TIDE-INDUCED VARIATIONS IN THE BACTERIAL COMMUNITY, AND IN THE PHYSICAL AND CHEMICAL PROPERTIES OF THE WATER COLUMN OF THE MONDEGO ESTUARY

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O bacterioplâncton é um componente chave da estrutura e função dos ecossistemas aquáticos. No entanto, o actual conhecimento dos controlos sobre a abundância e a actividade microbiana apenas evidenciam a sua complexidade. Nos estuários, a complexidade aumenta pelo facto de os parâmetros ambientais (salinidade, temperatura, pH, matéria orgânica e outros factores) serem extremamente variáveis.

O presente estudo investiga a dinâmica de três grupos de bactérias planctónicas envolvidos no ciclo de carbono (bactérias heterotróficas aeróbias, bactérias sulfato-redutoras e bactérias nitrato-redutoras) durante um ciclo de maré no estuário do Mondego.

Foi investigada a associação de diversos parâmetros físicos, químicos e biológicos na composição da comunidade bacteriana, utilizando métodos de análise multivariada, com o intuito de identificar as fontes de variabilidade na composição e dinâmica mareal da comunidade bacteriana no estuário do Mondego. A análise de componentes principais (ACP) permitiu identificar como fontes desta variabilidade, por um lado, as diferentes dinâmicas nos dois locais em estudo (Foz e Pranto), e por outro lado, os fluxos de maré de enchente e de vazante, pelos seus reflexos nos parâmetros ambientais.

Palavras-chave: bacterioplâncton, ecologia estuarina, distribuição mareal.

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The bacterioplankton is a key component of the structure and function of aquatic ecosystems. Yet, present understanding of the controls on microbial abundance and activity only highlights their complexity. In estuaries, the problem is further complicated by the high variability of environmental properties (salinity, temperature, pH, organic loading and other factors).
The present study investigates the dynamics of three main metabolic groups of planktonic bacteria involved in the cycling of organic matter (aerobic heterotrophic bacteria, sulphate-reducing bacteria, and nitrate-reducing bacteria), over one tidal cycle in the estuary of the Mondego.
The association of various physical, chemical and biological parameters with the composition of the bacterial community was assessed by multivariate analysis in order to identify key factors controlling the composition and tidal dynamics of the bacterial communities in the Mondego estuary. Principal component analysis (PCA) identified the sources of variability for the bacterial communities in the estuary, as being, on one hand, the different dynamics in the two stations under study (Foz and Pranto) and, on the other hand, the flood and ebb tide fluxes, by their effects in the environmental parameters.

Keywords: bacterioplankton, estuarine ecology, tidal variation.

INTRODUCTION

The estuary of the Mondego has been extensively studied in the aspects of nutrient dynamics (MARQUES *et al.*, 1997; PARDAL, 1998; AZEITEIRO, 1999; AZEITEIRO and MARQUES, 2000) and of the composition of the communities of estuarine invertebrates (PARDAL, 1998; AZEITEIRO and MARQUES, 1999; AZEITEIRO *et al.*, 1999 a, b). The bacterial community, vital in the various biogeochemical cycles (VALIELA, 1995) received, however, little attention. The present study emerged, therefore, from the need to analyse the environmental properties involved in the determination of the composition and dynamics of the bacterial community. It aimed also to the quantification of

different metabolic profiles relevant in the overall ecology of the estuary of the Mondego.

The Mondego estuary is an area under investigation as an eutrophication problem area (MARQUES *et al*., 1997; PARDAL, 1998; AZEITEIRO, 1999). Human impact on this estuary is essentially related to harbour activities, salt-works, aquaculture, and discharge of nutrients and chemicals from agricultural areas in the lower Mondego valley. Due to the extended residence time, the persistence of nutrients in the water column is relatively long, conducting to the eutrophication process taking place in the southern arm of the estuary (MARQUES *et al*., 1997; PARDAL, 1998; AZEITEIRO, 1999). It is, therefore, essential to monitor the physical and chemical conditions, and biological communities in order to have a clear perception of the potential modifications in the structure and function of the ecosystem, and to assess the reversibility of the ongoing environmental changes.

