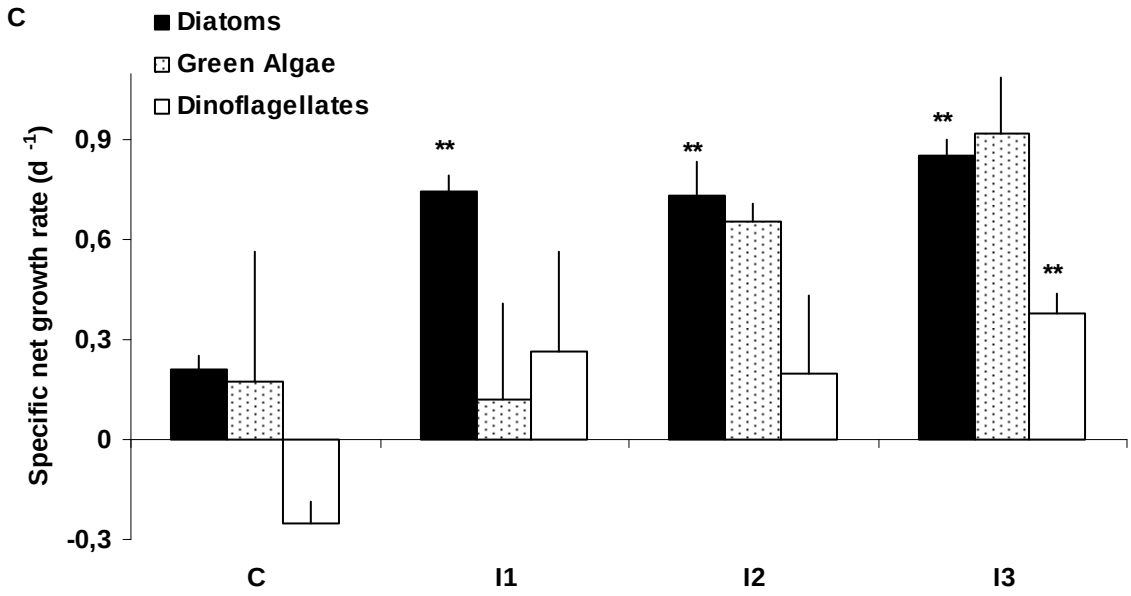

C
Diatoms
Green Algae
Dinoflagellates
Specific net growth rate (d -1)
0,9
0,6
0,3
0
-0,3
**
**
**
**
C
I1
I2
I3

Figure 6.5 (previous page) - A) Nitrate (N), phosphate (P) and silicate (Si) net consumption rates (μM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms, green algae and dinoflagellates based on abundance during the light enrichment experiment carried out in spring 2009. Control, I_1, I_2 and I_3 correspond to PAR exposures of 50, 70, 120 and 225 μmol photons m^{-2} s^{-1}, respectively (see Table 6.I). Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

Cyanobacteria growth rates displayed a high variability within replicates, so no trends can be deduced.

In the summer experiment, net consumption rates of nitrate (2.9 - 3.3 μM d^{-1}) and phosphate (1.1 - 1.2 μM d^{-1}) increased significantly in relation to the control (N – 1.1 μM d^{-1}; P – 0.8 μM d^{-1}) in all the treatments exposed to higher light intensities (Fig. 6.7A). Silicate consumption in treatment I_1 (20.2 μM d^{-1}) was also higher than in the control (25.1 μM d^{-1}). Community net growth rate (Fig. 6.7B) in treatment I_1 (0.4 d^{-1}) was significantly higher than in the control (0.3 d^{-1}), but growth rate in the treatment subjected to the highest light intensity (I_3), growth rate was lower (0.2 d^{-1}) than the control. Only cyanobacteria responded to light enrichment in the summer experiments, with higher net growth rates when subjected to a slightly higher light than I_m (treatment I_1, 0.3 d^{-1}; control 0.2 d^{-1}), but showing with negative growth rates under higher light intensities (treatments I_2 and I_3, -0.9 and -0.3 d^{-1}, respectively) (Fig. 6.7C).

During the autumn experiment, net consumption rates of nitrate (3.9 - 4.3 μM d^{-1}), phosphate (0.5 - 0.7 μM d^{-1}) and silicate (11.6 - 11.8 μM d^{-1}) were significantly higher in all the treatments exposed to higher light intensities than in the control (N – 1.6 μM d^{-1}; P – 0.3 μM d^{-1}; Si – 3.1 μM d^{-1}) (Fig. 6.8A). Community net growth rate also showed positive responses in relation to the control (0.3 d^{-1}) in treatments I_1 (0.5 d^{-1}) and I_2 (0.4 d^{-1}), but not in treatment I_3 (Fig. 6.8B). Considering specific phytoplankton groups (Fig. 6.8C), diatoms showed significantly higher net growth rates in all the light-enriched treatments (0.9 - 1.4 d^{-1}) in relation to the control (0.5 d^{-1}). Green algae net growth rates were also higher in all the treatments in relation to the control (-0.02 d^{-1}), although significantly higher growth rates were only found in treatment I_3 (0.6 d^{-1}).

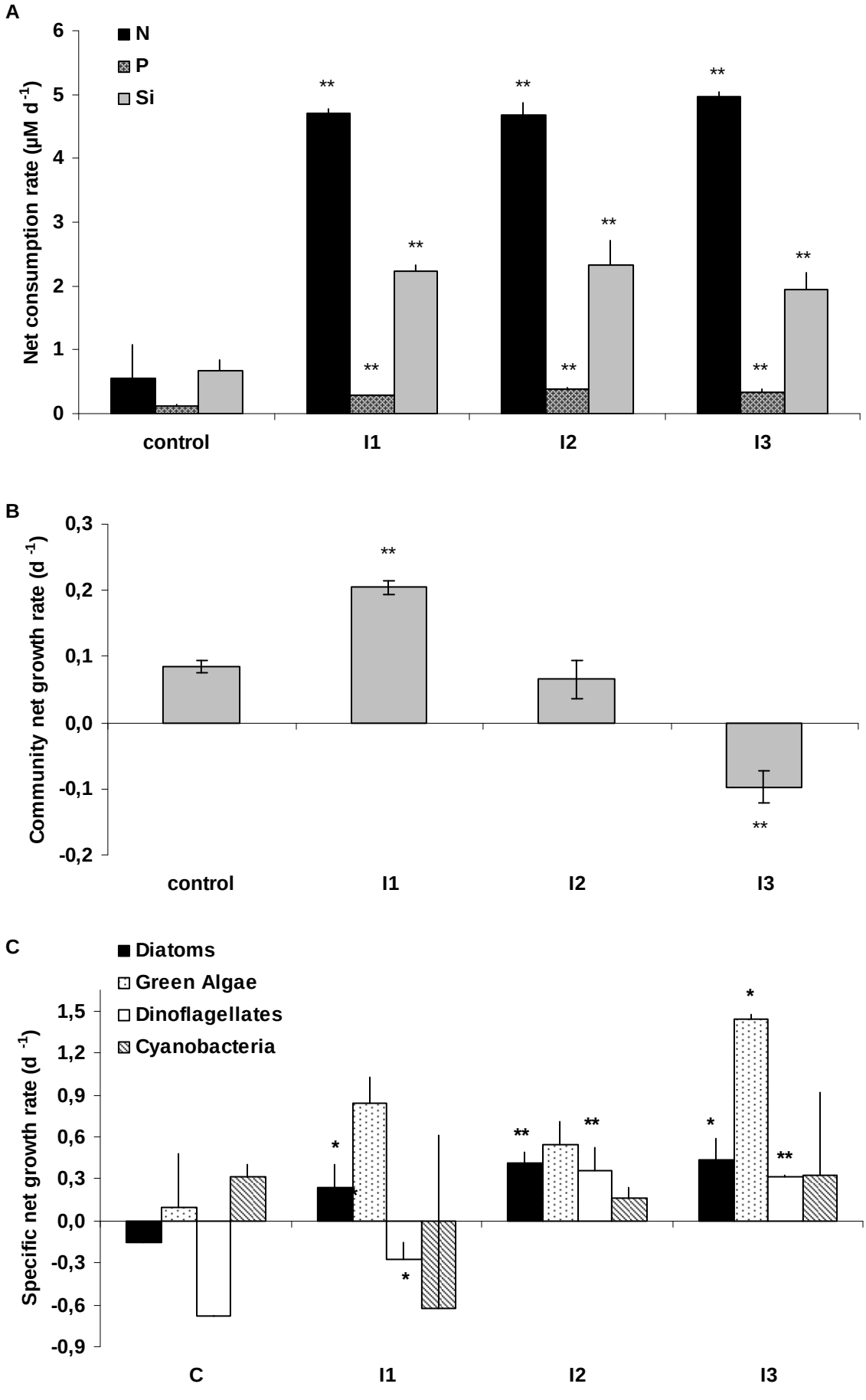

A
N
P
Si
Net consumption rate (µM d-1)
6
5
4
3
2
1
0
control
I1
I2
I3
B
Community net growth rate (d -1)
0,3
0,2
0,1
0,0
-0,1
-0,2
control
I1
I2
I3
C
Diatoms
Green Algae
Dinoflagellates
Cyanobacteria
Specific net growth rate (d -1)
1,5
1,2
0,9
0,6
0,3
0,0
-0,3
-0,6
-0,9
C
I1
I2
I3

Figure 6.6 (previous page) - A) Nitrate (N), phosphate (P) and silicate (Si) net consumption rates (µM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms, green algae, dinoflagellates and cyanobacteria based on abundance during the light enrichment experiment carried out in the spring-summer transition 2009. Control, I_1, I_2 and I_3 correspond to PAR exposures of 50, 70, 130 and 300 µmol photons m^{-2} s^{-1}, respectively (see Table 6.I). Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

6.4. Discussion

Phytoplankton production and net growth were clearly limited by light availability in the freshwater tidal zone of the Guadiana estuary. Phytoplankton growth was enhanced by PAR exposures ranging from 70 to 300 µmol photons m^{-2} s^{-1} and primary production was not photoinhibited at least up to 615 µmol photons m^{-2} s^{-1}.

Light limitation in nutrient-rich systems may either regulate the maximum attainable biomass in the system or stimulate physiological acclimation to low light conditions (Pennock and Sharp, 1986 and references therein). In most turbid systems, phytoplankton seems to be physiologically acclimated to low light, exhibiting a low light-saturated rate of biomass-specific primary production (P^B_S), low saturating irradiance (E_S) and high photosynthetic efficiency (α_S). However, this trend is not straightforward. P^B_S values are affected by nutrient concentration, temperature, cell size and light history (Falkowski, 1981 and references therein). Although minimum P^B_S values are characteristic of high latitudes and maximum P^B_S are typically found in tropical and subtropical waters (Finenko et al., 2002), a wide range of variability in photosynthetic parameters can be found in highly variable environments such as estuaries. In the Guadiana upper estuary, light-saturated rates of primary production were high (3.9 - 20.5 mg C (mg Chl)$^{-1}$ h^{-1}, mean 9.11 ± 4.97 mg C (mg Chl)$^{-1}$ h^{-1}) and comparable to rates in other turbid estuaries such as San Antonio Bay (3.0 - 22.9 mg C (mg Chl)$^{-1}$ h^{-1}: MacIntyre and Cullen, 1996) or the Neuse River estuary (0.14 – 33.9

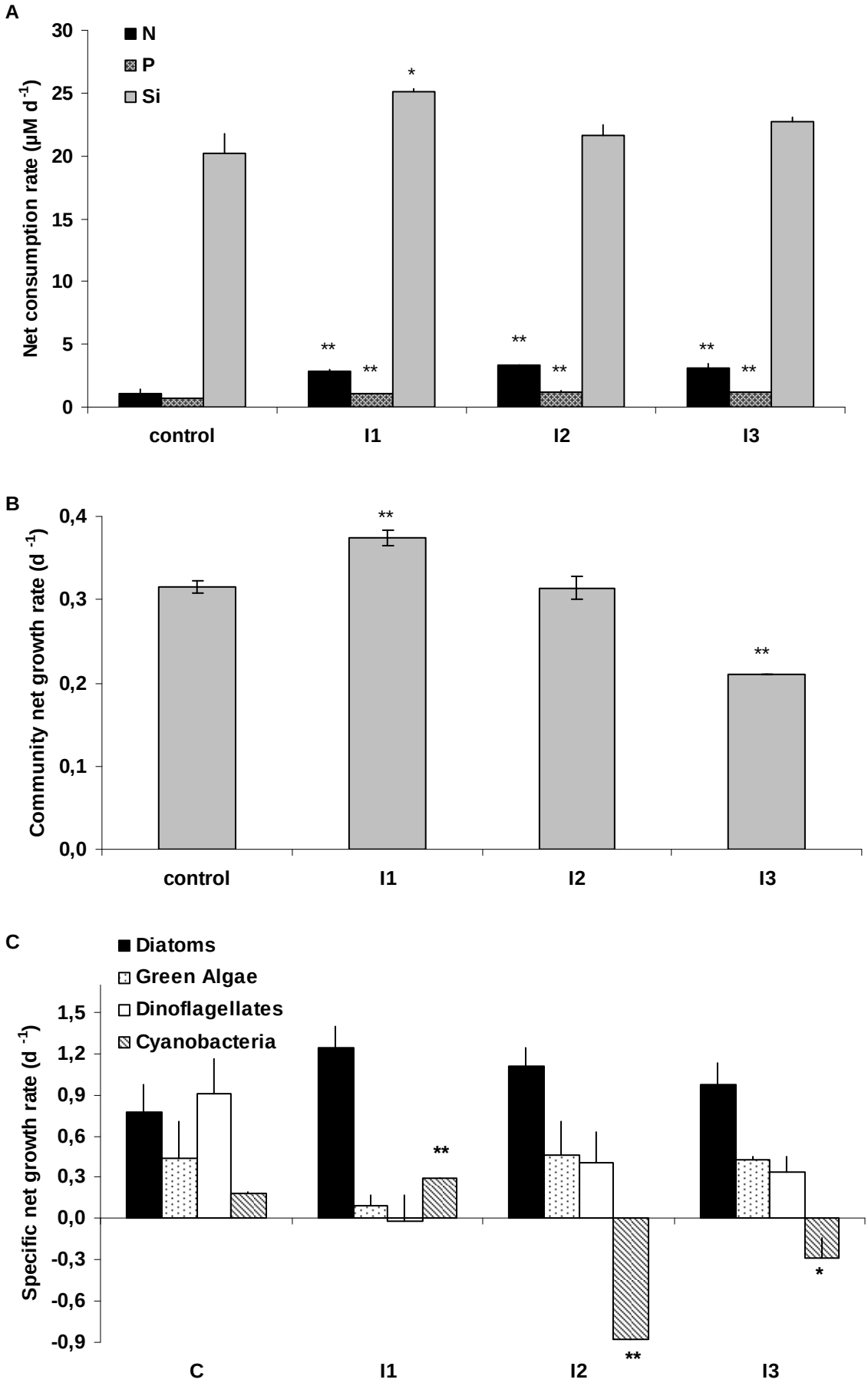

A
N
P
Si
Net consumption rate (µM d -1)
30
25
20
15
10
5
0
control
I1
I2
I3
B
Community net growth rate (d -1)
0,4
0,3
0,2
0,1
0,0
control
I1
I2
I3
C
Diatoms
Green Algae
Dinoflagellates
Cyanobacteria
Specific net growth rate (d -1)
1,5
1,2
0,9
0,6
0,3
0,0
-0,3
-0,6
-0,9
C
I1
I2
I3

Figure 6.7 (previous page) – A) Nitrate (N), phosphate (P) and silicate (Si) net consumption rates (μM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms, green algae, dinoflagellates and cyanobacteria based on abundance during the light enrichment experiment carried out in the summer 2009. Control, I_1, I_2 and I_3 correspond to PAR exposures of 50, 70, 130 and 300 μmol photons m^{-2} s^{-1}, respectively (see Table 6.I). Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

mg C (mg Chl)$^{-1}$ h^{-1}: Boyer et al., 1993), but also comparable to those found in clearer waters such as the Hudson estuary (4.0 - 22.0 mg C (mg Chl)$^{-1}$ h^{-1}: Malone and Neale, 1981) or the Gulf of Mexico (1.8 - 22.1 mg C (mg Chl)$^{-1}$ h^{-1}: Lohrenz et al., 1994).

On the other hand, P^B_S values in the Guadiana were higher than those estimated for other turbid systems, such as the Bay of Brest (1.61 - 8.88 mg C (mg Chl)$^{-1}$ h^{-1}: Claquin et al., 2010), the Black Sea (1 - 11 mg C (mg Chl)$^{-1}$ h^{-1}: Finenko et al., 2002), the Pas estuary (0.6 - 15.0 mg C (mg Chl)$^{-1}$ h^{-1}: Pérez and Canteras, 1993) or the Tagus estuary (1.0 - 8.4 mg C (mg Chl)$^{-1}$ h^{-1}: Gameiro, 2009). In these turbid environments, low P^B_S and E_S and high α_S values suggest that phytoplankton is acclimated to low light conditions. Furthermore, the occurrence of photoinhibition at low irradiances, as described for the Tagus estuary for PAR exposures of approx. 150 – 300 μmol photons m^{-2} s^{-1}, is another indicator of low light adapted phytoplankton cells (Gameiro, 2009). Nevertheless, photoinhibition of estuarine phytoplankton communities is seldom reported, given that the mixing depth usually exceeds the euphotic depth, and therefore cells spend considerable periods of time in the dark (Grobbelaar, 1995).

