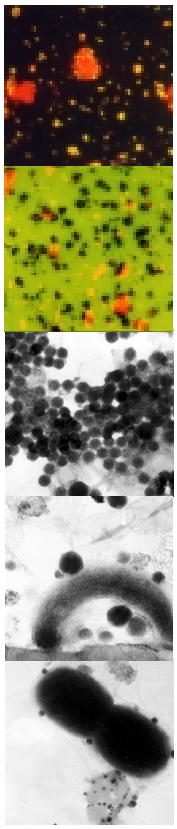
Universidade de Aveiro Departamento de Biologia
2001

Maria Adelaide de Pinho Almeida

Crescimento e mortalidade do bacterioplâncton em ambiente estuarino (Ria de Aveiro)



Universidade de Aveiro Departamento de Biologia
2001

Maria Adelaide de Pinho Almeida

Crescimento e mortalidade do bacterioplâncton em ambiente estuarino (Ria de Aveiro)

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Fernanda da Fátima Ribeiro Pereira de Saldanha Alcântara, Professora Associada Aposentada do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente

Prof. Dr. José Pereira da Costa Tavares
professor catedrático da Universidade de Aveiro

Prof. Dr. Milton Simões da Costa
professor catedrático do Departamento de Bioquímica da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Prof.ª Dr.ª Natércia Julieta Fernandes Teixeira Serra Rodeia
professora catedrática do Departamento de Biologia Vegetal da Faculdade de Ciências da Universidade de Lisboa

Prof. Dr. Amadeu Mortágua Velho da Maia Soares
professor associado com agregação da Universidade de Aveiro

Prof.ª Dr.ª Maria Helena da Cunha Soares Lopes Dias Moreira
professora associada da Universidade de Aveiro

Prof.ª Dr.ª Helena Maria Leitão Demigné Galvão
professora auxiliar da Faculdade de Ciências do Mar e do Ambiente da Universidade do Algarve

Prof.ª Dr.ª Fernanda da Fátima Ribeiro Pereira de Saldanha Alcântara
professora associada aposentada da Universidade de Aveiro

agradecimentos

É esta uma altura privilegiada para demonstrar o meu reconhecimento a todos aqueles que contribuíram para a realização deste trabalho.

À Doutora Fernanda Alcântara, Professora Associada do Departamento de Biologia da Universidade de Aveiro, orientadora desta tese, pelo seu sentido crítico, rigor científico, incentivo e amizade demonstrados ao longo de todo este trabalho.

À Licenciada Ângela Cunha, pela amizade, solidariedade e pelo espírito de equipa que sempre manifestou, em particular ao longo do trabalho laboratorial desenvolvido em conjunto.

Ao Professor Doutor Mikal Heldal do Department of Microbiology and Plant Physiology da Universidade de Bergen (Noruega) pela ajuda na montagem da técnica de pesquisa de vírus e pelo auxílio prestado na interpretação de alguns resultados experimentais.

Ao Professor Doutor Hans Hoppe do Institut für Meereskunde da Universidade de Kiel (Alemanha) por me ter proporcionado a possibilidade de participar no Programa de Mobilização "Microheterotrophs and Viruses" no Department of Microbiology and Plant Physiology da Universidade de Bergen, no qual tive a oportunidade de aprender e praticar técnicas de quantificação de vírus e de enumeração de bactérias activas.

Ao Doutor Klaus Gocke do Institut für Meereskunde da Universidade de Kiel (Alemanha) e ao Professor Doutor António Calado do Departamento de Biologia da Universidade de Aveiro pela ajuda na revisão dos manuscritos.

À Professora Doutora Marina Cunha do Departamento de Biologia da Universidade de Aveiro pela ajuda prestada no tratamento estatístico dos dados.

Às técnicas Helena Dias e Conceição Saraiva do Departamento de Biologia da Universidade de Aveiro, pela amizade e pelo auxílio no trabalho laboratorial.

Aos técnicos Rui Marques, Aldiro Pereira e Armando Costa do Departamento de Biologia da Universidade de Aveiro, pelo apoio prestado no trabalho de campo.

Aos Licenciados Clarinda Ferreira, Maria Miguel Manão, Miguel Moreira, Carla Fontes e Nuno Boucinha pelo auxílio no trabalho laboratorial.

Aos meus colegas de Departamento e aos meus amigos por todo o apoio e colaboração prestados nestes anos.

As últimas palavras são para o meu Marido e para os meus Filhos, cujo amor, carinho e apoio incondicional em todos os momentos, penso não conseguir jamais agradecer suficientemente.

resumo

O objectivo deste trabalho foi estudar o fluxo de materiais e de energia no bacteriopâncton do complexo sistema da Ria de Aveiro.

O estudo de campo permitiu caracterizar perfis longitudinais, verticais e tidais de abundância e de produtividade bacteriana, pondo em evidência alguns dos factores que controlam esses padrões de variação. Os ensaios laboratoriais foram realizados para caracterizar o potencial bacteriano de reactivação, o efeito das correntes tidais sobre a actividade do bacteriopâncton e a contribuição da infecção viral e da predação para a mortalidade bacteriana.

No estuário foram encontrados perfis longitudinais de abundância (bactérias totais 2,6-15,3 x 10^9 l^{-1} e bactérias activas 0,1-3,7 x 10^9 l^{-1}) e de produtividade (0,05-18,6 µg C l^{-1} h^{-1}) distintos, com máximos no estuário médio e mínimos no estuário inferior.

Na secção mais baixa do estuário a abundância e produtividade foram semelhantes ao longo da coluna de água mas, na zona marinha, a produtividade bacteriana foi significativamente mais elevada à superfície (até 0,5 m) que nas camadas mais profundas da coluna de água.