Bacteria have a very important role in planktonic marine microbial foodwebs (VALIELA, 1995). Bacteria comprise an important share of plankton biomass and their activities have a large impact on ecosystem metabolism and function (GASOL and DUARTE, 2000). The determination of the composition of bacterial populations, abundance of each metabolic group, and the discrimination of the different activities are essential in the understanding of pelagic ecology. Because of their ubiquity, diverse metabolic capabilities, and high enzymatic activity rates, bacterial communities play major biogeochemical roles, namely in carbon (VALIELA, 1995), sulphur (FAUQUE, 1995), iron (TORDELL *et al*., 1999) and nitrogen cycling (HERBERT, 1999).

The present and novel study investigates the dynamics of three main metabolic groups of bacteria, involved in the cycling of organic matter (aerobic heterotrophic bacteria, sulphate-reducing bacteria and nitrate-reducing bacteria), during a tidal cycle in the estuary of the river Mondego.

MATERIALS AND METHODS

Study site - The Mondego river, on the Western coast of Portugal, drains a hydrological basin of approximately 6.670 km^2 and its estuary (40º08' N 8º50' W; Figure 1) has an area of 3,3 km^2 and a water volume of 0,0075 km^3. Upstream, at about 5,5 km from the sea, the estuary is divided in two arms which converge near the mouth. The estuary is characterised by a tidal range of 0,35 to 3,3 m, average freshwater discharge of 8,5 x 10^9 $m^3.s^{-1}$, and average residence time of 2 d in the northern arm and 9 d in the southern arm (AZEITEIRO, 1999; AZEITEIRO and MARQUES, 2000). The south arm is almost totally silted in upstream areas, and consequently the water circulation depends on tides and, to a much smaller extent, on the freshwater discharge from a tributary - the Pranto River - which is controlled by a sluice located 3 Km from the confluence with the Mondego. Two sampling stations were located along the southern arm of the estuary: station 1 (S1) close to the mouth and station 2 (S2) near the Pranto.

Sampling program - Water samples (1 L) were collected at about 3 hour intervals over a tidal cycle that began at 9:15 and ended at 18:10, on the 15th of June 2000. Samples were collected at the water subsurface (*ca.* 0,3 m depth) and near-sediment surface at both stations (5 to 8 m and 0,5 to 2 m depth, respectively for S1and S2).

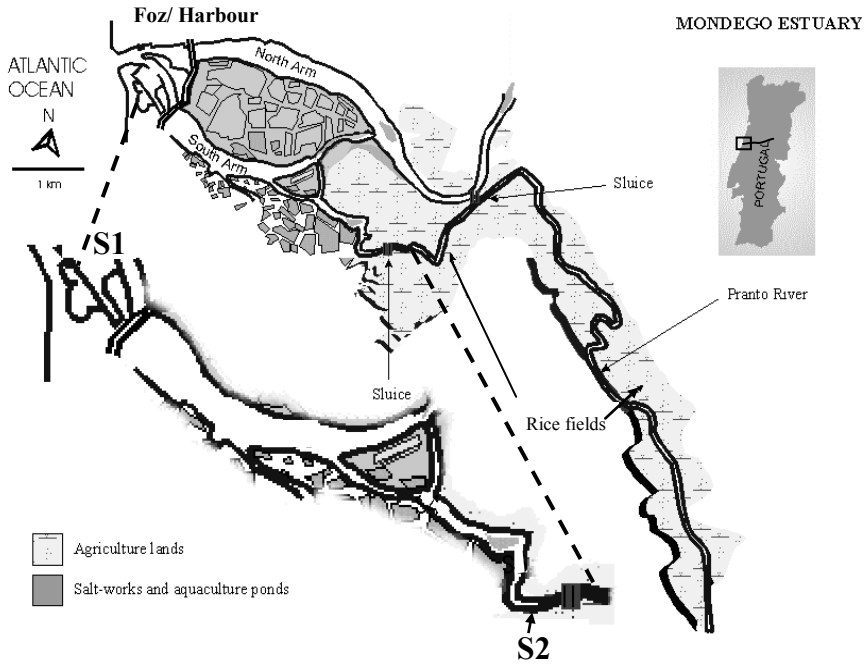

Figure 1. Map of the estuary of the Mondego river, showing the location of the sampling stations (S1, station 1; S2, station 2).