No relationships were found between photosynthetic parameters and the mean light intensity in the mixed layer. Mesotidal, semidiurnal tides and river runoff promote a continuous vertical mixing of the water column in the upper estuarine section, which is probably faster than phytoplankton photoacclimation rates. The same conclusions were drawn for the Delaware estuary, a turbid, nutrient-rich estuary regulated by light, where photoacclimation plays a minor role on the system's overall productivity (Pennock and Sharp, 1986). On the contrary, photoacclimation rates faster than mixing can be observed in other systems such as in the NE Mediterranean Sea

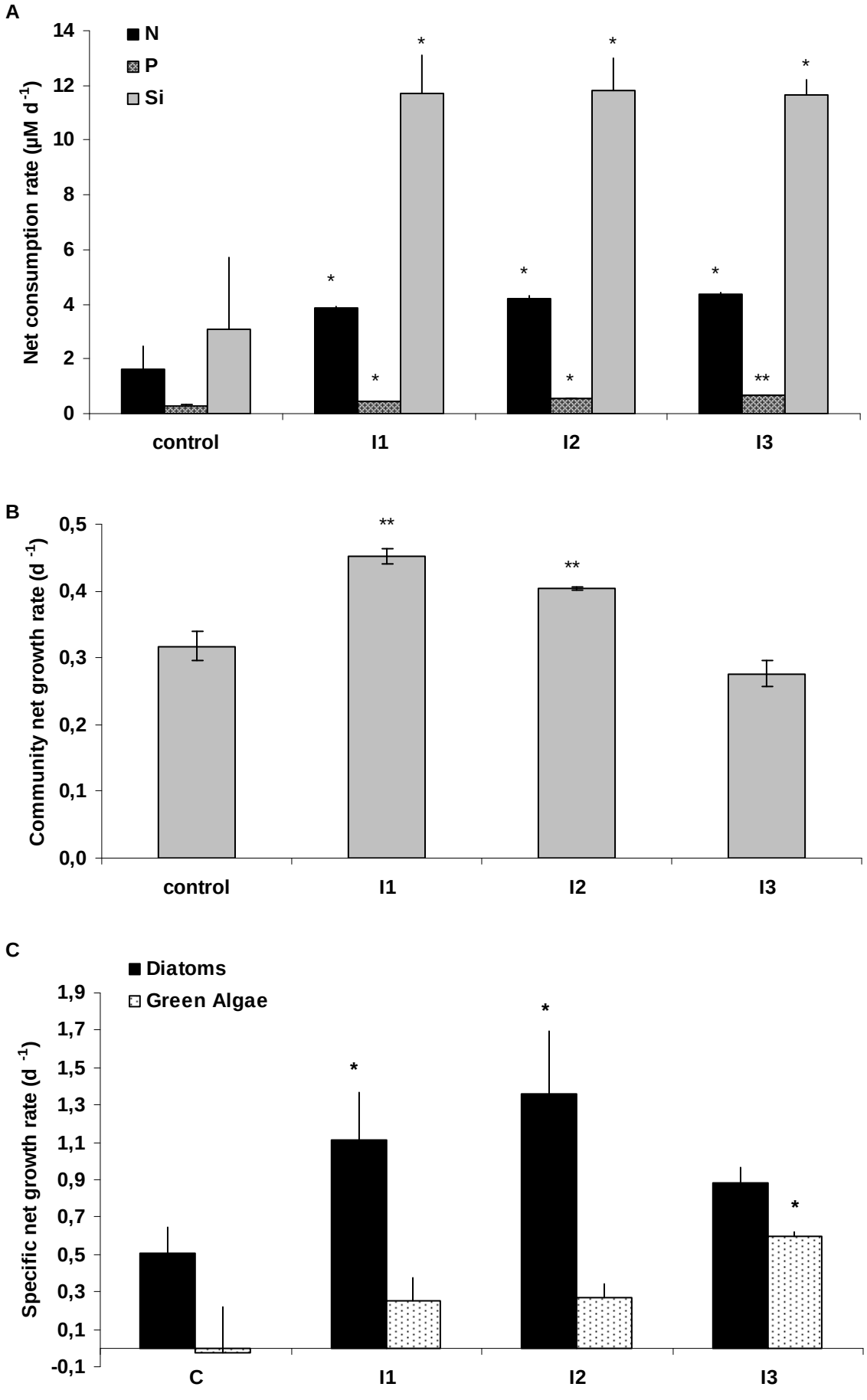

A
N
P
Si
Net consumption rate (µM d-1)
14
12
10
8
6
4
2
0
control
I1
I2
I3
B
Community net growth rate (d -1)
0,5
0,4
0,3
0,2
0,1
0,0
control
I1
I2
I3
C
Diatoms
Green Algae
Specific net growth rate (d -1)
1,9
1,7
1,5
1,3
1,1
0,9
0,7
0,5
0,3
0,1
-0,1
C
I1
I2
I3

Figure 6.8 (previous page) – A) Nitrate (N), phosphate (P) and silicate (Si) net consumption rates (μM d^{-1}), B) phytoplankton community net growth rates (d^{-1}) based on chlorophyll *a* concentrations, and C) specific net growth rates (d^{-1}) of diatoms and green algae based on abundance during the light enrichment experiment carried out in the autumn 2009. Control, I_1, I_2 and I_3 correspond to PAR exposures of 50, 90, 120 and 225 μmol photons m^{-2} s^{-1}, respectively (see Table 6.I). Vertical lines represent ± 1 S.D. Significant differences in the treatments in relation to the control are denoted by * ($p < 0.05$) or ** ($p < 0.01$) over the correspondent bar.

(Morán and Estrada, 1995). The effect of vertical mixing on photosynthesis is actually highly variable. Vertical mixing can enhance, reduce or have no effect on productivity (MacIntyre and Geider, 1996 and references therein). The long-term light enrichment experiments confirmed that phytoplankton is not acclimated to low-light conditions in the freshwater tidal reaches of the Guadiana estuary. Positive responses of phytoplankton community to light enrichment were obvious in all experiments, especially in the winter. In this experiment, initial phytoplankton community was undoubtedly light-limited, and light enrichment resulted in significant increases in chlorophyll *a* and diatom abundance, under all PAR exposures (90 - 225 μmol photons m^{-2} s^{-1}). Light usually regulates phytoplankton growth during the winter in many other estuarine and coastal systems (e.g., Pennock and Sharp, 1994; Maldonado et al., 1999; Ogilvie et al., 2003). Low phytoplankton biomass and abundance in the Guadiana estuary during this period (Domingues and Galvão, 2007; Domingues et al., 2007) can thus be attributed to light limitation, given that nutrient concentrations are usually not limiting (see Chapter 4).

In the other experiments, different responses were observed under different PAR intensities. Exposure to 70 - 90 μmol photons m^{-2} s^{-1} (I_1) promoted significant increases in biomass in all experiments, accompanied by significant increases in net growth rates of specific phytoplankton groups, usually diatoms. However, different responses in the community net growth rates (chlorophyll *a* concentration) and in net growth rates of specific phytoplankton groups (abundance) were observed under exposure to 120 - 130 μmol photons m^{-2} s^{-1} (I_2) and 225 - 300 μmol photons m^{-2} s^{-1} (I_3). Indeed, a decoupling between Chl*a* concentration and phytoplankton abundance was observed, with decreasing Chl*a* and increasing abundance of specific groups with increasing PAR intensities. Photoinhibition was not observed during the ^{14}C incubations, even for higher light intensities (up to 600 μmol photons m^{-2} s^{-1}), and

phytoplankton abundance and nutrient consumption under I_2 and I_3 were significantly higher in most of the experiments. Therefore, the decrease in Chl*a* concentration under higher light intensities may be attributed to a dilution of chlorophyll content by enhanced cell division or carbon production (Post et al., 1984). Indeed, chlorophyll *a* concentration depends on the physiological state of the cell, and it usually decreases with increasing light and nutrient stress (e.g., Zonneveld, 1998; Kruskopf and Flynn, 2005). These results clearly show that the use of chlorophyll *a* concentration as a proxy for phytoplankton biomass is not straightforward and may not reflect the variability of phytoplankton communities in natural systems (see Domingues et al., 2008, Chapter 2), especially in low-light environments (Buchanan et al., 2005). Furthermore, the highest PAR intensities used for the short-term (615 µmol photons m^{-2} s^{-1}) and the long-term (300 µmol photons m^{-2} s^{-1}) experiments are within the range of saturating light intensities described for estuarine phytoplankton, from 100 to 800 µmol photons m^{-2} s^{-1} (e.g., Fisher et al., 1982; Pennock and Sharp, 1986; Madariaga, 1995; Tillmann et al., 2000; Macedo et al., 2001; Kocum et al., 2002; Oviatt et al., 2002). Therefore, the occurrence of photoinhibition in the freshwater tidal reaches of the Guadiana estuary is unlikely.

Considering specific phytoplankton groups, diatoms showed the most prominent responses to light enrichment throughout the year. Except in the summer experiment, diatom net growth rates increased significantly in relation to the control under PAR exposures ranging between 70 and 300 µmol photons m^{-2} s^{-1}, simultaneously with significant increases in nutrient consumption, mainly Si. These results clearly show that diatom growth was light-limited. In the summer, no positive response was observed on net growth rates or on nutrient net consumption rates, most likely due to a strong nutrient limitation. Indeed, diatom growth limitation by nitrogen is evident in the Guadiana upper estuary especially in the spring and summer, when nitrate concentrations are lower than 20 µM (see Chapter 4). Furthermore, mean light intensity in the mixed layer did not show the characteristic seasonality of temperate latitudes, with higher light availability in the summer and lower in the winter, most likely due to the river flow regulation imposed by the Alqueva dam that results in a more constant river flow throughout the year. I_m in Alcoutim varied between 0.99 – 63.03 µmol photons m^{-2} s^{-1}, which is

lower than typical saturating irradiances described for diatoms (~30 to ~300 µmol photons m^{-2} s^{-1}: e.g., Blanchemain and Grizeau, 1996; Popovich and Gayoso, 1999; Fietz and Nicklisch, 2002).

Contrary to the other phytoplankton groups, picocyanobacteria growth rates increased only under exposure to 70 µmol photons m^{-2} s^{-1} (I_1), whilst higher PAR intensities (130 and 300 µmol photons m^{-2} s^{-1}) promoted a significant decrease in growth rates, suggesting photoinhibition. Indeed, cyanobacteria usually display low saturating irradiances, between 20 and 100 µmol photons m^{-2} s^{-1} (Andersson et al., 1994; Phlips and Badylak, 1996; Timmermans et al., 2005) and photoinhibition of picocyanobacteria has been observed for PAR irradiances between 60 and 300 µmol photons m^{-2} s^{-1} (Platt et al., 1983; Phlips and Badylak, 1996). These results suggest that cyanobacteria were the only group well acclimated to low light conditions in the Guadiana estuary.

Although not statistically significant, due to high variability within replicates, net growth rates of green algae increased with increasing irradiance, and the highest PAR exposures in the spring-summer transition (300 µmol photons m^{-2} s^{-1}) and autumn (225 µmol photons m^{-2} s^{-1}) experiments promoted significant increases on green algae net growth rates. Indeed, green algae are described as "sun" species (Raven and Richardson, 1986), but saturating irradiances vary greatly (e.g., ~60 - 400 µmol photons m^{-2} s^{-1} for *Scenedesmus* spp.: Senger and Fleishhacker, 1978; Flameling and Kromkamp, 1997). Dinoflagellates showed similar responses to those of green algae, although they were only observed during the productive period (from spring through summer). Only the highest PAR exposures (120-300 µmol photons m^{-2} s^{-1}) promoted significant positive responses of dinoflagellates. In the summer, however, nutrient limitation was probably too strong (see Chapter 4) and no changes in growth rates were observed under higher PAR exposures. Dinoflagellates are usually described as "shade" species (Raven and Richardson, 1986) and saturating irradiances for several dinoflagellate species grown in cultures vary between 70 and 114 µmol photons m^{-2} s^{-1} (Yamaguchi et al., 1997; Kim et al., 2004; Nagasoe et al., 2006; Matsubara et al., 2007). Our results, however, indicate that saturating irradiances of dinoflagellates in the freshwater tidal reaches of the Guadiana estuary

are most likely higher. The most abundant species in the Guadiana estuary, *Kryptoperidinium foliaceum*, is usually grown in cultures at 90 µmol photons m^{-2} s^{-1} (Figueroa et al., 2009; Domingues, unpublished data), but algae from a natural environment, not adapted to constant light conditions, will most likely exhibit a wide range of photosynthetic parameters. It is likely that saturating irradiances will vary intra- and inter-specifically, temporally and spatially. Additionally, the range of saturating intensities described for estuarine phytoplankton communities (100 - 800 µmol photons m^{-2} s^{-1}, see above) are higher than those described for algal species grown under controlled laboratorial conditions. This reflects that natural communities living in such unstable environments as turbid estuaries, rather than being adapted to low light conditions, are able to grow under a wide range of conditions and respond positively to variable light and nutrient conditions.

In conclusion, phytoplankton growth in the freshwater tidal reaches of the Guadiana estuary was light-limited throughout the year. In the summer, co-limitation by nitrogen masked the response to light enrichment. High rates of light-saturated primary production, high saturating irradiances and low photosynthetic efficiencies suggest that phytoplankton is not acclimated to low light conditions.

Chapter 7

Environmental drivers of phytoplankton in a turbid estuary: nutrient versus light limitation

Environmental drivers of phytoplankton in a turbid estuary: nutrient versus light limitation

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(submitted)

Abstract

Nutrients and light are typically considered the most important drivers of phytoplankton growth in estuaries. Given that phytoplankton plays a critical role in estuarine ecosystems, a comprehensive understanding of how phytoplankton is regulated is needed to properly assess the impacts of eutrophication and other natural or human-induced perturbations. The main goal of this work is to understand the relative importance of light and nutrients on phytoplankton succession and production in the Guadiana estuary, a sensitive and relatively pristine estuary, where anthropogenic pressures have been increasing in the last years. Sampling campaigns were conducted fortnightly during 2007 and 2008 in four locations covering the upper, middle and lower estuarine regions. Several abiotic and biotic variables were determined, including light availability, nutrient concentration and chlorophyll *a* concentration. Phytoplankton composition, abundance and biomass (biovolume) were determined using both epifluorescence and inverted microscopy.

Throughout 2007 and 2008, river flow controlled nitrate inputs and suspended particulate matter into the estuarine zone. Nitrogen was limiting to phytoplankton growth during 2008, with nitrate concentrations mostly <20 μM; in addition, phytoplankton abundance and biomass were significantly lower in 2008, although the same seasonal pattern was observed. The typical phytoplankton succession of temperate freshwater systems was observed in the upper and middle estuaries, with a diatom bloom in late spring/early summer, followed by a green algae bloom and finally a cyanobacteria summer bloom. Diatoms were the main component of phytoplankton biomass, whilst cyanobacteria dominated the community in terms of abundance. Light limitation probably occurred throughout the sampling period, but phytoplankton from the more turbid zones did not seem to be adapted to low light conditions. Primary production was in fact higher in the turbid regions, suggesting that phytoplankton growth was not regulated only by light, as described for other turbid estuaries; instead, nutrient availability probably played an equally important role in phytoplankton regulation in this turbid estuarine system.

Keywords: phytoplankton, nutrients, light, regulation, primary production, Guadiana estuary

7.1. Introduction

Estuaries have long been recognized as areas of high potential primary production, due to a significant riverine supply of nutrients, but the combination of other factors such as high turbidity and rapid flushing times may limit phytoplankton growth and therefore prevent the achievement of this potential (Joint and Pomroy, 1981). Nutrients and light are usually the most important bottom-up factors regulating phytoplankton growth, and their effects on estuarine phytoplankton dynamics have been addressed for a long time (e.g., Fisher et al., 1992; Mallin and Paerl, 1992). Nutrient availability has frequently outweighed all other factors as the main limiting factor of phytoplankton growth (Roelke et al., 1999 and references therein). Whilst in marine and freshwater systems nitrogen (N) and phosphorus (P), respectively, are widely accepted as the limiting nutrients, there is evidence of spatial and temporal variability of the limiting nutrient in estuaries, from P limitation in the winter, to silicon (Si) limitation of diatoms in the spring and N limitation in the summer (Fisher et al., 1999). These deeply-rooted dogmas have been questioned (Sterner, 2008), and in reality, the limiting nutrient may be species- or group-specific, given that the nutritional requirements of phytoplankton vary intra- and inter-specifically (Carpenter and Guillard, 1971).

Light availability has not yet received the same attention as nutrients as an environmental driver of phytoplankton, but in turbid ecosystems, light is of paramount importance (Cole and Cloern, 1984; Kromkamp and Peene, 1995; Kocum et al., 2002) and it may affect nutrient uptake (Litchman et al., 2004). Light availability in estuaries is regulated by turbidity, which in turn is mostly a consequence of suspended particulate matter. In addition, phytoplankton in turbid and nutrient-rich estuaries is more controlled by variations in SPM rather than the seasonal irradiance cycle (Adolf et al., 2006).

Both nutrient and light availability are highly variable within estuarine systems on temporal and spatial scales. A general seasonal pattern of phytoplankton growth limitation is limitation by light in the winter and by nitrogen in the summer. Spatial patterns may also occur, with P-limitation at the freshwater end and N-limitation at

the seaward end (Kocum et al., 2002 and references therein). The estuarine turbidity maximum zone may also be permanently light-limited (Irigoien and Castel, 1997).