Foi observado um padrão tidal de variação bastante nítido, nomeadamente na zona marinha, caracterizado por aumentos de densidade e produtividade perto da baixa mar e decréscimos na preia mar.

A variação sazonal do bacteriopâncton foi característica de um sistema temperado, com picos durante a estação quente e valores mais baixos nos meses frios.

Neste estuário, a maior parte das bactérias (cerca de 70 %) são aparentemente inactivas e podem não responder, em períodos curtos de 3 horas, à estimulação com nutrientes.

De entre os factores ambientais estudados, a temperatura e a salinidade foram as variáveis que melhor explicaram a variação bacteriana. A disponibilidade em substratos também foi um factor importante no controle da actividade bacteriana, nomeadamente na zona marinha do estuário. Embora durante a estação quente uma média de 21 % da produção primária seja potencialmente suficiente para suportar a produção bacteriana no estuário, a matéria orgânica aloctone pode suportar uma parte significativa do crescimento bacteriano, nomeadamente nos meses frios e na zona salobra. A contribuição da ressuspensão de sedimentos e da entrada de materiais particulados a partir da área de sapal pode ser considerada irrelevante para a actividade bacteriana.

A infecção viral e a predação exercem um controle forte sobre o bacteriopâncton da Ria. A contribuição da predação para a mortalidade bacteriana foi similar ao longo do estuário (69 % na zona marinha e 73 % na zona salobra) mas o controle por infecção viral (nas condições da experiência) na zona salobra atingiu quase o dobro (59 % na zona salobra e 36 % na zona marinha).

A capacidade de resposta rápida à variação das características da água põe em evidência a existência de reactividade bacteriana durante o ciclo tidal. Os diferentes perfis de variação apresentados pelas bactérias marinhas e salobras sugerem que a comunidade bacteriana pode variar ao longo do estuário.

Em face dos resultados apresentados conclui-se que o bacteriopâncton da Ria de Aveiro está sujeito a pressões físicas, químicas, nutricionais e biológicas que estão em diferente balanço nas zonas marinha e salobra do estuário.

abstract

The objective of this work was to study the fluxes of materials and energy at the level of the bacterioplankton in the complex system of Ria de Aveiro.

Field studies were undertaken in order to characterise longitudinal, vertical and tidal profiles of bacterioplankton abundance and productivity, putting in evidence some of the factors that control these profiles. Laboratory assays were performed to characterise the bacterial potential to loose or resume viability, the effect of tidal currents on bacterial activity and the contribution of viral infection and predation to bacterial mortality.

A distinct longitudinal profile of bacterial abundance (total bacteria 2.6-15.3 x 10^9 l^{-1} and active bacteria 0.1-3.7 x 10^9 l^{-1}) and productivity (0.05-18.6 µg C l^{-1} h^{-1}), with peaks at the mid-section, was observed in the estuary.

In the shallower section of the estuary, bacterial abundance and productivity were similar down the water column but in the marine zone bacterial productivity was significantly higher (3.3 times, on average) at the surface (down to 0.5 m) than in the deeper layers of the water column.

A clear pattern of tidal variation in density and productivity could be observed, namely in the marine zone. It was characterised by increasing density near low tide relatively to high tide.

The seasonal variation of baterioplankton was characteristic of a temperate system with peaks during the warm season and the lowest values during the cold months.

In this estuary, most of the bacteria (about 70 %) are apparently inactive and could not respond to nutrient amendments within the period of time of 3 hours.

Among the studied environmental factors, temperature and salinity were the variables that better explained bacterial variation. Substrate availability was also an important factor controlling bacterial activity, namely in the marine zone. Although during the warm season, an average of 21 % of the primary production was potentially sufficient to support the whole bacterial production in the estuary, allochthonous organic matter showed to support significant bacterial growth, namely in the cold months and in the brackish water zone. The contribution of sediment resuspension and of the input of particulate matter from the salt marshe area was irrelevant to bacterial activity.

Within the estuarine system, viral infection and predation exert strong control on the bacterioplankton compartment. The contribution of predation to bacterial mortality was similar throughout the system (69 % in the marine zone and 73 % in the brackish water zone) but the control by maximal viral infection (in the conditions of the experiment) almost doubled in the brackish water zone (59 % in brackish water and 36 % in marine water).

The capacity for short-term responses to changing water properties is evidence for reactivity during tidal cycles. The different profiles of marine and brackish water bacteria to these conditions suggest that bacterial assemblages are diverse within the estuary.

In face of the present results, it is concluded that the bacterioplankton of Ria de Aveiro evolves under physical, chemical, nutritional and biological pressures that are in different balance in the marine and brackish water zones.

CONTENTS

CHAPTER 1

INTRODUCTION

1. Marine and estuarine bacterioplankton

1.1. Ecological role of bacterioplankton

Bacteria are the most abundant and the most important biological component involved in the turnover (transformation and remineralisation) of organic matter in aquatic systems (Cho and Azam, 1990; Pomeroy *et al*., 1991). In coastal waters, heterotrophic bacteria often amounts to 10-30% of the living carbon biomass (Holligan *et al*., 1984, Cho and Azam, 1990) and may utilize as much as 40% of the carbon fixed by the primary producers (reviewed by Cole *et al.*, 1988; Cho and Azam, 1990; Ducklow and Carlson, 1992).