Determination of physical and chemical parameters - All samples were analysed *in situ* for salinity, temperature, dissolved oxygen, pH, E_h and conductivity. Samples were also analysed in the laboratory for their content in particulate organic matter, nitrate, nitrite, sulphate, phosphate and chlorophyll *a* (STRICKLAND and PARSONS, 1972).

Sampling of bacteria - Water samples (1 L) were collected into sterile glass flasks, filled to capacity, sealed with gas-tight rubber stoppers and immediately placed on ice until arriving at the laboratory (12-24 h later). These samples were obtained in parallel to those subjected to physical and chemical analysis.

Enumeration of viable bacteria - Numbers of viable aerobic chemoorganotrophic bacteria, sulphate-reducing bacteria, nitrate-reducing

bacteria, and iron-reducing bacteria were estimated through the Most Probable Number (MPN) method. Three or five replicates of series of 10-fold water dilutions, in appropriate selective liquid media (see below), were prepared in multiwell plates. Cultures were incubated at 37 ºC, in the dark, for 3 weeks (aerobes) or 6 weeks (anaerobes). The presence of bacteria was scored positive on the basis of turbidity or colour development in the case of iron sulphide precipitates, and confirmed by microscopic observation. The presence of nitrate-reducing bacteria was also confirmed by the production of nitrite or N_2. Growth of sulphate-, nitrate- and iron-reducing bacteria required strict anaerobic procedures that were used at all times (WIDDEL and BAK, 1992).

Growth media - The concentration of NaCl was adjusted to the average salinity of the estuary (20 g/L), and the pH was adjusted to 7 prior to autoclaving.

Media for aerobic heterotrophic bacteria: Two variants of the YPG medium were previously tested for growth of aerobic heterotrophic bacteria.: YPG containing yeast extract (2 g/L), bacto peptone (1 g/L) and glucose (2 g/L), and a 10 fold dilution of the YPG medium. The latter medium was intended for growth of oligotrophic bacteria. Undiluted YPG supported higher counts of aerobic bacteria and was adopted in this study.

Medium for sulphate-reducing bacteria (SRB): Three media were previously tested for growth of sulphate-reducing bacteria - a natural medium, a semi-defined medium VMN (BEECH and VITALIS, *unpublished*), and the saltwater WIDDEL and BAK multipurpose medium with trace elements and a vitamin mixture (WIDDEL and BAK, 1992). The natural medium was made up of water from the sampling site, which was filter-sterilised (0,22 μm pore diameter). Lactate (20 mM) was used as electron donor and carbon source, and sulphate (Na_2SO_4, 20 mM) as the terminal electron acceptor. Higher counts of SRB were obtained with VMN media, which was adopted for this work.

Medium for nitrate-reducing bacteria (NRB): The saltwater Widdel and Bak multipurpose medium was used adding nitrate ($NaNO_3$, 10 mM), instead of sulphate, as the terminal electron acceptor. Lactate was also added (20 mM).

Medium for iron-reducing bacteria (IRB): The FWA medium (LOVLEY and PHILLIPS, 1986) was tested for the growth of iron-reducing bacteria. Fe (III) was added at a final concentration of 10 mM.

Statistical analysis - Principal component analysis (PCA; SPAD 3.5, Cisia-Ceresta) was applied in order to identify the sources of variability for the bacterial communities under study, as well as the physical and the chemical parameters observed during the tidal cycle.

RESULTS

The patterns of variation of the measured environmental parameters, at each station and depth, are presented in figure 2. Salinity and conductivity values