Estuaries are valuable environments, both ecologically and economically (Underwood and Kromkamp, 1999), which have been increasingly subjected to eutrophication. The understanding of ecosystem functioning is thus imperative to predict, mitigate and/or prevent the adverse effects caused by anthropogenic pressures. Given that phytoplankton is a critical player in any aquatic ecosystem, due to its ecological function of primary production, a comprehensive understanding of how phytoplankton is regulated is needed to properly assess the impacts of eutrophication and other natural or human-induced perturbations.

The Guadiana estuary is located in a highly vulnerable region to climate change (IPCC, 2001) and it has been increasingly subjected to human disturbances, namely urban development. In addition, the large Alqueva dam restricts a significant amount of freshwater, promoting significant impacts on the estuarine ecosystem downriver. Phytoplankton succession in the Guadiana estuary, especially in the freshwater tidal zone, has been classically considered nutrient-regulated (Rocha et al., 2002), but the low light availability probably plays an important role on phytoplankton growth (Domingues et al., 2005). Recently, a trend of decreasing turbidity and decreasing chlorophyll has been observed for the Guadiana estuary, suggesting a shift from a potentially light-limited environment to a more nutrient-limited one (Barbosa, et al., 2010). The main goal of this study is, therefore, to understand the relative importance of light and nutrients on phytoplankton succession and production in the Guadiana estuary. To accomplish this goal, sampling campaigns along the estuarine salinity gradient were carried out, and several environmental drivers of phytoplankton were analysed, as well as phytoplankton composition, abundance and biomass. Additionally, we present the first estimates of primary production for this estuarine system.

7.2. Materials and Methods

7.2.1 Study site and sampling strategy

The Guadiana River arises in Spain, flows for 810 km and drains between SE Portugal and SW Spain (Fig. 7.1). Its last 70 km correspond to the estuarine zone, located in a Mediterranean climate area. The estuary is influenced by semidiurnal, mesotidal tides, and is usually partially stratified in its lower and middle sections, depending on river flow and tidal stage, and well mixed in the upper section. In the last years, intense damming has promoted a more regular but reduced river flow (2007-9: 22.2 ± 18.6 m^3s^{-1}), contrasting with sharp variations between dry and humid months (1995-2000: 333.0 ± 1095.9 m^3s^{-1}, http://snirh.pt) that used to occur before the Alqueva dam construction, 140 km from the river's mouth.

Sampling campaigns were performed fortnightly during 2007 and 2008 in four representative sampling stations in the Guadiana estuary: Mértola (70 km from river mouth) and Alcoutim (38 km) in the upper estuary; Foz de Odeleite (22 km, hereafter Odeleite) in the middle estuary; and Vila Real de Santo António (2 km from mouth, hereafter VRSA) in the lower estuary (Fig. 7.1).

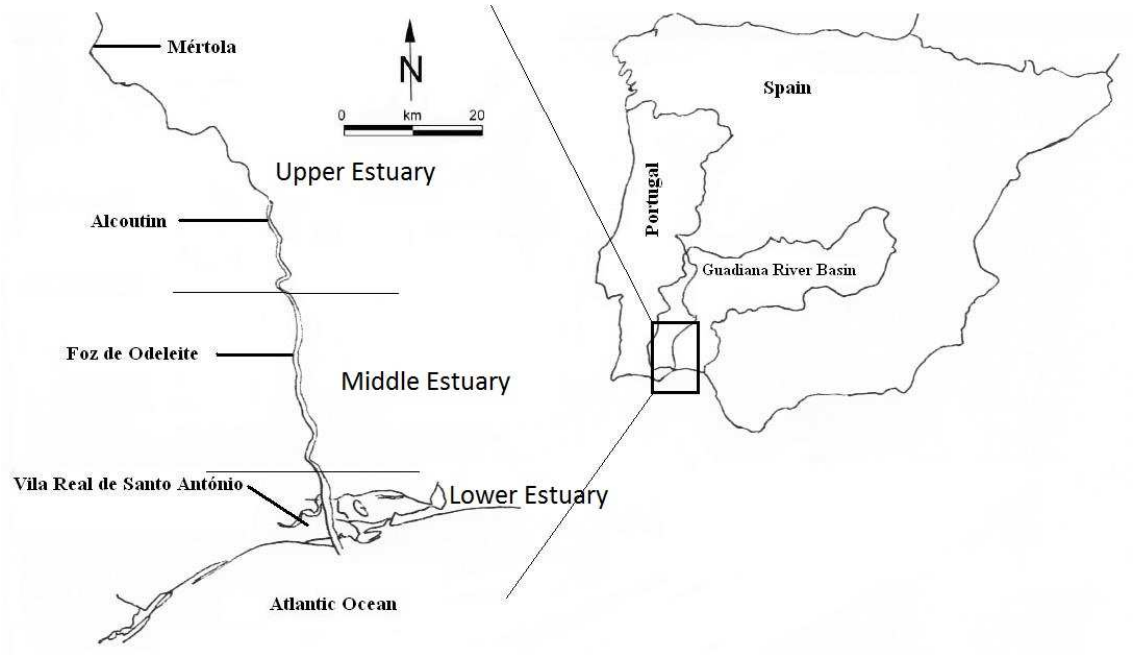

Figure 7.1 – Map of the Guadiana estuary and location of the sampling stations.

7.2.2 Physical-chemical variables

Data on daily river flow at Pulo do Lobo (85 km from river mouth), daily rainfall at Alcoutim and hourly solar radiation at São Brás de Alportel (50 km eastwards from Alcoutim) were obtained from the Portuguese National Water Institute public database (http://snirh.pt). Vertical profiles of water temperature and salinity were determined in situ using a YSI 556 MPS probe. Vertical profiles of photosynthetically active radiation (PAR) intensity were determined using a LI-COR radiometer and light extinction coefficient (k_e, m^{-1}) was calculated using an exponential function (eq. 7.1):

$$I_Z = I_0 e^{-K_e Z} \qquad \text{(eq. 7.1)}$$

where I_z is the light intensity at depth level Z (m) and I_0 is the light intensity at the surface. Hourly solar irradiance (W m^{-2}) was used to estimate the mean daily photosynthetically active radiation (PAR) at the surface (I_0), considering that PAR constitutes 45% of the total radiation reaching the water surface and a 4% reflection at the surface (Baker and Frouin, 1987). I_0 values were converted to µmol photons m^{-2}

s^{-1} multiplying by 4.587 mmol photons s^{-1} W^{-1} (Morel and Smith, 1974). Mean light intensity in the mixed layer for each sampling date (I_m, µmol photons m^{-2} s^{-1}) was subsequently calculated according to:

$$I_m = I_0(1-e^{(-K_eZ)})(K_eZ_m)^{-1} \qquad \text{(eq. 7.2)}$$

where Z_m (m) the depth of the mixed layer (Jumars, 1993). The mixed layer at stations Mértola, Alcoutim and Odeleite corresponded to the whole water column, since neither haline nor thermal stratification was observed. The mixing layer at VRSA was determined as the surface layer where salinity variations were <0.5 PSU. Euphotic zone depth (Z_{eu}, m) was calculated as $4.61/k_e$, assuming that irradiance at the bottom was 1% of surface irradiance. The ratio mixing depth:euphotic depth (Z_{mix}:Z_{eu}) was calculated as proposed by Cloern (1987). It is generally considered that when Z_{mix}:Z_{eu} values are higher than 5, i.e., the mixing depth is five times deeper than the euphotic depth, the development of phytoplankton blooms will be prevented, given that the cells will remain long periods below the euphotic zone. Light penetration in the water column was also measured with a Secchi disc (D_S, m) and light extinction coefficient was calculated as $k_e = C/Ds$, where C is a constant (C = 1.4 for euphotic depths ≥5 m: Holmes, 1970; C = 1.7 for euphotic depths <5 m: Poole and Atkins, 1929). An empirical relationship between Secchi disk measurements and light extinction coefficient (estimated using light data measured with the radiometer) was estimated using nonlinear regression.

Sub-superficial (approx. 0.5 m) water samples for determination of dissolved inorganic macronutrients were collected and immediately filtered through cellulose acetate filters (Whatman, nominal pore diameter = 0.2 µm) to acid-cleaned vials. Ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_4^{4-}) were determined immediately upon arrival to the lab, whilst samples for nitrate (NO_3^-) and nitrite (NO_2^-) where frozen (-20ºC) 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, phosphate and silicate, and an autoanalyzer Skalar for nitrate and nitrite.

Concentration of suspended particulate matter (SPM) was determined gravimetrically. For each sample, the analysis was made in duplicate. 250 mL were filtered onto pre-combusted (4 hours at 450ºC) glass fibre filters (Whatman GF/F, nominal pore diameter = 0.7 μm), dried at 50ºC for 24 hours and then weighed after cooling down to room temperature. Afterwards, the filters were combusted again to determine the concentration of particulate organic matter (POM).

7.2.3 Phytoplankton composition, abundance and biomass

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

Phytoplankton composition, abundance and biomass were determined using epifluorescence (Haas, 1982) and inverted microscopy (Utermöhl, 1958). Samples for enumeration of pico- (<2 μm) and nanophytoplankton (2 - 20 μm) were preserved with glutardialdehyde (final concentration 2%) immediately after collection, stained with proflavine and filtered (1 - 5 mL, depending on the amount of suspended matter) onto black polycarbonate membrane filters (Whatman, nominal 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 (-20ºC) in dark conditions, to minimize loss of autofluorescence. Enumeration was made at 787.5x magnification using an epifluorescence microscope (Leica DM LB). Samples for enumeration of microphytoplankton (>20 μm) were preserved with acid Lugol's solution (final concentration approx. 0.003%) immediately after collection, settled in sedimentation chambers (2-10 mL, depending on the amount of suspended matter; sedimentation time = 24 hours) and observed at 400x magnification with an inverted microscope (Zeiss Axiovert S100). Phytoplankton cells were identified, whenever possible, to species level. 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).

7.2.4 Primary production

The Steeman-Nielsen method (1952) was used to determine phytoplankton primary production in water samples collected throughout 2008. 50 mL aliquots were added to polycarbonate flasks and 100 µL (2 µCi) of ^{14}C-HCO_3^- were added to each flask. The sample flasks were incubated in triplicate under different light intensities (approx. 5, 83, 117, 302, 515 and 615 µmol photons m^{-2} s^{-1}) for 2 hours. Three dark flasks were also incubated and processed as the sample flasks. Incorporation of ^{14}C was stopped with 1 mL formaldehyde, and the samples were filtered onto nitrate cellulose filters (nominal pore diameter = 0.45 µm), which were placed inside 20 mL high-density polyethylene scintillation vials. The vials were subsequently placed inside a fume hood with HCl, to allow the release of inorganic carbon attached to the cells. 10 ml of scintillation liquid (Universol) were added to each vial, put in 4ºC overnight, and ^{14}C activity was subsequently measured on a scintillation counter (Beckman). Primary production was calculated as:

$$PP = \frac{(Rs - Rb)xDxWxCA}{RxN} \qquad \text{(eq. 7.3)}$$

where PP is phytoplankton primary production (mg C L^{-1} h^{-1}), Rs (dpm) is the activity in the sample, Rb (dpm) is the mean activity of the dark flasks, D (=1.05) is the isotopic discrimination, W (mg C L^{-1}) is the amount of dissolved inorganic carbon in the sample (obtained through alkalinity), CA is a correction factor (total sample volume/filtered volume), R (dpm) is the total activity of the ^{14}C added to each flask, and N (hours) is the incubation time. Alkalinity was determined by titration with HCl for non-freshwater samples (S > 1 PSU) (Parsons et al., 1984). A stepwise titration (Gran, 1950, 1952; Andersen, 2002) was used to determine alkalinity in freshwater samples (S < 1 PSU). Carbonate alkalinity was then converted to dissolved inorganic carbon and subsequently used in primary production determinations.

Primary production (PP, mg C L^{1-} h^{-1}) was converted to biomass-specific primary production (P^B = PP/Chl*a*, mg C (mg Chla)$^{-1}$ h^{-1}). The photosynthetic parameters were estimated using nonlinear regression fitting of equation 7.4 (Platt et al., 1980):

$$P^B = P^B{}_S(1-\exp(-\alpha.E_{PAR}/P^B{}_S)) \qquad \text{(eq. 7.4)}$$

where $P^B{}_S$ is the light-saturated rate of biomass-specific primary production (mg C (mg Chla)$^{-1}$ h^{-1}), α is the initial slope of the photosynthesis-irradiance curve (mg C (mg Chla)$^{-1}$ h^{-1} (µmol photons m^{-2} s^{-1})$^{-1}$) and E_{PAR} is the PAR irradiance during incubation (µmol photons m^{-2} s^{-1}). The saturating irradiance (E_S, µmol photons m^{-2} s^{-1}) was determined as $P^B{}_S$/ α.

Daily areal primary production (mg C m^{-2} d^{-1}) in the euphotic zone was also calculated for each sampling day using 0.1 m compartments. Volumetric primary production (mg C m^{-3} d^{-1}) was obtained dividing daily areal primary production by the euphotic depth. Volumetric production was then divided by chlorophyll *a* concentration to obtain the production to biomass ratio (P/B ratio).

7.2.5 Data analyses

Horizontal profiles abiotic and biotic variables were created using Surfer 8.01 software, using kriging (linear variogram model) as the gridding method. The occurrence of spatial differences was determined using analyses of variance (ANOVA) for normally distributed data and a Kruskall-Wallis ANOVA on ranks for other data. Normality of the data was assessed with a Kolmogorov-Smirnov test. Temporal variability was assessed using an unpaired t-test or a Mann-Whitney rank sum test, depending on the normality of the data. The strength of associations between variables was measured with Pearson's or Spearman's correlation, depending on the normality of the data.

ANOSIM, a multivariate technique was applied to examine the existence of significant inter-annual, seasonal and spatial patterns of environmental variables (I_m, K_e, Z_m:Z_{eu}, SPM, NO_3^-, PO_4^{3-}, SiO_4^{4-}), using the software Primer 5.2.1 (Primer-E Ltd.). The factors considered were: "Year" (2007, 2008, 2009); "Season" (spring = March,

April, May; summer = June, July, August, September; autumn = October, November; winter = January, February, December); and "Station" (Mértola, Alcoutim, Odeleite, VRSA). The similarity matrix was created after log(x+1) transformation of data and setting Euclidean distance as the similarity measure.

7.3. Results

7.3.1 Physical-chemical environment

Daily freshwater flow at Pulo do Lobo hydrometric station varied between 1.8 m^3 s^{-1} (July 2008) and 125.1 m^3 s^{-1} (February 2008) from 2007 through 2008, with a mean value of 25.2 ± 16.2 m^3 s^{-1} (Fig. 7.2). Freshwater flow during the sampling period showed significant interannual variability, with higher values in 2007 (35.6 ± 14.5 m^3 s^{-1}) and lower in 2008 (14.2 ± 9.1 m^3 s^{-1}). Overall, minimum values were measured in the summer months and maximum values during winter. Daily rainfall in Alcoutim from 2007 through 2008 varied between 0.0 and 57.20 mm, with a mean value of 1.19 ± 4.68 mm (Fig. 7.2). No interannual differences were found between 2007 and 2008. However, river flow and rainfall were positively correlated in 2007 (r^2 = 0.112, $p < 0.05$, n = 365), but not in 2008.

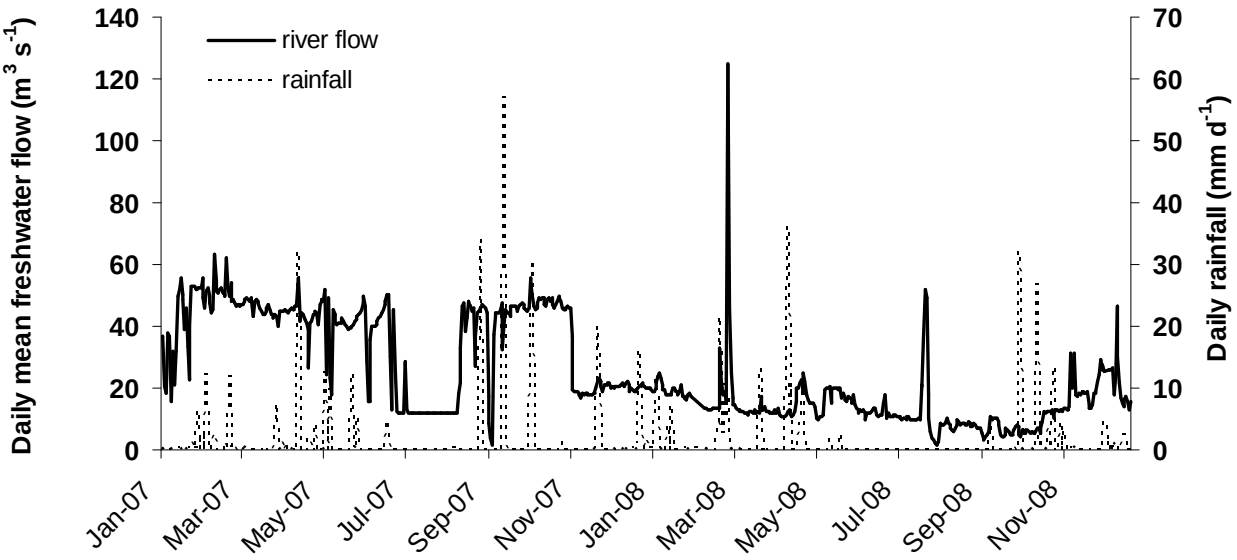

Figure 7.2 – Daily mean freshwater flow at Pulo do Lobo hydrometric station and daily rainfall (mm d^{-1}) in Alcoutim from 2007 through 2008 (data obtained from http://snirh.pt).