Heterotrophic bacteria are particularly skilled for organic matter transformation. They hydrolise dissolved and particulate organic matter, they can utilise substrates of difficult degradation and even use different allocthchtonous compounds as further sources of organic carbon. They convert dissolved organic carbon that would inevitably be lost to other members of the food web, into particulate carbon that becomes then potentially available to higher trophic levels (microbial loop). This capacity to the recover of dissolved organic carbon is significant in that it represents a link between the sources at different levels and higher consumers (Azam and Hodson, 1977; Azam, 1998; Pomeroy and Weibe, 1998). Moreover, studies in several aquatic environments have indicated that the microbial loop (phytoplankton $\rightarrow$ DOC $\rightarrow$ bacteria $\rightarrow$ protozoa $\rightarrow$metazoa) can processes roughly as much energy as the classical grazing food chain (phytoplankton $\rightarrow$ herbivore animals $\rightarrow$ carnivores animals) as reported by Riemann and Sondergaard (1986). Therefore the microbial loop has become increasingly recognized as an important component of the aquatic food web and it has been suggested that it must be explicitly represented in ecosystem models (Anderson, personal communication).

Through remineralisation of organic matter, bacteria regenerate nutrients *in situ* which are then used by the primary producers. Heterotrophic bacteria respire organic carbon to inorganic carbon at high rates, making bacterial respiration the major fraction of the total measured respiration in most aquatic systems (Williams, 1981). Cole *et al.* (1988) estimated, across a wide range of trophic conditions, that bacterial respiration is, at least, as larger as zooplankton respiration, reaching values up to 40% of the total planktonic respiration (Cole and Pace, 1995).

It has been shown that bacteria not only transform and respire organic matter, but they also contribute for the export of relatively recalcitrant compounds to the bottom layers of water basins (Smith *et al.*, 1992; Rivkin *et al.*, 1996). Export is supported by the occurrence of enzymatic hydrolysis without efficient uptake of the resulting hydrolysate (Smith *et al.*, 1992). Recently,

Stoderegger and Herndl (1998) showed that bacteria release up to 9% of the total carbon uptake in the form of difficult-to-degrade capsular material. The incorporation and respiration rates of this carbon material by bacterioplankton is three orders of magnitude lower than for glucose, suggesting that some forms of bacterial derived organic carbon may contribute substantially to the semilabile organic carbon pool in aquatic systems (Stoderegger and Herndl, 1998). Moreover, it has been shown that the small organisms that characterise the microbial loop (mainly bacteria), although generally grazed by organisms in the traditional food chain, may also aggregate and sink (Cho and Azam, 1988). So, the traditional view that carbon export is solely operated via large organisms requires revision (Anderson, personal communication).

Understanding the transformation and remineralization dual character of planktonic bacteria in aquatic systems is a central paradigm of contemporary microbial ecology (del Giorgio and Cole, 1998).

1.2. Contrasting properties of marine and estuarine bacterioplankton

Differences and similarities between ecosystems, communities, species and processes have always attracted the scientific community. Such comparative analyses produce valuable information about basic structures and possible functions of the ecosystems (Rieman and Christoffersen, 1993).

It is clear that physical, chemical and biological factors influence the microbial communities and operate with different strength in oceanic and estuarine environments. Nevertheless, the fundamental principles of the trophodynamic structure and functions are the same in oceanic and estuarine environments (Rieman and Christoffersen, 1993). Differences between ecosystems comprise the relative importance of various microorganisms or microbial processes (Rieman and Christoffersen, 1993). Autochthonous material, nutrient starvation (oligothrophic conditions), small cells, sodium growth requirement and seasonally-stable regime dominate in ocean systems (Cushing, 1989; Schultz and Ducklow, 2000), while allochtonous material, high nutrient concentrations (mesotrophic and eutrophic conditions), large cells, high bacterial diversity, strong-seasonal regime, steep physical, chemical and biological gradients are more important in estuarine ecosystems (Cushing, 1989; Rieman and Christoffersen, 1993; Thingstad *et al.*, 1999; Schultz and Ducklow, 2000). In addition, physical factors as wind forcing and water circulation are capable of changing estuarine conditions over a much smaller temporal scale than those occurring in the open ocean (Schultz and Ducklow, 2000). Moreover, the strong relationship between bacterial and primary production observed in the open ocean and in lakes (Cole *et al.*, 1988; Connan *et al.*, 1999) is not seen in estuarine systems (Ducklow and Kirchman, 1983; Wright and Coffin, 1984; Findlay *et al.*, 1991; Ducklow and Shiah, 1993). This absence of strong links could be due to differences in the metabolic capabilities of different bacterial communities found within the estuary according to space and time (Schultz and Ducklow, 2000).

1.3. Diversity of bacterioplankton

In general it has been observed a relatively low bacterial diversity in aquatic environments (Rehnstam *et al.*, 1993; Murray *et al.*, 1998). Pinhassi *et al.* (2000) found that a limited number of individual bacterial (typically 10 species) consistently represented the majority of the bacterial community in different marine areas. Moreover, the denaturing gel electrophoresis technique, recently applied to samples from aquatic environments, revealed a limited number of bands, while the same technique applied to a soil microbial community revealed a very high diversity (Murray *et al.*, 1996; Ovreas *et al.*, 1997; Ovreas *et al.*, 1998). A low or moderate bacterial diversity in aquatic samples has also been derived from theoretical considerations based on the number of bacteriophages versus bacterial numbers (Thingstad and Lignell, 1997). In aquatic systems, viral density is high and this implies that each potential host must be present also at high concentrations in order to allow efficient virus propagation (Wiggins and Alexander, 1985; Wilcox and Fuhrman, 1994). It was concluded that, in marine environments and at each site and moment, there is a limited number of dominant bacterial species, refuting the hypothesis of the presence of an infinite number of species at high densities (Pinhassi *et al.*, 2000). Moreover, it has been shown that most of the representative genera, widely distributed between different sea areas, show closely related isolates (Hagstrom *et al.*, 2000).