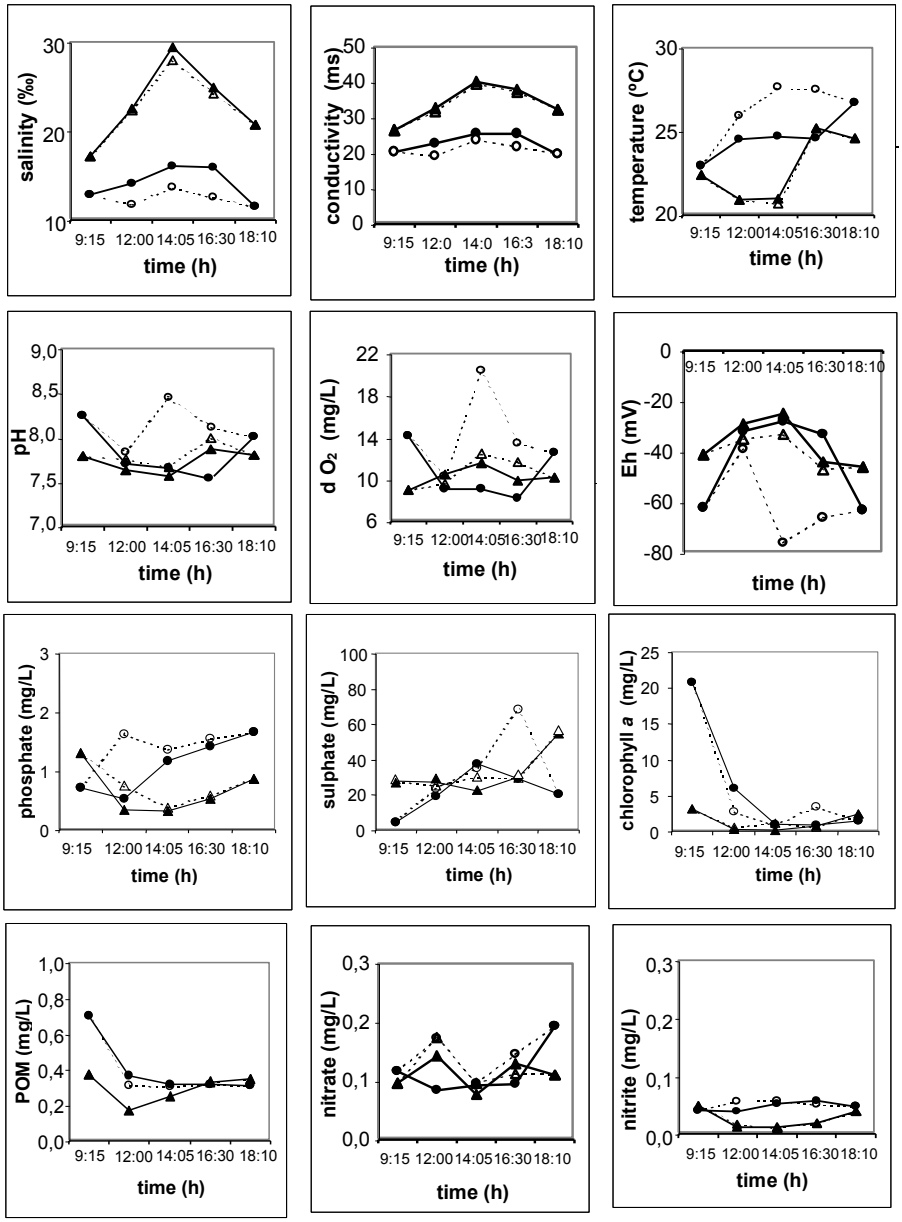

Figure 2. Changes in water physical, chemical and biological parameters at station 1 and station 2 during one tidal cycle. High tide was at 14h:05min. Legend: (△) station 1, subsurface water; (σ) station 1, near-sediment water; (μ) station 2, subsurface water; (λ) station 2, near-sediment water.

increased with flood tide and decreased with ebb tide over the tidal cycle, and were both greater at station 1 than at station 2. Both variables generally showed higher values near the sediment than in subsurface water. The temperature patterns at the two stations differed throughout the tidal cycle, even thought their initial values at 9:15 were similar. At station 1, the temperature decreased during flood tide (9:15 - 14:05) and increased at ebb tide (14:05 - 16:30), stabilising afterwards. The reverse pattern was observed in station 2, which showed a steady temperature rise, more pronounced at the subsurface water than near the sediment. Redox potential (E_h) also increased during the flood tide and decreased at ebb tide, except for station 2, at subsurface where a steep decrease in E_h values was registered from 12:00 till 14:05, followed by a slow rise of E_h values until 18:10. The concentration of dissolved oxygen at station 1 and in subsurface water of station 2 increased with the influx of saline water and decreased with outflow; however, near the interface with the sediment of station 2 the reverse was observed. pH values decreased during flooding (9:15 - 14:05) and increased during ebb tide, except for subsurface water of station 2; here, a strong increase of pH was observed at high tide (14:05) followed by a steady decrease in ebbing water.