Surface water temperature did not show significant spatial variability, although water temperature in the upper estuary (Mértola: 19.8 ± 4.8ºC, range 10.8 – 27.7ºC) displayed a larger range of values in relation to the lower estuary (VRSA: 19.1 ± 3.8ºC, range 12.7 – 25.3ºC). Water temperature seasonality was the expected for temperate regions, with higher values during summer (max 27.7ºC) and lower in the winter (min 10.7ºC). Salinity varied significantly among sampling stations. VRSA, in the lower estuary, registered the highest salinity (26.8 ± 6.9, range 11.6 – 37.3), while the lowest salinity values were measured in the upper estuary (Mértola and Alcoutim: 0.2 – 4.3). Significant interannual differences in salinity were also observed in the Guadiana estuary, with lower salinity values during 2007 (6.7 ± 10.4) in relation to 2008 (9.6 ± 12.8).

Mean light intensity in the mixed layer (I_m) showed significantly lower values ($p < 0.001$) in the middle estuary (Odeleite) and in the transition zone between the middle and the upper estuaries (Alcoutim) (Fig. 7.3A, Table 7.I). No significant inter-annual differences on I_m were found, but seasonal differences were detected, with lower I_m values in the winter. I_m varied between 4.3 µmol photons m^{-2} s^{-1} (middle estuary) and 499.6 µmol photons m^{-2} s^{-1} (lower estuary) (Table 7.I). Light extinction coefficient (k_e, Fig. 7.3B) and the ratio mixing depth to euphotic depth (Z_{mix}:Z_{eu}, Fig. 7.3C) were significantly higher in the middle estuary (max k_e = 9.5 m^{-1}, Z_{mix}:Z_{eu} = 20.5) and lower in the lower estuary (min k_e = 0.2 m^{-1}, Z_{mix}:Z_{eu} = 0.1). Z_{mix}:Z_{eu} at stations Odeleite (middle estuary) and Alcoutim (upper estuary) was, on average, higher than 5 (Table 7.I). The following empirical exponential relationship between Secchi depth (m) and k_e (m^{-1}) (obtained with the radiometer) was estimated through nonlinear regression: $k_e = 6.683 \exp(-1.507 D_S)$ (Fig. 7.4).

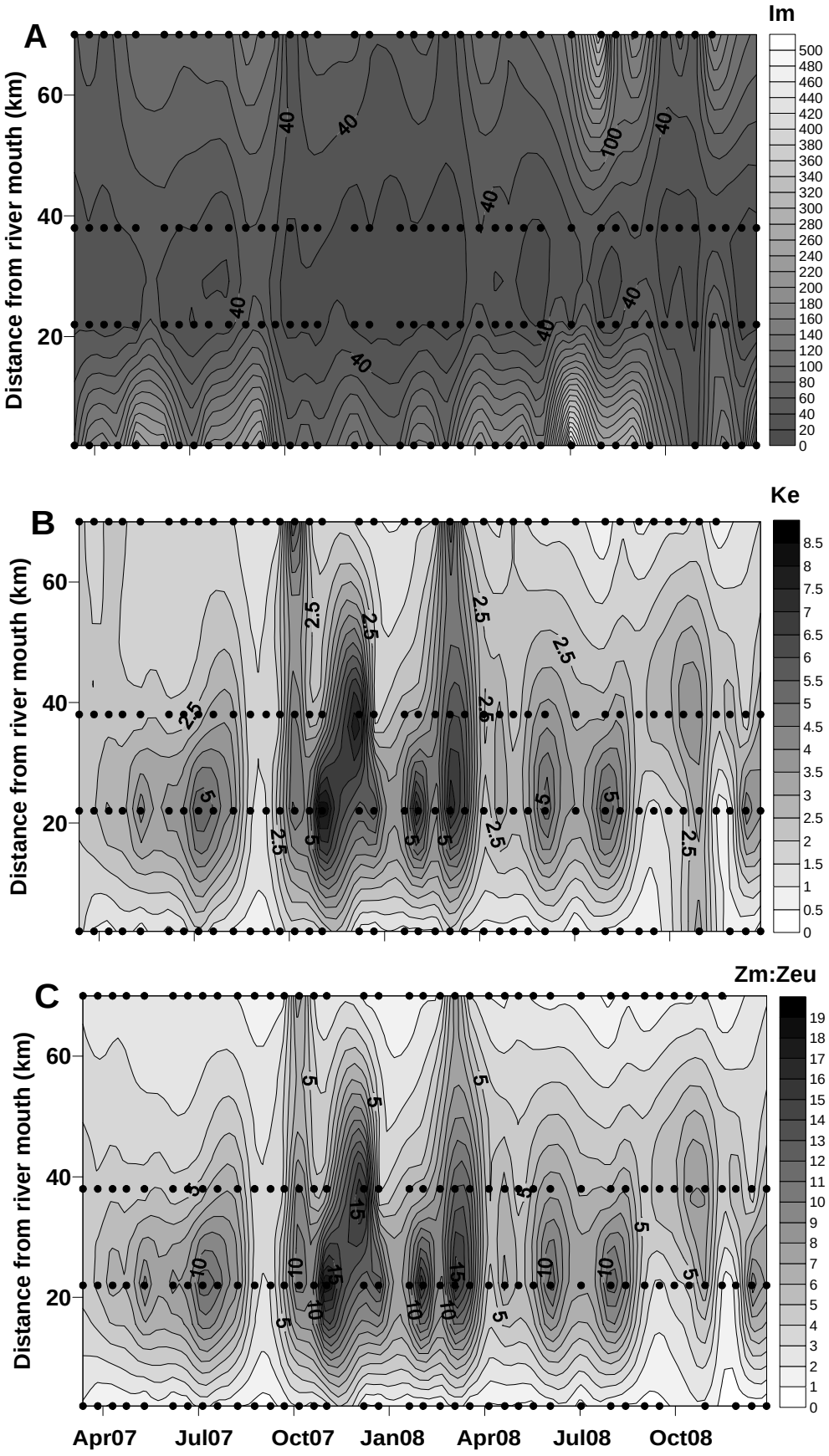

Figure 7.3 – Horizontal profiles of (A) mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}), (B) light extinction coefficient (k_e, m^{-1}), and (C) ratio mixing-depth to euphotic depth (Z_m:Z_{eu}) in the Guadiana estuary from 2007 through 2008.

Table 7.I – Mean values ± 1 standard deviation (mean ± SD), minimum (min) and maximum (max) values for mean light intensity in the mixed layer (I_m, µmol photons m^{-2} s^{-1}), light extinction coefficient (k_e, m^{-1}), mixing depth to euphotic depth ratio (Z_m:Z_{eu}), concentration of suspended particulate matter (SPM, mg L^{-1}), concentration of particulate organic matter (POM, mg L^{-1}), contribution of POM to SPM (%), concentration (µM) of ammonium (NH_4^+), nitrate (NO_3^-), silicate (SiO_4^{4-}) and orthophosphate (PO_4^{3-}), N:P and Si:N ratios in the sampling stations from 2007 through 2008 (nd = below detection limit).

	mean ± SD	min	max	mean ± SD	Min	max	mean ± SD	min	max
	I_m (µmol photons m^{-2} s^{-1})			**k_e (m^{-1})**			**Zm:Zeu**		
Mértola	91.5 ± 58.3	14.8	342.2	1.7 ± 1.2	0.4	6.8	2.2 ± 1.6	0.6	8.7
Alcoutim	39.6 ± 14.4	6.2	61.1	3.0 ± 1.4	1.2	8.6	6.0 ± 2.9	2.5	17.6
Odeleite	26.2 ± 21.0	4.3	98.7	3.9 ± 2.0	0.6	9.5	8.4 ± 4.4	1.3	20.5
VRSA	174.6 ± 89.6	21.1	499.6	1.0 ± 0.6	0.2	3.3	1.0 ± 0.5	0.1	2.2
	SPM (mg L^{-1})			**POM (mg L^{-1})**			**%POM/SPM**		
Mértola	15.2 ± 11.2	4.6	59.8	4.6 ± 2.6	0.0	10.6	42.1 ± 29.6	1.9	100.0
Alcoutim	35.9 ± 16.8	10.8	89.6	8.0 ± 6.1	0.4	31.4	24.6 ± 19.6	1.7	100.0
Odeleite	75.4 ± 31.3	25.0	141.2	14.4 ± 6.7	1.0	38.8	20.8 ± 11.0	2.6	65.9
VRSA	102.8 ± 28.2	42.2	185.6	29.8 ± 8.5	7.2	49.0	29.4 ± 6.8	17.1	49.9
	NH_4^+			**NO_3^-**			**PO_4^{3-}**		
Mértola	5.7 ± 9.5	nd	57.4	28.5 ± 23.1	0.1	99.0	1.9 ± 0.8	0.1	4.3
Alcoutim	3.1 ± 5.5	nd	33.6	32.8 ± 20.5	nd	93.6	2.2 ± 0.7	0.1	4.1
Odeleite	3.2 ± 4.3	nd	26.2	29.9 ± 16.9	3.6	80.8	1.9 ± 0.7	0.1	3.2
VRSA	6.7 ± 7.7	0.7	39.1	14.1 ± 15.3	0.5	86.0	1.3 ± 1.2	nd	6.8
	SiO_4^{4-}			**N:P**			**Si:N**		
Mértola	35.5 ± 23.9	1.3	100.8	20.6 ± 16.9	2.2	62.9	1.6 ± 1.6	0.0	6.8
Alcoutim	44.2 ± 30.8	3.3	125.7	18.8 ± 14.5	0.1	51.4	1.6 ± 1.3	0.0	5.8
Odeleite	36.8 ± 28.1	0.8	95.1	20.4 ± 15.1	5.4	56.7	1.4 ± 1.0	0.0	3.7
VRSA	14.6 ± 11.1	1.4	56.7	21.5 ± 21.5	5.5	98.7	1.0 ± 0.8	0.0	2.9

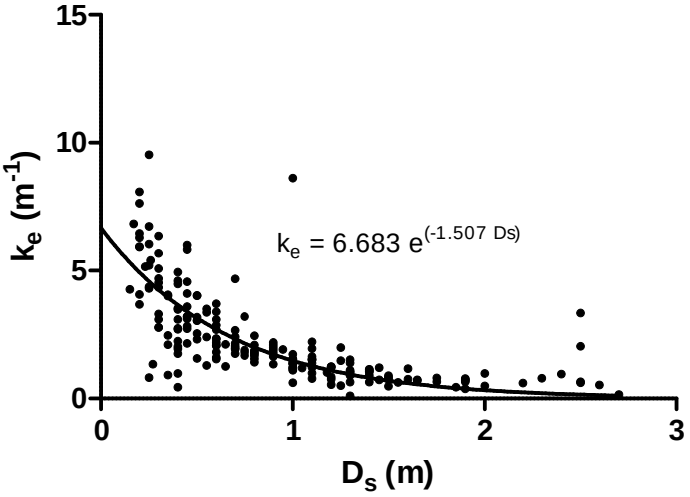

Figure 7.4 – Empirical exponential relationship between Secchi depth (D_S, m) and light extinction coefficient (k_e, m^{-1}), obtained through nonlinear regression (y_0 = 6.683 ± 0.4143; slope = 1.507 ± 0.1257; n = 194; R^2 = 0.5614).

Suspended particulate matter did not follow an inverse pattern in relation to light availability, presenting the highest values in the lower estuary (max 185.6 mg L^{-1}), where I_m was also the highest, and decreasing upriver (min 4.6 mg L^{-1} in Mértola) (Fig. 7.5A, Table 7.I). Significant correlations were obtained between SPM and light extinction coefficient in the upper (Mértola: r = 0.7470, p < 0.001; Alcoutim: r = 0.7252, p < 0.001) and middle estuaries (r = 0.5567, p < 0.001), but no relationship was found for the lower estuary (p > 0.05).

Most SPM was of inorganic origin. Particulate organic matter concentration presented the highest values in the lower estuary (29.8 ± 8.5 mg L^{-1}, range 7.2 – 49.0 mg L^{-1}) and the lowest concentrations in the upper estuary (Mértola: 4.6 ± 2.6 mg L^{-1}, range 0.0 – 10.6 mg L^{-1}) (Fig. 7.5B, Table 7.I). Mean contribution of POM to SPM varied between 42.1% ± 29.6% (range 1.9% - 100%) in Mértola and 20.8% ± 11.0% (range 2.6% - 65.9%) in Odeleite. Overall, POM contribution to SPM was higher in Mértola and VRSA (29.4% ± 6.8%, range 17.1% - 49.9%) than in the other stations (Fig. 7.5C, Table 7.I).

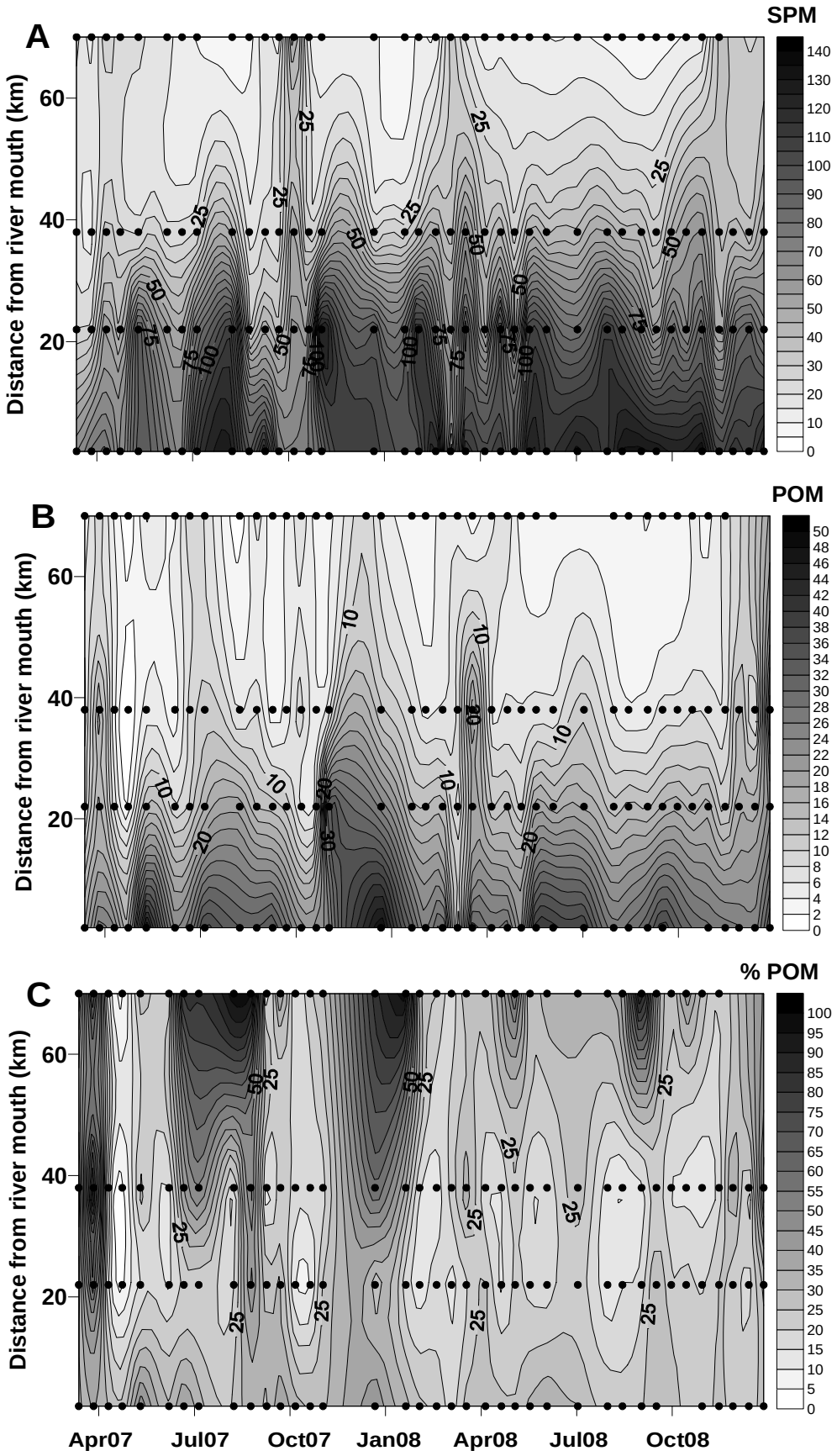

Figure 7.5 – Horizontal profiles of A) suspended particulate matter concentration (SPM, mg L^{-1}), B) particulate organic matter concentration (POM, mg L^{-1}) and C) contribution of POM to total SPM (%) in the Guadiana estuary from 2007 through 2008.