In estuarine environments it is expected a high bacterial diversity due to the mixing of seawater and freshwater and the transportation of particles from many sources, including salt marshes, mudflats, rivers and bottom sediments. Bible and Fletcher (1995) found that in the Chesapeake Bay the free-living bacteria composition differed considerably in the upper- and mid-bay regions. Moreover, Bie *et al.* (2001) showed that the composition of microbial assemblages varied along physical and chemical gradients. Murray *et al*. (1996) found that in two California estuaries the bacteria similarity in adjacent stations was greater than the similarity in distant stations. They suggested that differences in the quality of organic matter and in phytotypes along gradients may control the composition of bacterial assemblages. However, mixing of different communities may also contribute to the observed gradients (Murray *et al.*,1996).

It has been shown that bacterial composition of estuarine environments differ from oceanic waters (Martinez *et al.*, 1996; Hagstrom *et al.*, 2000). Hagstrom *et al.* (2000) studied the bacterial composition from marine and estuarine areas and found that the bacterial community of the Baltic estuary had a different overall composition at the genus level when compared to the open sea areas. The authors detected the lack of marine (salt tolerant) genera in the Northern Baltic and attributed this to the low salinity observed in this area (< 10 psu). In fact, Hofle and Bretar (1995, 1996) found the presence of *Pseudomonas* and *Shewanella* in the Baltic instead of species with salt requirement for growth (i.e. *Alteromonas*, *Roseobacter* and *Vibrio*) detected in open sea areas. Moreover, Methé *et al.* (1998) found high abundance of β-proteobacteria in freshwater lakes but the lack of these organisms in the sea and suggested that the presence of these bacteria in coastal

environments could be used as a marker for terrigenous inputs of bacteria in coastal waters (Hobbie, 1988). These community differences could be explained, in part, by different requirements for salt, but there are, probably, other factors determining the geographic distribution of different assemblages of bacterial species. Dynamic events like algal blooms, temperature change and upwelling have been also referred as important factors in the changing of the structural composition of bacterial communities (Fukami *et al.*, 1985; Hagstrom *et al*., 2000).

Differences in metabolic properties (e.g. bacterial production and substrate turnover) in different aquatic areas can be related to the distribution of specific bacterial populations (Hollibaugh, 1994; Martinez *et al.*, 1996; Murray *et al.*, 1996, Pinhassi *et al.*, 2000; Fandino *et al.*, 2001). In fact, Crump *et al.* (1999) found that the rapidly growing members of particle-attached estuarine bacteria were almost absent from the free-living or particle-attached bacteria of both coastal and riverine waters. On the other hand, the free-living estuarine bacteria were similar either to river or coastal bacterial members. Additionally, it has been proved that the higher cell specific activity of particle-attached-bacteria (Karmer and Herndl, 1992; Smith *et al.*, 1992) derives from the different phylogenetic identity of these bacteria when compared to free-living cells, rather than to substrate induction (DeLong *et al.*, 1993; Acinas *et al.*, 1999; Fandino *et al.*, 2001).

1.4. Bacterioplankton abundance and production

Since the 1970`s it has become increasingly clear that bacterioplankton makes a significant contribution to planktonic abundance (Holligan *et al.*, 1984; del Giorgio and Cole, 1998;) and productivity (Cole *et al.*, 1988; Goosen *et al.*, 1997) in aquatic systems.

Physical and chemical properties of the water column as well as the abundance and productivity of the bacterial community vary in aquatic systems according to the temperature range (Heinanen and Kuparinen, 1991; Di Siervi et al., 1995), depth of the water column (Pace and Cole, 1994; Talbot *et al.*, 1997) and, in estuaries, with the proximity to the sea (Almeida and Alcântara, 1992; Alcântara and Almeida, 1994; Goosen *et al.*, 1995; Goosen *et al.*, 1997; Kornas *et al.*, 1998) and with tidal water circulation (Almeida and Alcântara, 1992; Shiah and Ducklow, 1995; Hoppe *et al.*, 1996). In spite of the large ecological diversity among particular areas, common features of the bacterial community can be determined. Total and active bacterial numbers and productivity are generally highest in the warm season, in the photic zone, at the mid-upper estuarine sections as well as near low tide (Almeida and Alcântara, 1992; Alcântara and Almeida, 1994; Di Siervi *et al.*, 1995; Hoppe *et al.*, 1996; Goosen *et al.*, 1997).

1.4.1. Profiles of bacterioplankton abundance (total and active bacteria)

The levels of bacterial abundance in various aquatic systems (usually in the order of 10^8 to 10^9 cells l^{-1}) are relatively invariant when compared to the variation over 4-5 orders of magnitude