Phosphate levels were generally greater at station 2. The inflow of saline water at station 1 and near the sediment at station 2, decreased phosphate concentration. However, the subsurface water of station 2 showed an increase in phosphate levels at 12:00. Nitrate concentration increased from 9:15 till 12:00 and decreased to a minimum at 14:05 (high tide), except in subsurface water of station 2. At ebb tide, nitrate concentration increased at station 2, while it levelled out or slightly decreased at station 1. Nitrite exhibited a different variation pattern in station 1 and station 2. At station 1, nitrite concentration decreased during flooding (9:15 - 14:05) and increased afterwards till 18:10. The reverse was observed at station 2, where nitrite levels increased slightly until 14:05 and decreased at 18:10. Sulphate concentration was almost constant from 9:15 till 16:30 at station 1, increasing afterwards with ebb tide. At station 2, sulphate levels nearly mirrored the tidal cycle, increasing with the inflow and decreasing with the outflow (even though with a 3 hours delay at the subsurface water). POM concentration in the water column decreased in both sites from 9:15 till 12:00 (flood tide), and increased slightly (near-sediment water at station 1) or stabilised (station 2, both depths) thereafter. As with the physical and chemical parameters, the pattern of variation in abundance of chlorophyll *a*, appeared to be affected by the tidal cycle, showing a decrease with influx of saline water and a slight increase during flushing.

Aerobic chemoorganotrophic bacteria, nitrate-reducing bacteria (NRB) and sulphate-reducing bacteria (SRB) were detected in the estuary of the Mondego, but not iron-reducing bacteria.

Bacterial dynamics during the tidal cycle, at the two sampling stations, is shown in figure 3. The numbers of aerobic chemoorganotrophic bacteria, at

station 1, decreased (20 to 100 fold) during the inflow of saline water, to a minimum at high tide (14:05), and subsequently increased with flushing. At

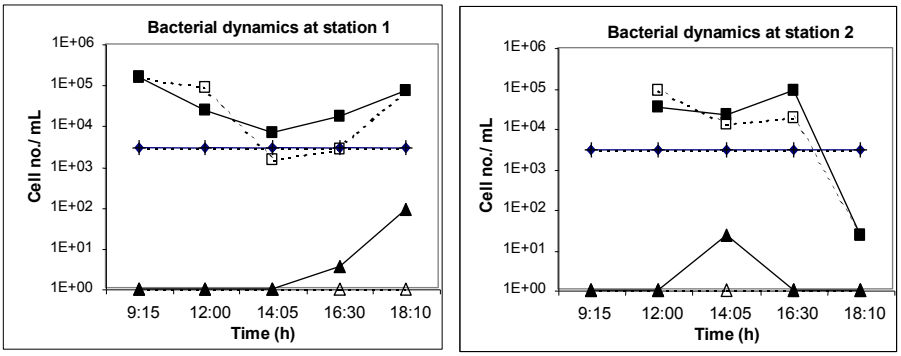

Figure 3. Changes in bacterial numbers at station 1 and station 2 during the tidal cycle. High tide was at 14h:05min. Legend: (ν) aerobic heterotrophic bacteria, near sediment; (□) aerobic heterotrophic bacteria, subsurface, (σ) SRB, near sediment; (Δ) SRB, subsurface (♦) NRB (note: this is a reference number as the number of NRB was determined to be always greater than $2{,}4x10^3$ cells/mL).

station 2, results also indicate a slight decrease in bacterial numbers with water inflow, followed by a steep decrease (up to 10^4 fold) from 16:30 till 18:10. SRB were only detected in deep water (interface with sediment) in station 1 (flushing period) and in station 2 (at high tide; Fig. 3). Our results, although still preliminary, determined that the number of NRB was greater than $2{,}4x10^3$ cells/mL at all studied sites, throughout the tidal cycle.