Except for ammonium, spatial and seasonal variability in nutrient concentration was consistent throughout the sampling period. Spatially, higher nutrient concentrations were determined in the upper estuary, decreasing downriver. Seasonally, higher nutrient concentrations were detected in the winter and spring and lower in the summer. Throughout the sampling period, nitrate concentration varied between 14.1 ± 15.3 μM in VRSA and 32.8 ± 20.5 μM in Alcoutim and spatial variability was detected, with higher values in the upper estuary (Mértola: 28.5 ± 23.1 μM) and lower in the lower estuary (Fig. 7.6A, Table 7.I). Nitrate also varied interannually, with higher concentrations in 2007 (35.6 ± 23.9 μM) in relation to 2008 (18.8 ± 12.4 μM). Ammonium (NH_4^+) varied between undetectable values and 57.4 μM, and presented higher values in Mértola (5.7 ± 9.5 μM) and VRSA (6.7 ± 7.7 μM), and lower in the other stations. Maxima in ammonium concentration were detected occasionally in all sampling stations, but neither seasonal nor interannual variability was evident (Table 7.I). Overall, nitrate was the main component of total dissolved inorganic nitrogen (DIN), with contributions ranging from 64.7% ± 24.7% in VRSA to 88.4% ± 18.8% in Alcoutim. Ammonium contribution to DIN varied between 11.6% ± 18.8% in Alcoutim and 35.3% ± 24.7% in VRSA. Phosphate and silicate showed significantly higher values in the upper estuary (P: 2.2 ± 0.7 μM; Si: 44.2 ± 30.8 μM) in relation to the lower estuary (P: 1.3 ± 1.2 μM; Si: 14.6 ± 11.1 μM), but no significant interannual differences were observed (Fig. 7.6B, 7.6C; Table 7.I).

Nutrient ratios (N:P and Si:N) did not show significant spatial differences along the Guadiana estuary. Mean N:P ratio varied between 19.0 ± 14.7 in Alcoutim and 21.9 ± 21.8 in VRSA and ranged between 0.1 and 99.0. Interannual differences were detected in N:P ratio, with higher values, usually >16, during 2007 and lower values, mostly <16, during 2008. Si:N ratio showed the opposite behaviour, with values lower than 1 during 2007 and higher than 1 during 2008. Mean Si:N varied between 1.0 ± 0.8 in VRSA and 1.6 ± 1.6 in Mértola, with a minimum of 0.0 and a maximum of 6.8 (Table 7.I).

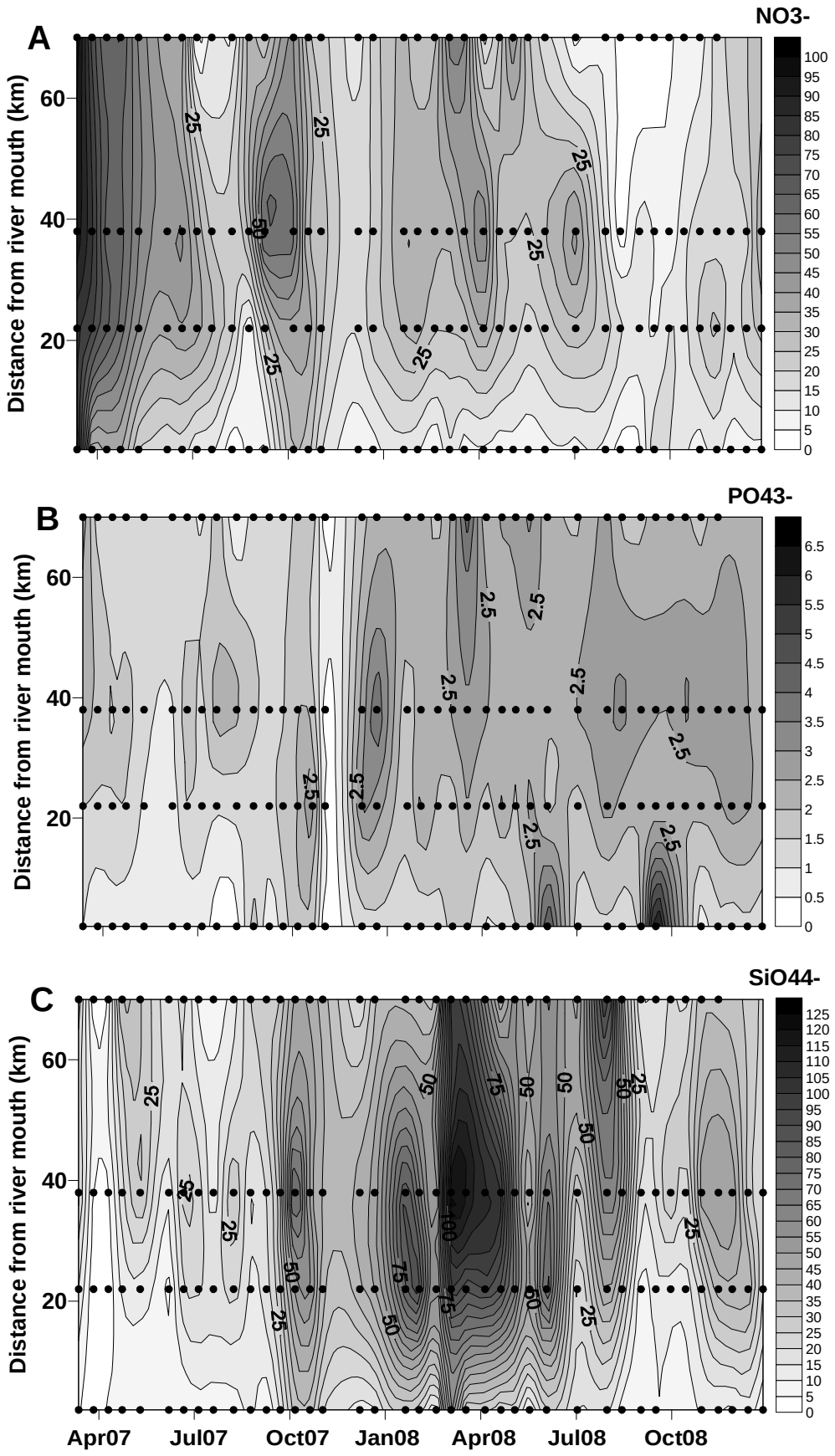

Figure 7.6 – Horizontal profiles of A) nitrate (NO_3^-), B) phosphate (PO_4^{3-}) and C) silicate (SiO_4^{4-}) concentration (µM) in the Guadiana estuary from 2007 through 2008.

Overall, significant spatial differences were observed in the distribution of environmental variables, as revealed by ANOSIM. R-values were the highest between the upper and the lower estuaries (R-values: 0.968 and 0.971), indicating a high dissimilarity between these locations. Year (R = 0.089) and Season (R = 0.052) presented R values close to 0, indicating a high degree of inter-annual and seasonal similarity.

7.3.2 Phytoplankton

Chlorophyll a concentration varied between undetectable values and 16.0 µg L^{-1}., with significantly higher values in Alcoutim (6.7 ± 3.0 µg L^{-1}) and lower in VRSA (1.6 ± 1.4 µg L^{-1}). Overall, higher values were observed in the summer (Fig. 7.7A, Table 7.II*).* Total phytoplankton abundance from 2007 through 2008 varied between 0.04 x 10^5 and 1390 x 10^5 cells L^{-1} (Fig. 7.8A, Table 7.II). Although slightly higher in Mértola, phytoplankton abundance did not show significant spatial differences throughout the sampling period. Total phytoplankton biomass varied between 0.1 and 3162 µg C L^{-1} (Fig. 7.8B, Table 7.II). Significantly lower biomass values were determined in the lower estuary in relation to the middle and upper estuarine sections. The ratio carbon to chlorophyll *a* (C:Chl) ranged between 1.1 and 586.9 mg C mg Chl^{-1}. The highest values were determined in the summer and a clear decrease on C:Chl from the upper (86.7 ± 160.4 mg C mg Chl^{-1}) to the lower estuary (37.7 ± 49.9 mg C mg Chl^{-1}) was observed (Fig. 7.7B, Table 7.II).

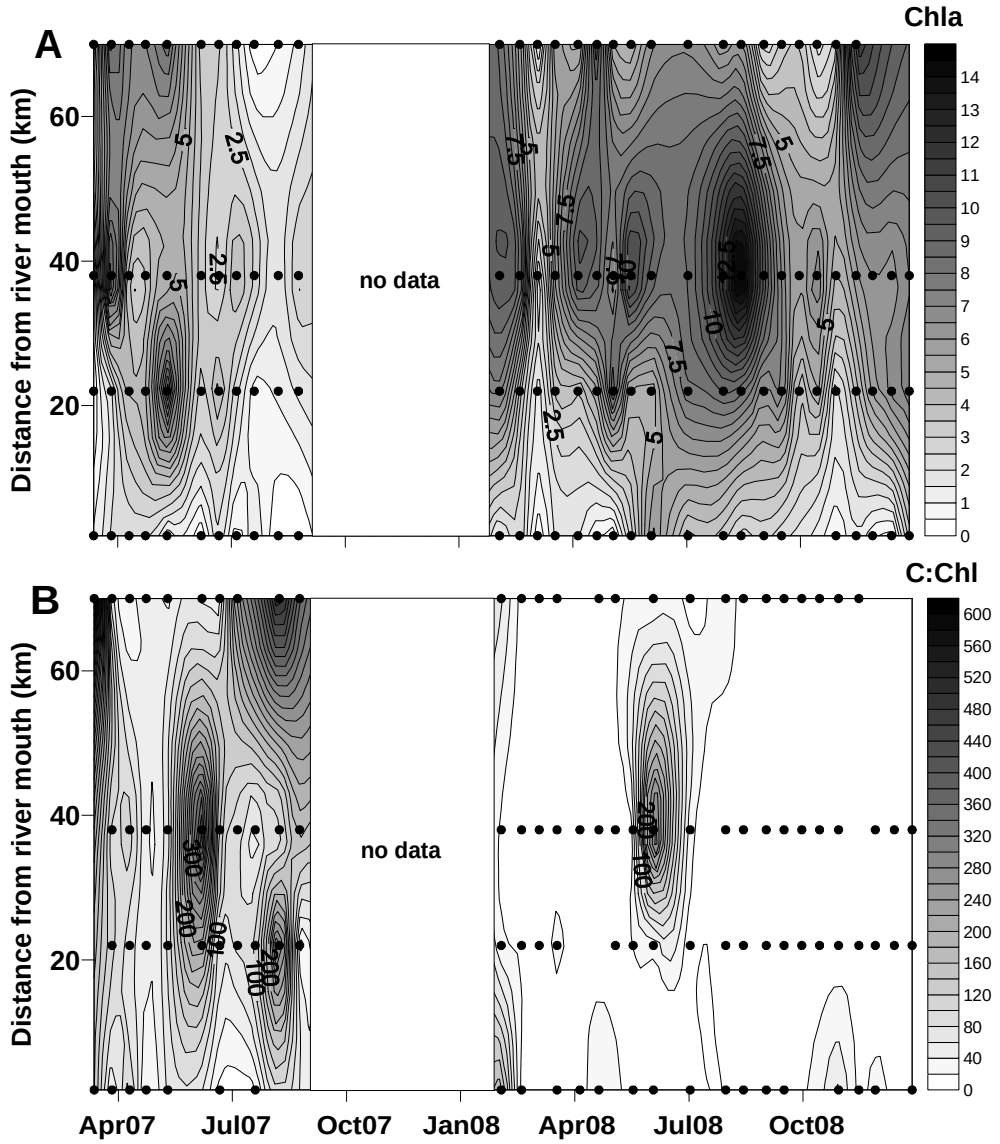

Figure 7.7 – Horizontal profiles of A) chlorophyll a concentration (µg L^{-1}) and B) carbon to chlorophyll ratio in the Guadiana estuary from 2007 through 2008.

Diatoms, green algae and cyanobacteria were the most important phytoplankton groups. Diatoms were the main component of the phytoplankton community in terms of biomass, representing, on average, from 33% (VRSA) to 73% (Odeleite) of the total biomass. Except for the lower estuary, cyanobacteria were, on average, the most abundant group, with contributions to total phytoplankton abundance ranging between 51% (Alcoutim) and 85% (Odeleite). Seasonally, diatoms were more abundant in the spring/early summer, reaching a maximum abundance of 72.4 x 10^5 cells L^{-1} in the spring 2007 in Odeleite and maximum biomass of 3,150 µg C L^{-1} in the spring 2008 in Alcoutim (Figs. 7.9A, 7.10A, Table 7.II). Green algae presented the highest abundances in late spring/early summer, with a maximum of 31.4 x 10^5 cells

L^{-1} in the upper estuary, whilst biomass reached 940 µg C L^{-1} in late spring 2008 in Alcoutim (Figs. 7.9B, 7.10B, Table 7.II). Cyanobacteria dominated in terms of abundance during summer months in all the sampling stations. Maximum cyanobacteria abundance and biomass were 1260 x 10^5 cells L^{-1} and 41 µg C L^{-1}, respectively, in August 2007 in Mértola (Figs.7.9C, 7.10C, Table 7.II).

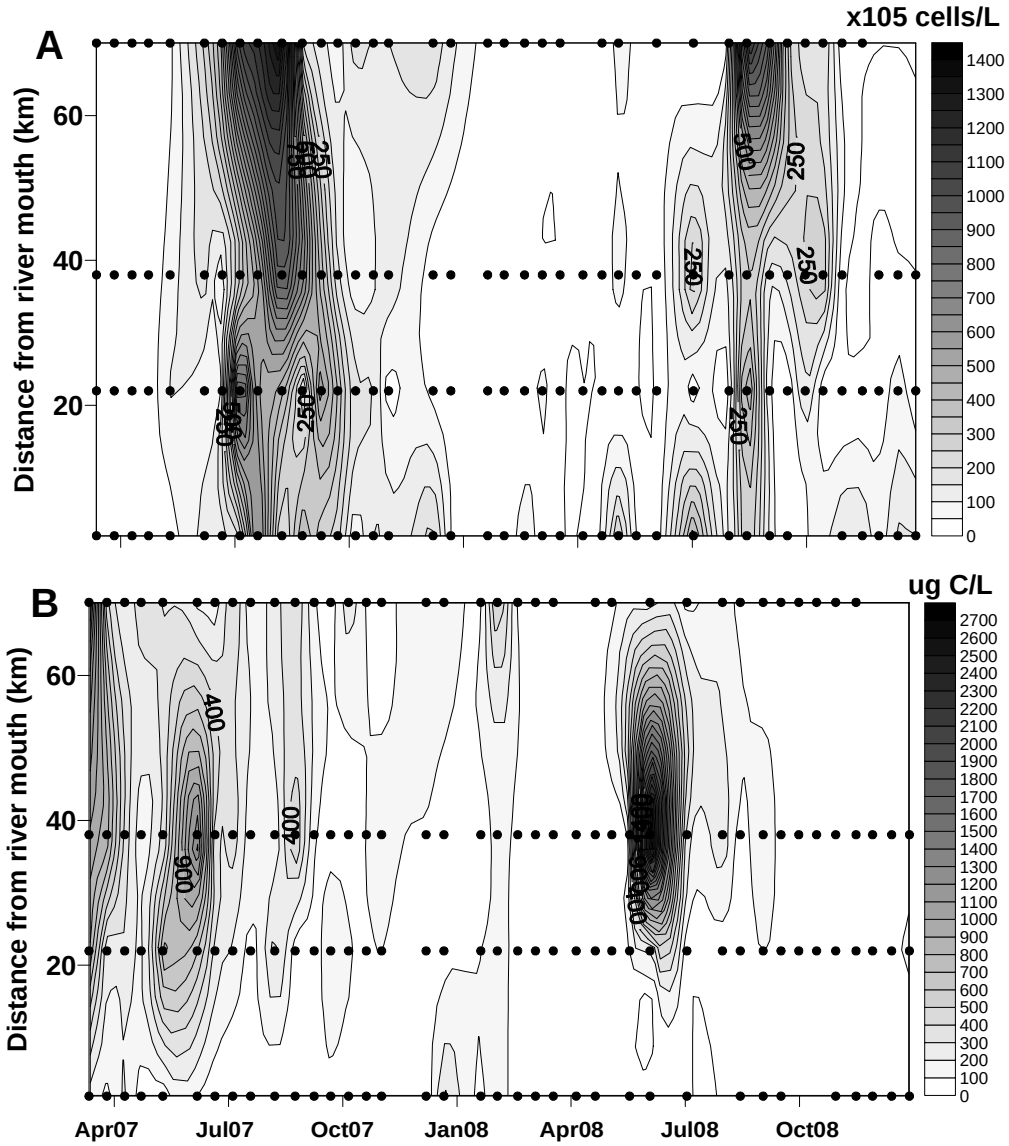

Figure 7.8 – Horizontal profiles of A) total phytoplankton abundance (x10^5 cells L^{-1}) and B) total phytoplankton biomass (µg L^{-1}) in the Guadiana estuary from 2007 through 2008.