in activity measurements (Chin-Leo and Brenner, 1992; del Giorgio *et al.*, 1996; Hoppe *et al.*, 1996). Bacterial density, however, can vary greatly in different environments. Estuarine waters are 1-3 orders of magnitude richer in bacterioplankton than the open ocean (Hall and Vincent, 1990; Pomeroy *et al.*, 1994; Bianchi and Giuliano, 1996; Talbot *et al.*, 1997; Bouvy *et al.*, 1998; Kornas *et al.*, 1998; Borsheim, 2000). Bacterial abundance in the ocean seldom exceeds 2 x 10^9 cells l^{-1}, whereas it commonly does so in estuaries (Ducklow, 1992). The lower limit of the range reported for the open ocean reach values in the order of 10^7 cells l^{-1} (Hall and Vincent, 1990; Pomeroy *et al.*, 1994; Bianchi and Giuliano, 1996). The upper limit of the range has been observed in temperate estuaries and is in the order of 10^{10} cells l^{-1} (Hoppe *et al.*, 1996; Amon and Brenner, 1998; Cunha *et al.*, 2000). In the open ocean, bacterial abundance exhibits clear vertical profiles of variation according to the decrease of cell density below the photic zone (Bianchi and Giuliano, 1996; Borsheim, 2000; Bode *et al.*, 2001). In coastal waters, bacterial density is, in general, highest in surface water (Heinanen, 1991) but in shallow estuarine systems bacterial abundance can be homogeneous down the water column (reviewed by Ducklow and Shiah, 1993) or even higher near the bottom (Amon and Benner, 1998). Total bacterial numbers in open oceanic waters and in coastal waters decrease, in general, in the cold season by a factor of 4 to 14 relatively to the warm season (Hoppe, 1978; Sommmaruga and Conde, 1997; Yanada *et al.*, 2000). In estuarine systems, distinct longitudinal profiles of variation are generally characterised by increases in bacterial abundance from the outer to the upper- (Palumbo and Fergunson, 1978; Goosen *et al.*, 1997; Kornas *et al.*, 1998) or mid- (Wright and Coffin, 1983; Bordalo *et al.*, 1998; Cunha *et al.*, 2000) estuarine sections. In these systems the variation associated to tidal currents is generally characterised by increasing bacterial density near low tide compared to high tide (Shiah and Ducklow, 1995; Hoppe *et al.*, 1996).

It has been shown that only a fraction of the total bacterial number is metabolically active (del Giorgio *et al.*, 1996; Ullrich *et al.*, 1996; Sommaruga and Conde, 1997; Sherr *et al.*, 1999; Choi *et al.*, 1999; Bernan *et al.*, 2001). Consequently, the total bacterial number may not be a useful ecological parameter, since only the fraction of metabolically active bacteria are responsible for growth, nutrient uptake and transformation of organic carbon substrates. On the other hand, this fraction may also be selectively grazed (Sherr *et al.*, 1992; del Giorgio *et al.*, 1996) or infected by viruses (Proctor and Fuhrman, 1990). Total bacterial numbers yield valuable information on standing stock without however reflecting the real metabolic activity (review by Es Van and Mayer-Reil, 1982). It is possible that the number of active cells is more variable among systems than the total number of bacteria, and that this variation is masked by a rather large and constant pool of inactive bacteria (del Giorgio and Scarborough, 1995). Therefore, it may be pertinent to distinguish between active and inactive bacteria in an ecosystem.

Several methods have been developed to estimate the fraction of active bacteria in aquatic environments. Among them, those involving microscope direct counts (Tabor and Neihof, 1984; Rodriguez *et al.*, 1992) and, more recently, flow cytometry (Porter *et al.*, 1995; Button *et al.*, 1996) have been preferred to the more complex and time-consuming microautoradiography procedure (Meyer-Reil, 1978; Tabor and Neihof 1982). Direct viable count methods, based on cell enlargement response, suffer however from methodological limitations when applied to natural samples (Joux and LeBaron, 1997; Ullrich *et al.*, 1999; Choi *et al.*, 1999). Direct viable count methods based on the addition of a single antibiotic (nalidix acid), or of an antibiotic cocktail, require the addition of supplementary substrates (yeast extract) and, when applied to complex communities have limitations due to the variable response of the different bacteria to the antibiotic, making the enlargement response difficult to evaluate (Thorsen *et al.*, 1992). On the other hand, direct methods based on determinations of the activity of the electron transport system, predominantly following the use of the tetrazolium salt 2-p-iodo-phenyl-3-p-nitrophenyl-5 phenyltetrazolium chloride (INT) (Zimmermann *et al.*, 1978) and, more recently, of a fluorochrome version, 5-cyano-2,3 ditolyl tetrazolium chloride (CTC) (Rodriguez *et al.*, 1992) underestimates the fraction of active bacteria (Ullrich *et al.*, 1999; Choi *et al.*, 1999). Underestimation is due to the toxicity of the redox compound to bacteria (Ullrich *et al.*, 1996) and to the fact that not all bacteria can effectively reduce CTC (Smith and McFeters, 1997). More recently, other methods have been proposed to distinguish inactive from active bacteria using fluorogenic compounds, such as TOPRO-1, methods that identify non-viable cells with membrane and cell wall integrity compromised (Williams *et al.*, 1998). Universal 16S rRNA-targeted oligonucleotide probes have been also used to identify bacterial cells as potentially metabolically active (Karmer and Fuhrman, 1997).

Although few studies have analysed in detail the variability of the abundance and proportion of active bacteria, the general pattern is that, despite the different methods used, in most aquatic systems only a small fraction of bacteria is metabolically active (del Giorgio and Scarborough, 1995; Zweifel and Hagstrom, 1995; Choi *et al*., 1999; Sherr *et al.*, 1999). Data from the literature compiled by del Giorgio and Scarborough (1995) indicate that the proportion of active bacteria increases from <5% in the most oligotrophic open ocean areas to >50% in estuarine environments. Active bacterial number seems to vary considerably more than total number of bacteria. Over a broad range of aquatic systems, the active bacterial number varied by four orders of magnitude, whereas the number of total bacteria varied by three orders of magnitude (del Giorgio and Scarborough, 1995). Active bacteria number show a distinct minimum during the cold season (about an order of magnitude bellow warm season values) but the annual variation in the fraction of active bacteria is not so distinct (Hoppe, 1978; Sommmaruga and Conde, 1997; Bernan *et al.*, 2001). The highest values of active bacteria number are frequently observed in surface water

but the proportion of active bacteria either decreases with depth (Novitsky, 1983; Simek, 1986) or varies irregularly down the water column (Bianchi and Giuliano, 1996).