PCA of physical, chemical and biological factors *versus* samples matrix, taking into account the space of the first two axes of variability (65,90 %), showed a clear opposition along the first axis (45,29 %) between the two sampling stations (irrespectively of depth). Samples characterised by higher temperature, pH, E_h, phosphate and nitrite levels corresponded largely to those taken from station 2, and samples mainly characterised by higher salinity and conductivity, corresponded to samples collected from station 1. Therefore, the variability along the first axis includes a very pronounced spatial gradient (Fig. 4).

Along the second axis of variability (20.61 %), it is possible to recognise a distinction between samples collected during the flood tide, which are characterised by higher aerobic heterotrophic bacterial counts, chlorophyll *a* and POM levels, and samples collected during high tide and ebb tide, which are characterised by higher levels of dO_2. Therefore the variability along the second axis corresponds primarily to a spatio-temporal (tidal) gradient (Fig. 4).

As a whole, the sample projection in the space of the first two axes of variability made it possible to determine a spatial gradient that separates the two stations, and a temporal gradient that recognises the tidal cycle in both stations.

The third principal factor (12,41 %) recognised an opposition between sites with high levels of nitrate and sites with high levels of sulphate (not shown).

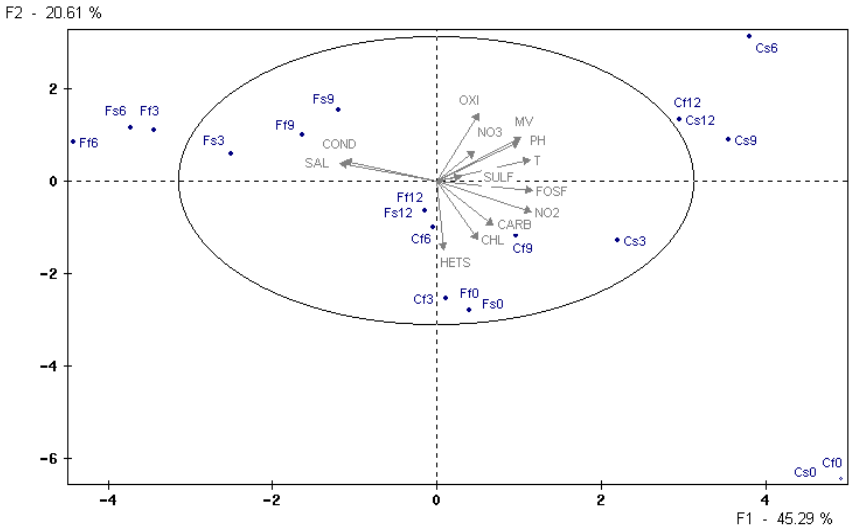

Figure 4 - Projection of the principal components 1 and 2 (F1 and F2) for the PCA of physical, chemical and biological factors *versus* samples matrix during the tidal cycle on the 15th June 1999 (SPAD 3.5, Cisia-Ceresta).
Descriptors: salinity (SAL), conductivity (COND), temperature (T), pH (PH), E_h (MV), dissolved oxygen (OXI), particulate organic matter (CARB), nitrate (NO3), nitrite (NO2), sulphate (SULF), phosphate (FOSF) and chlorophyll *a* (CHL), MPN of heterotrophic bacteria (HETS). Ff - station 1 (F=Foz) near sediment (f=bottom); Fs - station 1 (F=Foz) subsurface (s=subsurface); Cf - station 2 (C=Pranto) near sediment (f=bottom); Cs - station 2 (C=Pranto) subsurface (s=subsurface); numbers 0, 3, 6, 9, 12 correspond to sampling times 9:15, 12:00, 14:05, 16:30 and 18:10.

DISCUSSION

The present study showed that bacterial dynamics in the estuary of the Mondego, as well as the environmental physical and chemical parameters, are dominated by the spatial structure of the estuary and, to a less extent, by the tidal pattern, during a tidal cycle (from the multivariate analysis). Similar distribution patterns were found in annual cycle studies in the same estuary with physical, chemical parameters and biological parameters (AZEITEIRO, 1999; AZEITEIRO and MARQUES, 2000), namely zooplankton (AZEITEIRO and MARQUES, 1999; AZEITEIRO *et al*., 2000).