Table 7.II - Mean values ± 1 standard deviation (mean ± SD), minimum (min) and maximum (max) values of chlorophyll *a* concentration (Chl*a*, µg L^{-1}), ratio carbon to chlorophyll *a* (C:Chl, mg C mg Chl^{-1}), abundance (x 10^5 cells L^{-1}) and biomass (µg C L^{-1}) of the phytoplankton community and of specific groups (diatoms, green algae and cyanobacteria) in the sampling stations from 2007 through 2008 (nd = not detected).

	mean ± SD	min	max	mean ± SD	min	max
	Chl*a*			C:Chl		
Mértola	4.8 ± 3.0	nd	11.7	86.7 ± 160.4	3.4	586.9
Alcoutim	6.7 ± 3.8	nd	16.0	56.6 ± 102.4	1.1	453.8
Odeleite	4.6 ± 2.7	nd	10.1	47.4 ± 75.4	2.5	342.3
VRSA	1.6 ± 1.4	nd	6.4	37.7 ± 49.9	1.1	180.6
	Phytoplankton abundance			Phytoplankton biomass		
Mértola	241 ± 387	1.85	1390	191 ± 280	20	1585
Alcoutim	134 ± 208	0.17	997	270 ± 555	5	3162
Odeleite	114 ± 211	0.19	1020	139 ± 195	0.1	839
VRSA	137 ± 152	0.04	661	47 ± 70	0.3	326
	Diatom abundance			Diatom biomass		
Mértola	2.04 ± 2.83	0.1	12.30	119 ± 268	1.1	157
Alcoutim	4.41 ± 6.35	nd	33.8	221 ± 521	nd	3150
Odeleite	4.32 ± 11.7	nd	72.4	110 ± 178	nd	790
VRSA	0.27 ± 0.49	nd	2.73	28.2 ± 66.4	nd	326
	Green algae abundance			Green algae biomass		
Mértola	3.71 ± 6.40	nd	31.4	13.3 ± 41.5	nd	245
Alcoutim	1.93 ± 2.71	nd	10.7	26.8 ± 150	nd	940
Odeleite	2.50 ± 5.20	nd	21.0	2.7 ± 6.3	nd	33.7
VRSA	0.33 ± 0.99	nd	5.26	1.1 ± 3.9	nd	326
	Cyanobacteria abundance			Cyanobacteria biomass		
Mértola	223 ± 371	nd	1260	5.6 ± 10.5	nd	40.7
Alcoutim	123 ± 205	nd	973	2.6 ± 6.7	nd	39.4
Odeleite	103 ± 209	nd	1000	1.1 ± 2.4	nd	11.8
VRSA	133 ± 150	nd	654	1.5 ± 1.8	nd	7.7

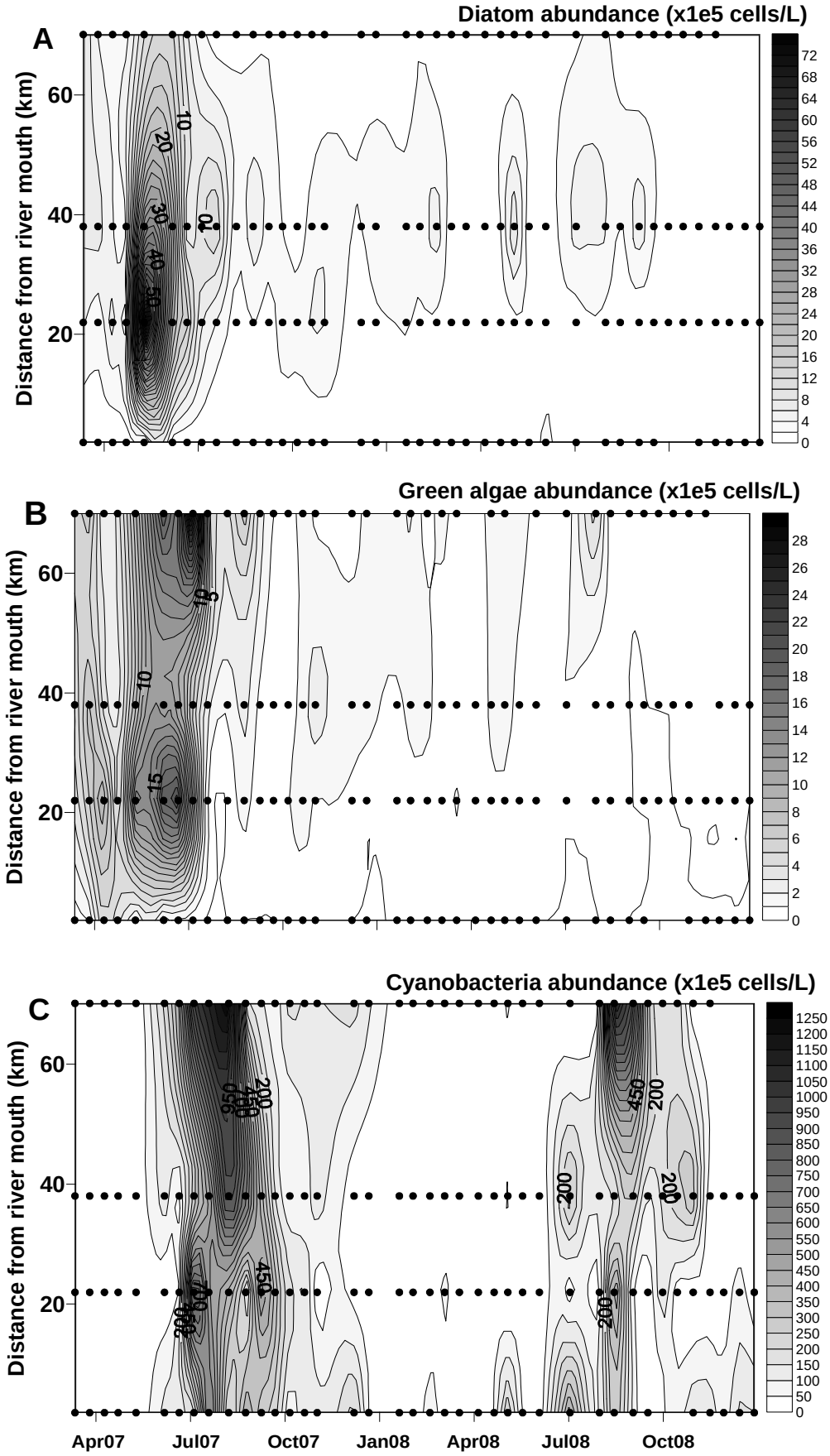

Figure 7.9 – Horizontal profiles of abundance (x10^5 cells L^{-1}) of specific phytoplankton groups in the Guadiana estuary from 2007 through 2008: A) diatoms, B) green algae and C) cyanobacteria.

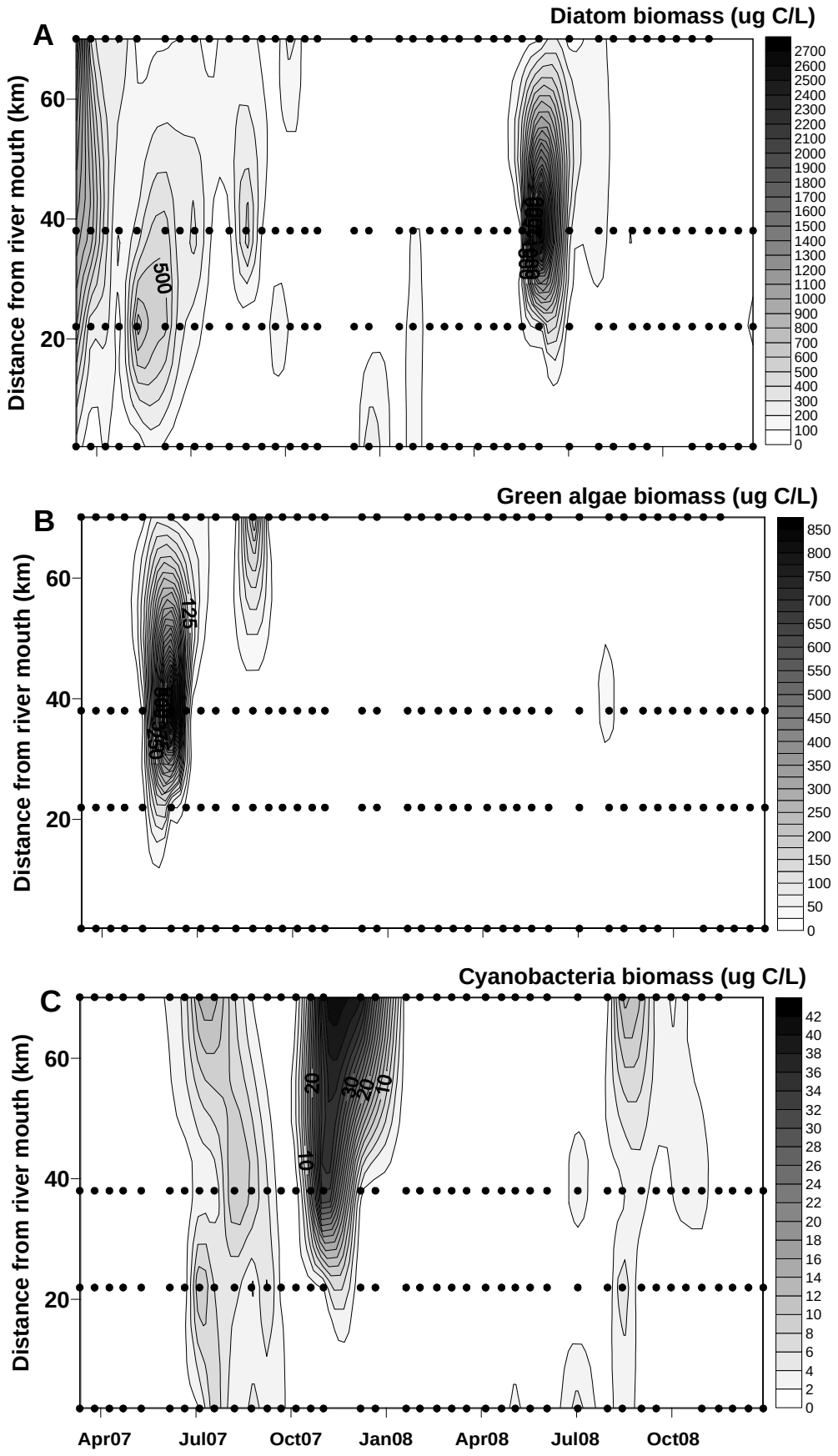

Figure 7.10 – Horizontal profiles of biomass (μg L^{-1}) of specific phytoplankton groups in the Guadiana estuary from 2007 through 2008: A) diatoms, B) green algae and C) cyanobacteria.

7.3.3 Primary production

Photosynthesis-irradiance (P-E) curves obtained from ^{14}C incubations show that the rate of photosynthesis normalized to chlorophyll (P^B) was significantly higher upriver (Mértola) than in the other sampling stations (Fig. 7.11, Table 7.III). Maximum values were observed in the summer, when the estimated light-saturated rate of photosynthesis (P^B_S) reached 69 mg C (mg Chl)$^{-1}$ h^{-1}. In the other stations, P^B_S did not surpass 20 mg C (mg Chl)$^{-1}$ h^{-1} and remained mostly below 10 mg C (mg Chl)$^{-1}$ h^{-1} throughout the year (Fig. 7.11, Table 7.III). Saturating irradiances (E_S) also presented higher values in the upper estuary (Alcoutim: max 2252 µmol photons m^{-2} s^{-1}) and lower downriver (VRSA: max 443 µmol photons m^{-2} s^{-1}) (Fig. 7.11, Table 7.III). Overall, a decreasing trend from the upper to the lower estuary was observed for the photosynthetic rate and saturating irradiance.

Likewise, areal primary production in the Guadiana estuary was higher in the upper estuary (22.3 – 1138.8 mg C m^{-2} d^{-1}) and lower in the middle (9.6 – 446.4 mg C m^{-2} d^{-1}) and lower estuaries (42.9 – 824.6 mg C m^{-2} d^{-1}). The highest values of primary production were detected during summer (Fig. 7.12A, Table 7.IV). Volumetric primary production was higher in the stations with the shallowest euphotic depths, Alcoutim (23.2 - 443.3 mg C m^{-3} d^{-1}) and Odeleite (16.1 – 353.4 mg C m^{-3} d^{-1}), and lower in VRSA (9.4 – 91.8 mg C m^{-3} d^{-1}), where euphotic depth was the highest (Fig. 7.12B, Table 7.IV). The average production to biomass (P/B) ratio was similar in all sampling stations (26.0 ± 28.9 mg C mg Chl^{-1} d^{-1}, 27.3 ± 15.9 mg C mg Chl^{-1} d^{-1}, 25.9 ± 14.6 mg C mg Chl^{-1} d^{-1} and 24.6 ± 20.0 mg C mg Chl^{-1} d^{-1} in Mértola, Alcoutim, Odeleite and VRSA, respectively), but P/B in Alcoutim and Odeleite was slightly higher than in the other stations throughout 2008 (Fig. 7.12C, Table 7.IV).

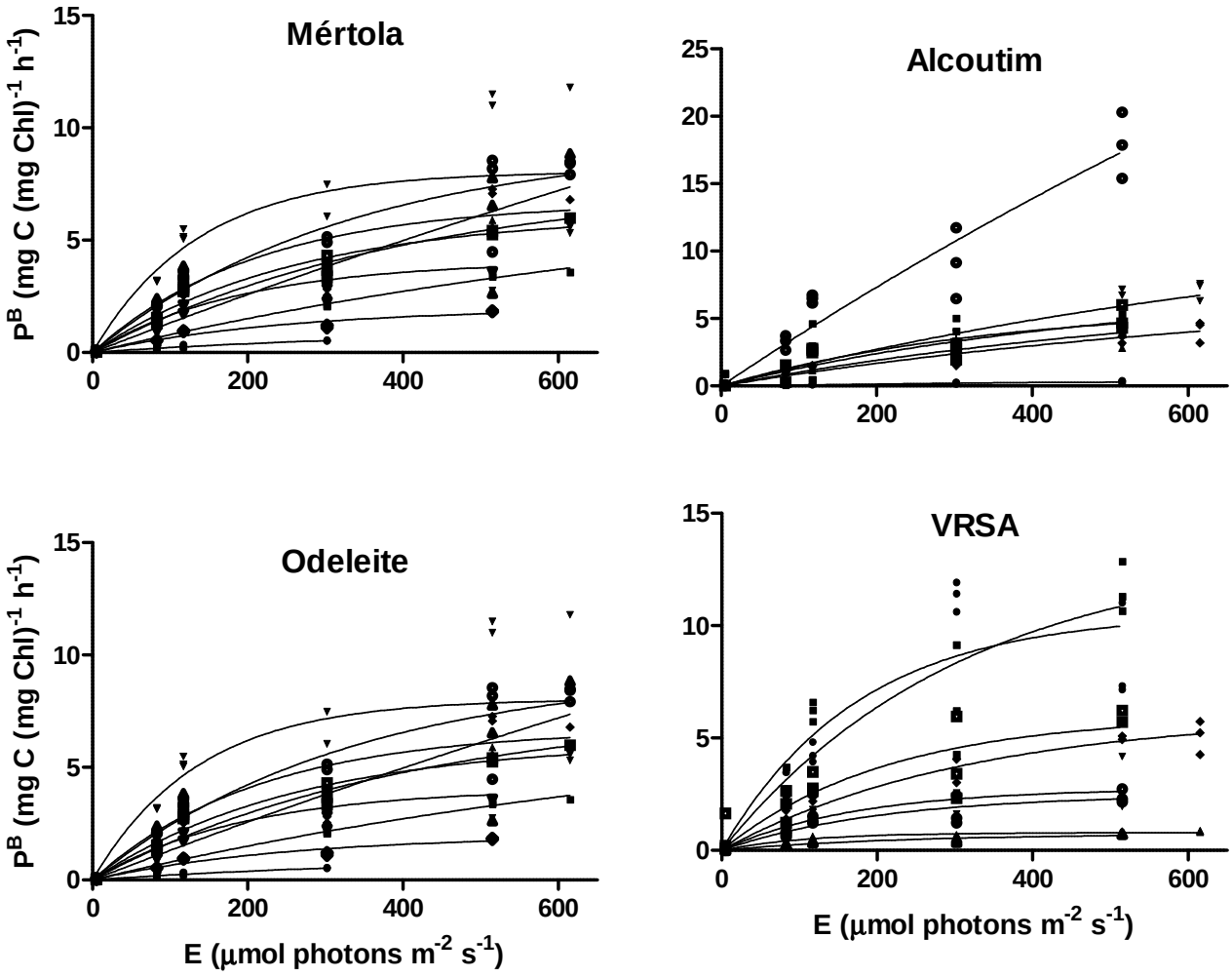

Figure 7.11 – Photosynthesis-irradiance (P-E) curves for ^{14}C incubations under PAR from water samples collected in several sampling stations in the Guadiana estuary throughout 2008. Nonlinear regressions were obtained by fitting values to equation 7.4.