Although a large fraction of bacteria has been considered as metabolically inactive, it is well known that inactive bacteria may be dead, dormant, or slowly growing (Morita, 1997; Schut *et al.*, 1997; Sherr *et al.*, 1999). Choi *et al.* (1999) demonstrated that a substantial fraction (30 to 85%) of the apparently inactive marine bacteria, in relation to the detected activity of the electron transport system, can become active within 1 to 2 days after addition of organic matter and/or increase of water temperature. Bernan *et al.* (2001) found also that some bacteria, which appear to be inactive, can become active when stimulated by substrate addition, even though cell division is inhibited. This result suggests that a significant proportion of less active bacteria have the capacity to change their physiological state becoming metabolically active under favorable growth conditions.

1.4.2. Profiles of bacterioplankton production

Biomass production by heterotrophic bacteria is, from an ecological viewpoint, secondary production and reflects the overall bacterial response to the prevailing ecological conditions. The current methods for measuring bacterial production are simple to perform and are a convenient way to follow bacterial growth over time (Bell and Kuparinen, 1984), or over changing geographical or local conditions (Pace and Cole, 1994). On the other hand, comparison of bacterial production and primary production is a well-accepted indication of the trophic state of a system. It is known that ecosystems in which bacterial production is in excess of net primary production are dominated by allochthonous inputs of organic carbon and are decidedly heterotrophic. Contrarily, systems in which bacterial production is in deficit of net primary production are characterised as autotrophic (Cole and Pace, 1995).

In spite of the fact that only a small proportion of the total bacteria are active under natural conditions, their production in various aquatic environments has been found to average 20% of primary production (Williams, 1981; Cole *et al.*, 1988), and about twice the production of the macrozooplankton (Cole *et al.*, 1988).

Biomass productivity by heterotrophic bacterioplankton in aquatic systems is in the range of 0.0003-26.2 µg C l^{-1} h^{-1} (Shiah and Ducklow, 1995; Jellett *et al.*, 1996; Bouvy *et al.*, 1998; Gasol *et al.*, 1998; Hoppe *et al.*, 1998; Kisand and Noges, 1998, Shiah *et al.*, 1999). In estuarine systems the values are often high when compared to the adjacent coastal areas and open sea (Ducklow, 1992; reviewed by Ducklow and Shiah, 1993; Di Siervi *et al.*, 1995). Ducklow and Carlson (1992) compilated data from various marine habitats and found means ranging from 1.8 to 5.6 µg C l^{-1} h^{-1} in estuaries and from 0.5 to 0.6 µg C l^{-1} h^{-1} in open ocean. The highest values have been registered during summer months and the lowest during the winter in both estuarine and oceanic waters

(Kuosa and Kivi, 1989; Di Siervi *et al.*, 1995). Vertical profiles of bacterial production in oceanic waters have been characterised by decreasing values from surface to the deeper water layers (Jellet *et al.*, 1996; Talbot *et al.*, 1997). In estuarine systems bacterial production is, in general, higher at surface water (Heinanen, 1991; Fuks *et al.*, 1994) but in shallow estuaries bacterial productivity can be similar throughout the water column (Ducklow and Shiah, 1993) or even higher near the bottom sediment (Amon and Benner, 1998). It is most frequent bacterial productivity to increase up to 10 times from the lower to the mid-upper sections of the estuary (Goosen *et al.*, 1997; Sanudo-Wilhelmy and Taylor, 1999; Cunha *et al.*, 2000). In these systems, the highest values of bacterial productivity have been observed near low tide (Shiah and Ducklow, 1995; Hoppe *et al.*, 1996; Cunha *et al.*, 2000).

Several authors reported good correspondence between variations in bacterial and primary production in several ecosystems (Kuosa and Kivi, 1989; Fuks *et al.*, 1994; Lignell, 1990; Panzenbock *et al.*, 2000). Cole et al. (1988) examined the data from 70 studies on production of bacterial biomass, and found that bacterial production was 20% of primary production, on a volumetric basis. This value agrees well with the earlier estimation by Williams (1981) and with other direct studies in lakes and marine systems (Reinheimer, 1981; Fernandez *et al.*, 1994; Connan *et al.*, 1999; Panzenbock *et al.*, 2000). When the entire water column is considered, bacterial production is more significant, averaging 31% of primary production in the data from the 70 referred studies in lakes and coastal marine systems (Cole *et al.*, 1988). In deep water columns where the depth is many times greater than the photic zone, the importance of bacterial production may be higher (Cole *et al.*, 1988). On shorter time and space scales the ratio of bacterial production to primary production can, however, vary greatly (Bano *et al.*, 1997; Witek *et al.*, 1997; Bano *et al.*, 1998; Shiah *et al.*, 2001). Depth profiles of secondary and primary production were found to correspond poorly (Pace and Cole, 1994). Changes in bacterial and phytoplankton production within years were not closely related (Hoch and Kirchman, 1993) although total values covaried over the years Cole *et al.*, 1988).

2. Factors of variation of bacterioplankton growth

Since a large fraction of carbon in aquatic systems is now thought to flow through bacteria, the knowledge of the factors controlling bacterioplankton production is relevant to the understanding of biogeochemical cycles functioning and, particularly, to the prediction of their evolution after perturbation.