Station 1, near the mouth of the estuary, showed the effects of saline intrusion in the fluctuation of salinity, redox potential, conductivity and dissolved oxygen. It also showed a dilution pattern in pH, temperature, phosphate, nitrite, POM,

chlorophyll *a*, and number of heterotrophic bacteria. The different patterns of sulphate, pH and redox potential, between the near-sediment and subsurface water, suggest that the inflow of saline water at the mouth of the estuary is wedged, which agrees with reports by GONÇALVES (1992).

Station 2 showed effects of tidal intrusion in the values of salinity, conductivity, sulphate and phosphate levels, while it exhibited a dilution effect on POM, chlorophyll *a* and number of heterotrophic bacteria (only between 12:00 and 14:05). The different variation patterns of pH, redox potential and dissolved oxygen observed in subsurface and near-sediment water may be related to the chemical environment at station 2: this is subjected to inputs of freshwater (through leakage over the sluice) which leaches highly fertilised, iron-rich agricultural fields, facts that may affect the phosphate and ionic dynamics of the receiving stream, in station 2 (PEREIRA, 1999). This aspect needs to be further addressed at in the laboratory.

The observed intrusion *versus* dilution effect of saline water was also evident from the observation of the multivariate analysis plot (namely PC2). PC3 clearly separated samples collected from subsurface and near-sediment water at station 2 (not represented in the plot), which were characterised, respectively, by high nitrate levels and high sulphate levels.

The different environmental characteristics of the two stations determined the distinct patterns observed for the bacterioplankton. PC2 indicated a close relationship between the abundance of aerobic chemoorganotrophic bacteria, chlorophyll *a* and POM (corroborated by positive correlation between them), and opposed the latter three variables to dO_2 (negatively correlated with those variables). A close relationship between bacterial and phytoplankton (represented by chlorophyll *a*) compartments is often reported in aquatic systems, less clearly in estuaries where the allochtonous supply of organic matter is appreciable (DUCKLOW, 1999; CHATILA *et al*., 1999; JUGNIA *et al*., 1999). The opposition to variable dO_2 suggests that more oxygen was being used than produced in the system. A possible explanation to this would be an increase of microbial activity by heterotrophs, consuming carbon resources and oxygen, and/or using carbon resources under low dO_2 conditions (as supported by PC2).

The results of PCA suggest that NRB activity may be of great relevance in the cycling of carbon in the estuary of Mondego, particularly in station 2 (supported both by PC1 and PC3, and levels of nitrite), even though their abundance was only determined to be "greater than $2{,}4 \times 10^3$ cells/mL" in all sites. Although NRB are mainly active under anaerobic conditions, they can also be active under aerobiosis (ZUMFT, 1997), and this might have caused some overlapping of NRB with MPN counts of aerobic heterotrophic bacteria. This is supported by the fact that MPN counts of aerobic heterotrophs were positively correlated with nitrite levels.

SRB were detected in very low numbers during the tidal cycle, their presence being closely related with the increase of sulphate concentration (and supported

by PC3). The fluctuation of SRB and sulphate concentration in the water column appeared to result from sediment ressuspension induced by the water influx and outflow during the tidal cycle, as they coincided with high tide at station 2, and ebb tide at station 1 (N. DUARTE, *pers. com*.). The opposition revealed by PC3 (see Results), suggested a possible competition between SRB and NRB for carbon sources in the estuary. This may, however, be limited to the water interface with the sediment and the sediment itself where both groups of bacteria are most active (TESKE *et al*., 1998; HERBERT, 1999; VALIELA, 1995).

The present study indicates that the three groups of heterotrophic bacteria investigated, particularly the NRB and the aerobic heterotrophs, were involved in the cycling of carbon in the water column of estuary of the Mondego, their specific relevance to this cycle being dependent on the particular physicochemical and biological environment.

Principal components PC4 and PC5 (not shown) were not strongly explained by any of the biological or physicochemical variables measured in the present study. Future studies will look at these unanswered questions.

In situ dynamics of the various bacterial groups remains mostly unknown. Further studies using molecular biology methods (DGGE of 16S rDNA sequences) will help elucidating the diversity of phylogenetic groups and which represent the allochtonous and autochthonous populations in the estuarine transition environments.

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