Table 7.III - Photosynthetic parameters of phytoplankton from the Guadiana estuary, estimated using nonlinear regression fitting of equation 4. Values presented are mean ± 1 standard deviation, minimum and maximum values of light-saturated rate of photosynthesis (P^B_S, mg C (mg Chl)$^{-1}$ h^{-1}), saturating irradiance (E_S, µmol photons m^{-2} s^{-1}), and range of determination coefficients (R^2) for the nonlinear regressions (n = 13 – 18).

		upper estuary		middle estuary	lower estuary
		Mértola	**Alcoutim**	**Odeleite**	**VRSA**
P^B_S	**mean ± 1 SD**	16.00 ± 23.93	9.11 ± 4.97	7.31 ± 4.26	5.62 ± 4.74
	min	0.54	3.89	0.44	0.73
	max	69.57	20.52	13.52	13.45
E_S	**mean ± 1 SD**	801.3 ± 469.7	662.6 ± 650.6	471.1 ± 499.9	244.7 ± 106.4
	min	344.3	2.2	10.4	109.7
	max	1795.0	2252.0	1731.0	442.8
R^2		0.6392 – 0.9163	0.8793 – 0.9848	0.6331 – 0.9894	0.7163 – 0.9263

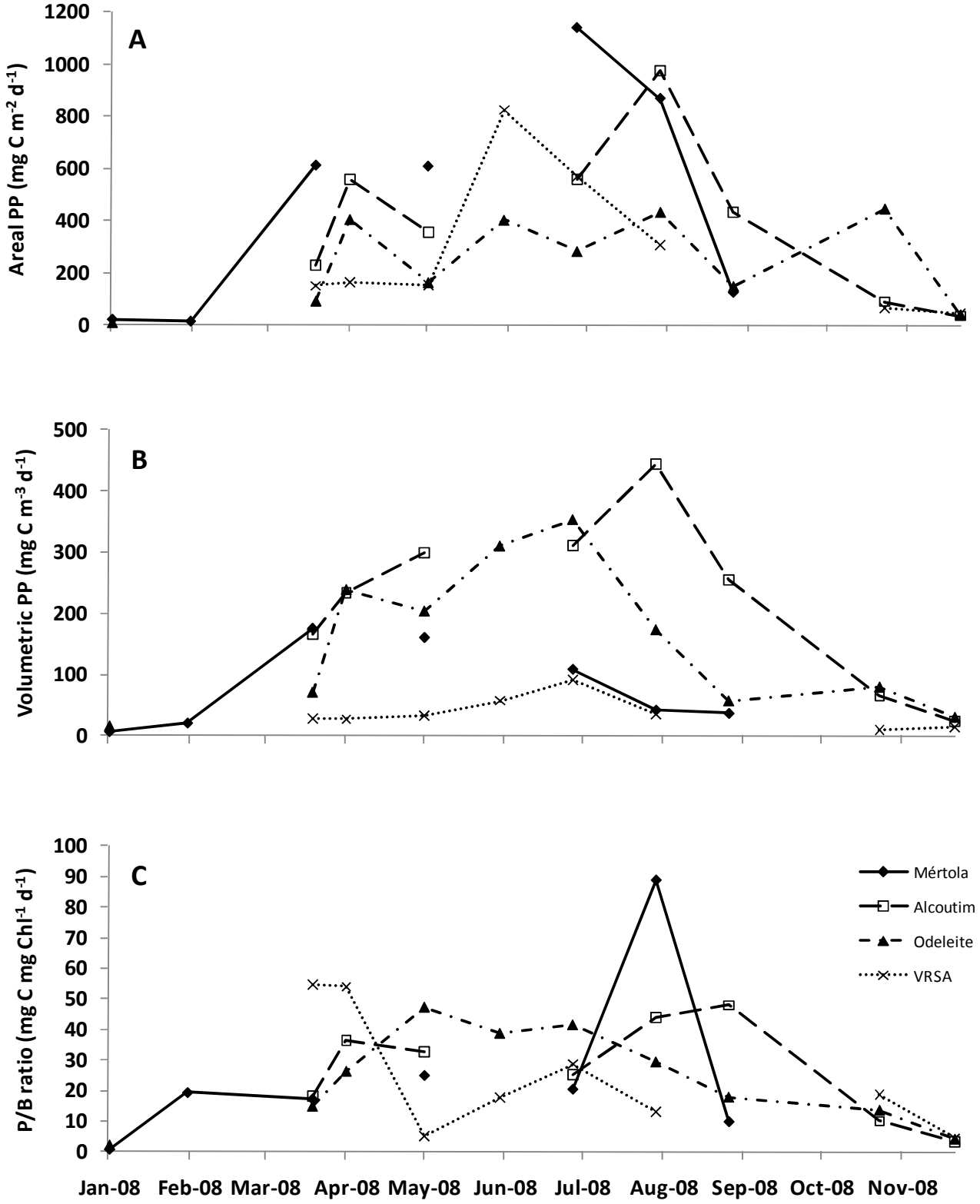

Figure 7.12 – A) Daily areal primary production (mg C m^{-2} d^{-1}), B) daily volumetric primary production (mg C m^{-3} d^{-1}) and C) P/B ratio (mg C mg Chl^{-1} d^{-1}) along the Guadiana estuary throughout 2008.

Table IV – Daily areal primary production (areal PP, mg C m^{-2} d^{-1}), daily volumetric primary production (volume PP, mg C m^{-3} d^{-1}) and production to biomass ratio (P/B, mg C mg Chl^{-1} d^{-1}) in the Guadiana estuary throughout 2008.

	Mértola			Alcoutim		
	Areal PP	**Volume PP**	**P/B**	**Areal PP**	**Volume PP**	**P/B**
28 Jan	22.3	7.4	0.9			
27 Feb	14.9	21.3	19.4			
15 Apr	613.0	174.9	17.3	231.6	165.4	18.2
28 Apr				560.7	233.6	36.5
28 May	608.2	160.1	25.0	358.0	298.3	32.8
26 Jun						
24 Jul	1138.8	108.5	20.5	560.7	311.5	25.3
25 Aug	867.8	142.3	88.9	975.1	443.3	43.9
22 Sep	125.1	36.8	9.9	431.8	254.0	47.9
19 Nov				90.3	64.5	10.1
18 Dec				34.8	23.2	3.4
	Odeleite			**VRSA**		
	Areal PP	**Volume PP**	**P/B**	**Areal PP**	**Volume PP**	**P/B**
28 Jan	9.6	16.1	2.1			
27 Feb						
15 Apr	92.3	71.0	14.8	150.7	27.4	54.8
28 Apr	406.1	238.9	26.3	164.5	27.0	54.0
28 May	163.1	203.8	47.4	154.0	32.8	5.1
26 Jun	403.2	310.1	38.8	824.6	56.9	17.8
24 Jul	282.7	353.4	41.6	569.2	91.8	28.7
25 Aug	433.5	173.4	29.4	307.4	35.3	13.1
22 Sep	148.1	57.0	17.8			
19 Nov	446.4	79.7	13.5	65.9	9.4	18.8
18 Dec	39.1	30.0	4.0	42.9	14.3	4.5

7.4. Discussion

7.4.1 Hydrological conditions

The interactions between river flow and tidal regime have significant impacts on the availability of light, nutrients and other resources. River flow in the Guadiana estuary usually displays significant inter-annual variability, given that the climate in southeast Portugal/southwest Spain alternates between dry and wet years and water retention in dams further impacts the amount of freshwater reaching the estuarine zone. River flow in the Guadiana estuary used to be characterized by winter maxima above 2,000 m^3 s^{-1}, but during and after the construction of the Alqueva dam, freshwater flow decreased significantly, especially during winter (Barbosa et al., 2010). From 2007 through 2008 winter maxima did not surpass 125 m^3 s^{-1}. Significant interannual variability in river flow was observed, with higher values during 2007. However, daily rainfall during 2007 was not higher than during 2008, and whilst rainfall and river flow were positively correlated during 2007, no correlation was found in 2008, indicating a clear regulation of river flow by the dam. Regarding temperature and salinity, both variables were within the range of values described for the Guadiana estuary. Salinity in the upper estuary, by definition a freshwater zone, reached 4.3 in the summer in Alcoutim. Oligohaline conditions had already been observed in this location in previous years, in association with decreased freshwater flow (e.g., Domingues et al., 2007).

7.4.2 Variability of light and nutrients

The occurrence of spatial and temporal variability of light availability in the Guadiana estuary was evident throughout the sampling period and it has already been related to variability in turbidity (Domingues and Galvão, 2007), which in turn is controlled by suspended particulate matter. Suspended particulate matter (SPM) in estuaries is mainly driven by river flow, waves, wind, tidal regime and water residence time (see Guinder et al., 2009 and references therein). In the Guadiana estuary, the main source of SPM is the river itself, so seasonal variability in SPM is

observed, with higher values during periods of high river discharge (autumn and winter). Like in many turbid estuaries, light attenuation in the water column is mainly controlled by SPM (e.g., Cloern, 1987; Guinder et al., 2009), but only in the upper and middle estuarine sections. In the lower estuary the highest SPM values (42.2 – 185.6 mg L^{-1}) and the lowest light extinction coefficients (0.2 – 3.3 m^{-1}) were measured simultaneously, reflecting the dependence of light attenuation on the chemical composition of the suspended material. Indeed, suspended sediments in this region are mainly composed by quartz, which do not contribute to light attenuation in the water column, whilst in the middle and upper estuaries SPM is mostly dominated by clays (Machado et al., 2007), which play an important role in light absorption. In other estuarine systems, the lowest SPM concentrations are usually measured in the lower estuarine reaches (Calliari et al., 2005), given that SPM is generally of riverine origin. Both SPM and the light extinction coefficient were within the range of values previously described for the Guadiana estuary (e.g., Domingues et al., 2007; Domingues and Galvão, 2007) and for other turbid estuaries such as the Westerschelde (Kromkamp et al., 1995), the Colne (Kocum et al., 2002), Río de la Plata (Calliari et al., 2005) and Bahía Blanca (Guinder et al., 2009).

The high productivity usually associated with estuarine ecosystems is in part attributed to the occurrence of high concentrations of organic matter that sustain heterotrophic communities (Cloern, 1987). Organic matter may enter or be created in estuaries from industrial and urban effluents, natural vegetation, biological material processing and other diffuse sources (Boyes and Elliott, 2006). In the Guadiana estuary, the concentration of particulate organic matter (POM) was particularly high in the lower estuary (29.8 ± 8.5 mg L^{-1}, range 7.2 – 49.0 mg L^{-1}), where the anthropogenic influence is the highest. These values are higher than those described for other estuaries, such as Bahía Blanca estuary (max 24.3 mg L^{-1}: Guinder et al., 2009) and Río de la Plata estuary (means 5.3 – 11.2 mg L^{-1}: Calliari et al., 2005). POM values in the middle (14.4 ± 6.7 mg L^{-1}) and upper estuaries (Mértola: 4.6 ± 2.6 mg L^{-1}; Alcoutim 8.0 ± 6.1 mg L^{-1}), where the anthropogenic pressure is much lower, were significantly lower than in VRSA. This pattern in POM variability reflects the importance of allochthonous sources of organic matter in the Guadiana estuary. In addition, no correlations were found between POM and phytoplankton biomass,

indicating that phytoplankton represents a negligible fraction of POM. Phytoplankton may account for only a small fraction of the total organic matter, but its contribution to bioavailable organic matter can be much higher (Sobczak et al., 2002). This fact was observed in the Sacramento-San Joaquin River delta and evidenced the strong food-chain linkage between phytoplankton and the pelagic food web (Sobczak et al., 2002).

The availability of photosynthetically active radiation (PAR) in the Guadiana estuary throughout the sampling period was lower in the middle estuary (Odeleite) and in the transition zone between the middle and upper estuaries (Alcoutim), where the estuarine turbidity maximum is usually located. Mean light intensity in the mixed layer (I_m) in these locations did not surpass 100 µmol photons m^{-2} s^{-1}. Although this value is higher than Riley's critical value of 42 µmol photons m^{-2} s^{-1} below which net growth of phytoplankton does not occur (Riley, 1957), it is much lower than saturating light intensities referred for estuarine phytoplankton communities (100 - 800 µmol photons m^{-2} s^{-1}: e.g., Fisher et al., 1982; Pennock and Sharp, 1986; Madariaga, 1995; Tillmann et al., 2000; Macedo et al., 2001; Kocum et al., 2002; Oviatt et al., 2002). Therefore, the occurrence of light-limited growth of phytoplankton in these locations is a strong possibility. Furthermore, the mixing depth in Alcoutim and Odeleite was generally more than 5 times the euphotic depth, suggesting that net growth of phytoplankton could not be sustained (Cloern, 1987). In well mixed estuaries such as the Guadiana, Z_{mix}:Z_{eu} follows the contours of bathymetry (Cloern, 1987), as in the Colne estuary, where the highest Z_{mix}:Z_{eu} was measured in the deeper, clearer waters in the lower estuary, and not in the turbid, shallow freshwater reaches (Kocum et al., 2002). Therefore, the higher Z_{mix}:Z_{eu} measured in Alcoutim and Odeleite may be the result of the higher mixing depth in these locations (9.4 and 9.9 m, respectively), in relation to Mértola (5.9 m) and VRSA (1.0 - 5.0 m). However, the highest turbidity was measured in Odeleite, decreasing upriver, so it is not clear if Z_{mix}:Z_{eu} ratios are controlled by bathymetry, turbidity or both (Domingues and Galvão, 2007). Considering both I_m and Z_{mix}:Z_{eu}, it is probable that phytoplankton growth was potentially light-limited in Alcoutim and Odeleite, but not in Mértola and VRSA. However, considering the saturating light intensities described for estuarine phytoplankton, from 100 to 800 µmol photons m^{-2} s^{-}, it is possible that

growth was light-limited in the whole estuary. Permanent light limitation in Alcoutim was confirmed by light enrichment experiments, under PAR exposures ranging between 90 and 225 µmol photons m^{-2} s^{-1} (see Chapter 6).

Furthermore, the determination of the mean light intensity in the mixed layer poses some methodological problems. The light extinction coefficient (K_e) and the depth of the mixed layer (Z_{mix}), two variables necessary to calculate I_m, are usually considered constant throughout the day and in the same sampling station, respectively, but in reality K_e varies along the day with tidal phase and river flow (e.g., Kromkamp et al., 1995), and Z_{mix} varies along the channel's cross-section and also with tidal phase. K_e is mainly regulated by SPM concentration, which may vary significantly along the semidiurnal tidal cycle. In the Guadiana estuary, significantly higher SPM values were measured during flood and lower SPM occurred during low tide (Domingues et al., 2010), which may promote a wide range of K_e values over the semidiurnal cycle. For instance, during a winter 2008 spring tide, K_e values ranged between 2.0 m^{-1} during low tide and 4.7 m^{-1} 6 hours later, during flood (Domingues et al., 2010). Considering Z_{mix} = 9.4 m and I_0 = 1500 µmol photons m^{-2} s^{-1}, I_m calculation based on K_e = 2.0 m^{-1} would be 80 µmol photons m^{-2} s^{-1}, whilst using K_e = 4.7 m^{-1}, I_m is 34 µmol photons m^{-2} s^{-1}. Furthermore, I_m is usually calculated using the light intensity profiles measured during sampling; if sampling is conducted in the early morning when incident solar radiation is lower, I_m will be lower than if measurements were taken in the afternoon; likewise, sampling around noon will result in higher I_m values. Since these isolated estimates are taken as a proxy for the whole day, the mean light availability in the mixed layer over the light period may be severely under- or overestimated. For instance, the isolated measurement we made on December 18th in Alcoutim at 10 A.M. with I_0 = 162 µmol photons m^{-2} s^{-1} and K_e = 3.1 m^{-1} resulted in I_m = 5.6 µmol photons m^{-2} s^{-1}. Considering the whole light period (11 hours) and the incident solar radiation for each hour (obtained in http://snirh.pt) that ranged between 6 and 890 µmol photons m^{-2} s^{-1}, mean daily I_m would be 12 µmol photons m^{-2} s^{-1}, which is twice the I_m value obtained with our isolated measurement in the morning.

The spatial and seasonal variability of nutrient concentrations observed during 2007 and 2008 were the typical for the Guadiana estuary. The occurrence of higher

nutrient concentrations in the upper estuary, and during periods of higher river discharge (autumn and winter), suggests that the main nutrient source is the river itself (Domingues and Galvão, 2007) and supports the small influence of anthropogenic nutrient sources. Only in the lower estuary the effect of anthropogenic pressures was detected, with the highest POM and ammonium concentrations. Indeed, nutrient concentrations in the Guadiana estuary (NO_3^- 0.0 - 99.0 μM, PO_4^{3-} 0.0 - 6.8 μM) during 2007 and 2008 were lower than those found in eutrophic or slightly eutrophic estuaries such as the Scheldt, Netherlands (DIN 70 - 600 μM, PO_4^{3-} 3 - 20 μM: Kromkamp and Peene, 1995), the Colne, UK (NO_3^- 5.75 – 564 μM: Kocum et al., 2002), the Rhine, Netherlands (mean NO_3^- 270 μM, mean PO_4^{3-} 11 μM: Schaub and Gieskes, 1991), the Pearl, China (mean NO_3^- 80 μM: Yin et al., 2001), the Gironde, France (mean NO_3^- 140 μM: Cabeçadas et al., 1999), or the Tagus, Portugal (DIN 0.2 – 182.4 μM, PO_4^{3-} 0.1 – 19.1 μM: Gameiro et al., 2010). Nutrient concentrations in the Guadiana during 2007 and 2008 were also lower than during the period 1996 - 2005 (NO_3^- 0.0 – 250.3 μM: Barbosa et al., 2010), probably as a consequence of increased water and sediment retention in the Alqueva dam. Conversely, nitrate concentrations in the Guadiana were more close to those found in oligotrophic estuaries such as the Conwy, UK (NO_3^- 27.7 ± 8.1 μM: Dong et al., 2006), reflecting the relatively preserved/pristine nature of the Guadiana estuary (Vasconcelos et al., 2007). Whilst nitrate and silicate availability has been positively correlated to river flow and rainfall, as in other estuaries (e.g., Mallin et al., 1991), the lack of seasonal or inter-annual patterns in phosphate and ammonium concentrations reflects the dependency of NH_4^+ and PO_4^{3-} availability on biological sources and sedimentary fluxes (Barbosa et al., 2010).