The main factors regulating bacterial growth include different variables namely nutritional (availability of organic and inorganic substrates), physical (e.g. temperature, water circulation), chemical (e.g. salinity), and biological (predation and viral lysis). Surprisingly, however, the

qualitative and quantitative relationships among these factors are still poorly understood (Shiah and Ducklow, 1995; Dufour and Torréton, 1996; Pomeroy and Wiebe, 2001).

2.1 Availability of organic and inorganic nutrients

Substrate supply has long been recognized as a dominant factor in regulating bacterioplankton abundance and production in aquatic systems (Ducklow and Carlson, 1992; Fuhrman, 1992; Shiah *et al.*, 1999). Several studies have suggested, however, that the strength of the bottom-up control might be system- dependent and might also change within a system (Wiebe *et al.*, 1992; Ducklow and Shiah, 1993; Bernan *et al.*, 1994; Shiah and Ducklow, 1995; Shiah *et al.*, 1999).

The strong positive correlation between phytoplankton and bacterioplankton biomass and bacterial production usually observed in field and enclosure studies suggests that phytoplankton may be an important autochthonous source of bacterial growth substrates (Williams, 1990; White *et al.*, 1991, Panzenbock *et al.*, 2000). Phytoplankton can directly supply bacteria with organic matter, through exudation from healthy cells and lysis of senescent and dead cells (Vadstein *et al.*, 1993; Panzenbock *et al.*, 2000). Indirect supply occurs via viral lysis or grazing by herbivorous zooplankton (Peduzzi and Herndl, 1992; Stom *et al.*, 1997; Bratbak *et al.*, 1998; Noble and Fuhrman, 1999; Hasegawa *et al.*, 2000).

In coastal and estuarine systems, bacterial growth substrates frequently derive from additional sources such as river transported materials, terrestrial runoff, antropogenic discharges, benthic fluxes and sediment resuspension. It has been found that non-phytoplankton or allochthonous organic matter supplies mediate, to a large extent, bacterioplankton growth in coastal and estuarine systems (Lee *et al.*, 2001). Findlay *et al.* (1992), for example, estimated that the amount of allochthonous carbon inputs needed to support bacterial productivity in the Hudson Estuary was three to six times greater than the net carbon fixed by phytoplankton. Other authors (Coveney and Wetzel, 1995) arrived at identical conclusion with respect to Lawrence Lake. The original allochthonous compounds had undergone, however, partial degradation and transformation before entering the aquatic system, and were thus less labile than the autochthonous substrates originated from phytoplankton production (Hobbie, 1988). It has been suggested therefore that allochthonous organic matter provide stability to bacterioplankton, in the sense that the large pool of recalcitrant organic matter supports continuous slow growth, independent of the intermittent growth associated with the less constant presence of autochthonous labile organic matter (Wetzel, 1984; Hobbie, 1988).

The concentration of inorganic substrates is also a factor of control of bacterial production (Toolan *et al.*, 1991; Thingstad *et al.*, 1993; Torréton *et al.*, 2000; Ferrier-Pagès and Furla, 2001). It is not yet clear, however, to what extent the inorganic or organic substrates may limit bacterioplankton production (Toolan *et al.*, 1991). While availability of organic carbon has

traditionally been thought of as a key factor in the limitation of bacterial growth, the high inorganic nutrients requirements (Vadstein *et al.*, 1988) and the high percentage of uptake traceable to bacteria (Currie and Kalff, 1984) suggest that the inorganic nutrients supply could also limit bacterial abundance and production. In fact, several studies have shown that bacterial growth increases with increasing availability in inorganic nutrients (Kroer, 1993; Pace and Cole, 1996; Wikner *et al.*, 1999; Torréton *et al.*, 2000). Moreover, heterotrophic bacteria have been shown to compete successfully with phytoplankton for inorganic nutrients (Currie and Kalff, 1984; Thingstad *et al.*, 1993). Blackburn *et al.* (1998) showed that bacteria have nutrient uptake potentials around 100 times faster than that of phytoplankton. At low concentrations, the competitive advantage of bacterioplankton over phytoplankton for inorganic nutrients is a consensual idea (Dufour and Berland, 1999; Torréton *et al.*, 2000).

2.2 Water properties

The growth of bacteria in aquatic systems is affected by *in situ* temperature, as concluded from the generally found positive correlation between bacterial production and temperature (Shiah and Ducklow, 1994; Kirchman and Rich, 1997; Shiah and Ducklow, 1997). It has been shown that the different temperature optima for bacterioplankton growth reflect the *in situ* temperature range (Simon and Wunsch, 1998). Recent studies have demonstrated that the variation in the rates of bacterial activity in eutrophic and mesotrophic ecosystems might primarily be regulated by temperature, with substrate supply playing a lesser role (Bernan *et al.*, 1994; Griffith *et al.*, 1994; Shiah and Ducklow, 1995). In oligotrophic systems the reverse situation occurs, with substrate supply playing a higher role on regulation of bacterial growth than temperature (Shiah *et al.*, 1999). Wiebe and Pomeroy (1992) have clearly demonstrated reciprocal interactions of temperature and substrate concentration in the control of bacterial growth: bacteria could grow rapidly at low (<10°C) temperature and high (micromolar) substrate concentration, or at high (>10°C) temperature and low (nanomolar) substrate concentrations, but not at low temperatures and low substrate concentration.