7.4.3. Variability of phytoplankton composition and production

Phytoplankton in the Guadiana estuary, particularly in the freshwater tidal zone, usually exhibits a marked seasonal succession, clearly related to nutrient availability. In the spring, with high N:P and high Si, a diatom bloom occurs, followed by a decrease in Si concentration and the development of green algae. Finally, with low N:P and low Si, cyanobacteria dominate the community throughout the summer

(Rocha et al., 2002; Domingues et al., 2005). In terms of biomass, this cycle is a unimodal one, with spring maxima corresponding to the diatom bloom, which is typical of other temperate estuaries (e.g., Andersson et al., 1994; Dugdale et al., 2007). During 2007 and 2008, phytoplankton exhibited a unimodal cycle with a biomass maximum in late spring/early summer, slightly later than usually observed in the Guadiana estuary (e.g., Domingues et al., 2005). Diatoms were the main component of biomass throughout the year in all the sampling stations, as in other temperate estuaries (e.g., Popovich and Marcovecchio, 2008), whilst cyanobacteria dominated in terms of cell numbers in the summer, especially in the upper and middle estuaries.

Diatoms bloomed in the middle and upper estuaries in late spring/early summer, and reached a maximum abundance of 7.2 x 10^6 cells L^{-1}. Maximum diatom abundances in previous years were higher (see Domingues et al., 2005, 2007; Domingues and Galvão, 2007; Barbosa et al., 2010) and this decrease throughout the last years has been accompanied by a decrease in river flow, especially during winter due to the flow regulation by the Alqueva dam, and consequently a decrease in nutrient concentrations, particularly silicon and nitrogen (see Barbosa et al., 2010). In 2007, nitrate concentrations before and during the diatom bloom (May - June) in the upper and middle estuaries were higher than the critical value of 20 μM referred in Chapter 4, below which nitrate becomes limiting to phytoplankton growth. In addition, Z_{mix}:Z_{eu} was lower than the critical value of 5 in the upper estuary, but higher than 8 in the middle estuary, where the diatom bloom was more pronounced. The onset of the green algae bloom that followed the diatom bloom in early summer also occurred under high Z_{mix}:Z_{eu} in the middle estuary and high nitrate concentrations. Therefore, it is clear that phytoplankton blooms may develop in the Guadiana estuary even when the mixed layer is more than 5 times the euphotic layer. Two hypothesis may explain this: importation of phytoplankton from areas with lower Z_{mix}:Z_{eu} and/or adaptation of phytoplankton to low light levels (see Irigoien and Castel, 1997 and references therein). The importation of phytoplankton from the upper estuary is a strong possibility, given that the phytoplankton identified in the samples were mostly freshwater species, and the maintenance of a regular river flow by the Alqueva dam results in a constant supply of freshwater to the estuarine zone. On the contrary, the adaptation of phytoplankton to low light availability is unlikely,

given that high rates of light-saturated photosynthesis (P_B^S) and high saturating light intensities (E_S) were observed. High P_B^S and high E_S values are usually found in cells adapted to clear waters, but also in other turbid estuaries such as San Antonio Bay (3.0 - 22.9 mg C (mg Chl)$^{-1}$ h^{-1}: MacIntyre and Cullen, 1996) or the Neuse River estuary (0.14 – 33.9 mg C (mg Chl)$^{-1}$ h^{-1}: Boyer et al., 1993). On the contrary, in other turbid environments such as the Tagus estuary (1.0 - 8.4 mg C (mg Chl)$^{-1}$ h^{-1}: Gameiro, 2009), low P_B^S and E_S values and the occurrence of photoinhibition at low irradiances (150 – 300 μmol photons m^{-2} s^{-1}) suggest that phytoplankton is acclimated to low light conditions. However, the ecological interpretation of P-E responses may be difficult; ideally, P-E curves should provide information on the photosynthetic state of the sample at the moment of collection. But unless incubation time is only a few minutes, some acclimation of cells will always happen during incubation (Sakshaug et al., 1997). Overall, both the P-E curves and light enrichment experiments carried out in Alcoutim (see Chapter 6) suggest that phytoplankton growth is light-limited in the middle and upper estuaries.

During 2008, the same seasonal pattern in phytoplankton succession was observed, but diatom and green algae abundance were significantly lower than during 2007. No significant interannual differences were observed in light availability, but nitrate concentration in 2008 was significantly lower than in 2007, and in many occasions, nitrate was <20 μM, so phytoplankton growth was most likely N-limited. Nutrient and light enrichment experiments carried out throughout 2008 and 2009 confirmed that phytoplankton growth was light-limited throughout the year and that diatom and green algae growth was nitrogen-limited during the productive period (see Chapters 4 and 6). Comparing nutrient concentrations and light availability in the Guadiana estuary in the last years (see Barbosa et al., 2010) it is possible that light limitation had always occurred in the middle and upper estuaries, but has now been surpassed by nitrogen limitation that started only recently, due to a reduction in river flow and consequently a decrease on nutrient inputs to the estuarine zone. This shift from a light-limited environment to a more nutrient-limited one had already been predicted for the Guadiana upper estuary (Barbosa et al., 2010).

Picocyanobacteria showed the same pattern as in previous years, blooming in the summer in the upper, middle and lower estuaries with a maximum abundance of 1.3

x 10^8 cells L^{-1}, similar to previous years (Domingues and Galvão, 2007). The summer dominance of picocyanobacteria under N-limitation in polyhaline regions has also been observed in other estuaries such as Chesapeake Bay (e.g., Fisher et al., 1988; Malone et al., 1991). However, no signal of the cyanobacteria summer blooms was observed on neither total biomass nor chlorophyll *a*, given that cyanobacteria population was mostly composed of coccoid pico-sized cells. The reduced contribution of cyanobacteria to total biomass and the regular presence of toxic cyanobacteria genera had already been observed in the Guadiana estuary (Domingues et al., 2005), but cyanobacteria blooms would not have been detected using chlorophyll *a* as a proxy for phytoplankton biomass (see Domingues et al., 2008, Chapter 2).

Indeed, the use of chlorophyll *a* concentration as a substitute for phytoplankton biomass is deeply-rooted, but its application is not straightforward, because it may overlook blooms of pico- and small nanophytoplankton and overestimate the importance of microphytoplankton (Domingues et al., 2008, see Chapter 2). A carbon to chlorophyll *a* (C:Chl) ratio, typically between 30 and 50 (e.g., Legendre et al., 1999) is usually applied to convert chlorophyll into biomass. However, C:Chl is highly variable intra- and inter-specifically and it also depends on the physiological state of the cell (Chan, 1980; Zonneveld, 1998; Kruskopf and Flynn, 2005; Putland and Iverson, 2007). Therefore, C:Chl may exhibit a wide temporal and spatial variability, which will complicate the use of chlorophyll *a* concentration as a proxy for phytoplankton biomass (see Domingues et al., 2008, Chapter 2). Indeed, C:Chl in the Guadiana estuary showed significant temporal and spatial variability, ranging between 1.1 and 586.9 mg C mg Chl^{-1}. The higher C:Chl values observed in the summer were probably the result of higher light and lower nutrient availability in the water column that promoted a decrease in the cellular chlorophyll *a* content and thus higher C:Chl values (e.g., Zonneveld, 1998; Kruskopf and Flynn, 2005). In addition, a significant spatial gradient was found, with higher C:Chl values in the upper estuary, decreasing downriver, reflecting the higher phytoplankton biomass found in the freshwater tidal zone. Unlike other turbid estuaries, where phytoplankton biomass decreases in the landward direction where turbidity is higher (e.g., Calliari et al., 2005; Popovich and Marcovecchio, 2008), phytoplankton

biomass and chlorophyll *a* concentration in the Guadiana estuary were higher in Alcoutim, close to the estuarine turbidity maximum, which was probably a consequence of the occurrence of larger phytoplankton cells. Indeed, biomass maxima in Alcoutim were due to the resuspension of large pennate diatoms (*Navicula* and *Pleurosigma*) from the bottom.

Overall, the middle and upper estuaries could be considered relatively homogeneous in terms of phytoplankton and environmental variables. The main differences were found in the lower estuary, where nutrient concentration, particularly N and Si, and phytoplankton abundance and biomass were significantly lower, evidencing a reduced riverine influence but a much stronger impact of coastal waters. This pattern is observed in other lower estuarine zones, such as in the Colne estuary (Kocum et al., 2002) or the Bahía Blanca estuary (Popovich and Marcovecchio, 2008).

Daily areal primary production in the Guadiana estuary varied between ≈10 and ≈1,140 mg C m^{-2} d^{-1}, the lowest values in the winter and the highest in the summer, concurrent with higher water temperature and light availability. Overall, primary production is highly variable across estuaries: mean production values range from 20 - 40 mg C m^{-2} d^{-1} (e.g., van Es, 1977; Kocum et al., 2002) up to 2,000 – 4,000 mg C m^{-2} d^{-1} (e.g., Malone et al., 1996; Thompson, 1998; Adolf et al., 2006), and the same estuary may alternate between low and high productivities (e.g., 90 – 1800 mg C m^{-2} d^{-1}: Mortazavi et al., 2000; 5 – 1,880 mg C m^{-2} d^{-1}: Azevedo et al., 2006).

A clear horizontal gradient on areal primary production was also observed, with higher values in the upper estuary and lower in the middle and lower estuaries, contrary to most turbid estuarine systems, where production is higher in the less turbid regions (e.g., Pennock and Sharp, 1986; Mallin et al., 1991). In the Neuse River lower estuary, for instance, downstream and upstream effluents and agricultural runoff and a shallow, well-mixed water column, contribute to a much higher productivity (60.9 – 2,766.4 mg C m^{-2} d^{-1}: Mallin et al., 1991) than in the Guadiana lower estuary (42.9 – 824.6 mg C m^{-2} d^{-1}). The higher levels of primary production in the turbid upper estuary may be due to the importation of cells from less turbid riverine locations upriver, but also due to higher nutrient concentrations in this region. Indeed, whilst light is considered the major regulator of phytoplankton growth in turbid estuaries with elevated nutrient inputs (Cloern, 1987; Alpine and

Cloern, 1988; Kocum et al., 2002), nutrients in the Guadiana estuary, particularly nitrogen, are not plentiful and may in fact limit phytoplankton growth, especially during spring and summer (see Chapter 4). Therefore, light and nutrients are equally important in phytoplankton regulation, and the occurrence of higher primary productivity in the upper estuary, contrary to the pattern for most turbid estuaries, is a consequence of the higher nutrient concentrations in this region in relation to the lower estuary.

Volumetric primary production and the production to biomass (P/B) ratio showed a different pattern in relation to areal production, with higher values in Odeleite and Alcoutim, the locations with the lowest euphotic depths, lowest light availability and highest Z_{mix}:Z_{eu} ratios. P/B ratio normalizes production across the range of phytoplankton biomass and is a realistic physiological indicator (Platt and Filion, 1973; Yoshiyama and Sharp, 2006). The higher P/B values in these locations suggest that the phytoplankton communities from these turbid regions are more efficient in utilizing the available resources, even under constant light limitation and occasional nutrient limitation.

Chapter 8

Final Remarks

8.1 The problems

This work aimed to understand how phytoplankton composition and growth in the Guadiana estuary is regulated by environmental drivers. Both sampling campaigns and laboratorial experiments were conducted to answer to six main questions:

a) How can phytoplankton be used as a biological quality element in coastal ecosystems (Chapter 2)?

b) How are phytoplankton and their environmental drivers affected by semidiurnal and spring-neap tidal cycles in the freshwater tidal zone of the Guadiana estuary (Chapter 3)?

c) Which is(are) the limiting nutrient(s) for phytoplankton growth in the freshwater tidal zone of the Guadiana estuary and how does(do) it(they) vary with the seasonal cycle (Chapter 4)?

d) How does ammonium affect nitrate uptake and phytoplankton growth and composition in the freshwater tidal zone of the Guadiana estuary (Chapter 5)?

e) Does light limitation occur in the Guadiana upper estuary throughout the seasonal cycle and is phytoplankton adapted to a low light environment (Chapter 6)?

f) Overall, how important is nutrient and light availability for phytoplankton succession and production in the Guadiana estuary (Chapter 7)?

8.2 The answers

In Chapter 2 we demonstrated that the use of phytoplankton as a biological quality element to assess the ecological status of coastal ecosystems will pose some constraints. The major problem encountered is related to the deeply-rooted used of chlorophyll *a* concentration as a proxy for phytoplankton biomass and even phytoplankton abundance, because chlorophyll *a* content within the cell may vary tremendously with the cells' physiological state. Indeed, a wide range of carbon to chlorophyll (C:Chl) ratios can be found in aquatic ecosystems, and the Guadiana estuary was no exception (C:Chl range 1.1 - 586.9 mg C mg Chl^{-1}: see Chapter 7). In addition, the use of chlorophyll *a* as a substitute of biomass may overlook blooms of pico- and small nanophytoplankton and overestimate the importance of large microphytoplankton. For instance, in the Guadiana estuary, cyanobacteria represent the major fraction of phytoplankton total abundance during the productive period; however, due to their small size, there is usually no signal of cyanobacteria blooms in chlorophyll *a* concentration. Given that microscopy techniques are time-consuming and require a well-trained observer, their use in monitoring programs is not cost-effective. Alternatively, we proposed the use of other techniques, such as remote sensing and chemotaxonomic analysis, as supplements in phytoplankton monitoring programs.

Tidally-induced variability of phytoplankton and their environmental drivers in the freshwater tidal zone of the Guadiana estuary were analysed in Chapter 3. We observed that the water was vertically and horizontally homogeneous, showing no evidence of haline or thermal water column stratification, and no significant tidally-induced differences were found for most physical-chemical variables. Some tidally-induced differences were observed in suspended particulate matter concentration and chlorophyll *a* concentration, related to seasonal and fortnightly variability in river flow and tidal currents, respectively. Overall, the differences detected were not as considerable as those observed in the lower estuary. However, the occurrence of tidally-induced variability in some seasons reflects that thorough sampling programs to study estuarine tidal dynamics should be conducted throughout the year; occasional sampling will not reflect the typical variability of these systems.

On an annual scale, nutrient and light availability in the Guadiana estuary, particularly in the freshwater tidal zone, were mostly regulated by river flow (Chapter 7). River flow controlled nutrient, particularly nitrogen, and suspended particulate matter (SPM) inputs into the estuarine zone. SPM was, in turn, the main regulator of light extinction in the middle and upper estuaries zones, controlling, therefore, light availability. Comparison of light and nutrient availability with other estuaries suggested that both light and nutrient limitation occurred throughout the year or seasonally, especially in the more turbid estuarine regions.

Nutrient (Chaptes 4 and 5) and light (Chapter 6) enrichment experiments confirmed the occurrence of resource limitation in the freshwater tidal reaches of the Guadiana estuary. In addition, enrichment experiments proved to be a solid strategy to infer on nutrient and light limitation of phytoplankton growth, although the interpretation of the outcomes of such experiments may not always be straightforward. Overall, phytoplankton in the Guadiana upper estuary was light-limited throughout the year and nitrogen-limited during the productive period.

Diatoms and green algae were the most nutrient-limited phytoplankton, responding significantly to nitrogen additions. Although nitrate was the main nitrogenous source for phytoplankton in the Guadiana estuary, an overall preference for ammonium was observed. Indeed, nitrate consumption decreased with increasing ammonium concentrations and uptake. However, different groups demonstrated different preferences in relation to their nitrogen source. Green algae and cyanobacteria preferred ammonium, whilst diatoms preferred nitrate. Increased anthropogenic inputs of ammonium and increased water and sediment retention behind dams, leading to reduced nitrate inputs to the estuarine zone, will possibly promote a shift on phytoplankton community composition towards the dominance of small-sized, ammonium-preferring groups.

Regarding light limitation, phytoplankton community was not acclimated to the low light conditions that prevail in the Guadiana upper estuary and light limitation occurred throughout the year. Diatoms were the most light-limited group, whilst cyanobacteria seemed to be more acclimated to low light. Contrary to other turbid estuaries, primary production was higher in the more turbid regions, where light availability was the lowest, but nutrient concentrations, although occasionally

limiting, were the highest. Therefore, phytoplankton in such turbid regions were the most efficient in using limiting resources.

8.3 The future

By the end of any research project, some questions were answered but many others are raised. This work was no exception, and several questions on phytoplankton dynamics in the Guadiana estuary remain unsolved. The interactive effects of light and nutrients on phytoplankton growth is one of the most immediate concerns. Although we concluded that both resources were limiting for phytoplankton, only simultaneous manipulations of light and nutrients can detect which one is the most limiting resource. In addition, the role of top-down processes has never been addressed in the Guadiana estuary. Considering that phytoplankton dynamics is regulated by interactions between bottom-up and top-down processes, this line of research is relevant to understand and predict phytoplankton variability. Finally, considering the predicted changes in global climate and the fact that the Guadiana estuary is located in a highly sensitive area to climate change, the evaluation of potential increases in CO_2, ultraviolet radiation and temperature on phytoplankton and ecosystem dynamics is pressing. The interactions between these atmospheric variables and other environmental variables, such as nutrient and PAR availability, is a pertinent subject that remains poorly studied in the world's ecosystems (Beardall et al., 2009). Furthermore, most studies on the effects of increased UV radiation and CO_2 on phytoplankton have been conducted with unialgal cultures; the impacts on natural phytoplankton communities are still poorly recognized (Sobrino et al., 2009).

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