Salinity acts as a selecting agent of the bacterial strains that may proliferate in an ecosystem (Campbell, 1983; Rheinheimer, 1985). For example, bacteria with a salt requirement for growth will not be able to grow in estuarine environments with a large impact of freshwater (Campbell, 1983). On the other hand, it is unlikely that inflowing freshwater bacterial populations may survive within an estuary (Valdés and Albright, 1981; Painchaud *et al.*, 1987). Stahl *et al.* (1992) showed that phylogenetically distinct species of *Caulobacter* occur in freshwater when compared to saline environments. In estuarine systems, in general, bacterioplankton exhibit higher abundance and activity at low salinities with decreasing values towards higher salinity values (Palumbo and Fergunson, 1978; Murrell *et al.*, 1999). Peaks of bacterial abundance and activity

may occur, however, at intermediate salinities (Wright and Coffin, 1983; Fuks *et al.*, 1991; Chin-Leo and Benner, 1992; Cunha *et al.*, 2000).

It is well known that phytoplankton is negatively affected by high light intensities prevailing at the surface water, but there are only a few reports of light effects on bacterioplankton. Photoinhibion of heterotrophic bacteria has been reported (Pakulsky *et al.*, 1998; Pausz and Herndl, 1999; Mousseau *et al.*, 2000). Herndl *et al.* (1993) found that bacterial production at the Adriatic Sea surface was inhibited by UV-radiation up to 40 %. The authors observed that this inhibition could be detected down to 5 m. Bacteria below the photic zone were as sensitive to light as bacteria in the euphotic zone, suggesting that bacteria are, in general, not adapted to UV-radiation (Lindell and Edling, 1996). However, in spite of the decrease bacterial activity by photoinhibition, photolysis of recalcitrant organic matter can stimulate bacterial growth (Wetzel *et al.*, 1995; Miller and Moran, 1997; Bano *et al.*, 1997; Bushaw-Newton and Moran, 1999).

It has been found that UV-radiation can reduce phytoplankton photosynthesis (Helbling *et al.*, 1996a) and growth (Jokiel and York, 1984), enhance phytoplankton exudation (Zlotnik and Dubinsky, 1989; Feuillade *et al.*, 1990) and even modify the structure of the phytoplankton community (Helbling *et al.*, 1996b). Contrarily to bacteria, phytoplankton may respond to light stress with different strategies including decrease of metabolism, increase in the production of protective pigments and by migration to deeper water (Karentz *et al.*, 1994).

Solar irradiation, on the other hand, is a major cause of decline in viral infectivity in surface waters (Wommack *et al.*, 1996; Noble and Fuhrman, 1997), reducing virus-mediated mortality of bacterio- and phytoplankton.

The indirect effects of light on the bacterioplankton are certainly complex.

2.3. Water circulation

The circulation of estuarine water is driven by a complex interplay of mechanisms, all of which have been shown to be important in regulating biological processes (Painchaud *et al.*, 1987; Ducklow and Shiah, 1993). Circulation is capable of changing the conditions of the ecosystem over a much smaller temporal scale than that occurring in the open ocean (Schultz and Ducklow, 2000). In estuarine systems circulation may have a large effect on the abundance and production of the bacterial community as it supplies allochthonous organic matter, concentrates and conserves within the estuary locally produced organic matter and provides mechanisms for longer-term coupling of bacterial production and authochonous sources of organic matter (Ducklow and Shiah, 1993).

In estuaries, water circulation is conditioned by tidal currents, river discharges and wind. Tidal currents move plankton populations back and forward creating complex circulation patterns. For example, Wright and Coffin (1983) studied bacterial distribution in the Essex estuary and showed a peak in abundance in the mid-estuary, flanked by lower numbers up- and downstream.

The authors explained this pattern of variation as due to the tidal regime which allowed bacterial populations to be retained within the estuary over several tidal cycles, and exposed them to contact with enriched salt marsh waters. Tidal currents also interfere with vertical distribution patterns in estuaries. Tidal currents can, for example, destratify the water column (Haas, 1977), stimulating total bacterial production (Ducklow, 1982).

2.4 Mortality through infection and predation

The discovery that the abundance of free viruses ($< 10^7$ to $> 10^{11}$ viruses l^{-1}) can exceed that of planktonic bacteria by 1 to 2 orders of magnitude (Proctor and Fuhrman, 1990; Jiang and Paul, 1994; Maranger and Bird, 1995; Weinbauer and Peduzzi, 1995a) motivated the research effort on the impact of viral infection on aquatic microorganisms.

Numerous studies on a wide variety of aquatic ecosystems have indicated that bacteriophages can be important in controlling bacterial growth (Proctor and Fuhrman, 1991; Bratbak *et al.*, 1992; Fuhrman and Noble, 1995; Maranger and Bird, 1995; Weinbauer and Peduzzi, 1995b). It has been shown that up to 30% of planktonic bacteria were infected by litic viruses (Fuhrman and Suttle, 1993; Suttle, 1994). Estimates of bacterial mortality due to viral lysis indicated that phages can be responsible for as little as 1% up to 100% of the observed bacterial mortality (Proctor and Fuhrman, 1991; Bratbak *et al.*, 1992; Steward *et al.*, 1992; Bratbak *et al.*, 1993; Proctor *et al.*, 1993; Suttle, 1994; Fuhrman and Noble, 1995; Hennes *et al.*, 1995; Weinbauer and Peduzzi, 1995b), depending on environmental conditions and host community structure (reviewed by Wommack and Colwell, 2000). In coastal waters the virus-mediated bacterial mortality was estimated to represent a value similar to grazing by protozoa (Fuhrman and Noble, 1995). Likewise, in an eutrophic lake, viruses caused large bacterial mortality and their contribution to mortality increased with depth since protozoa were absent in the anoxic zone (Weinbauer and Hofle, 1998).

Viruses may also influence the structure of bacterial communities since they are host specific (Suttle *et al.*, 1990; Thingstad *et al.*, 1993; Wommack *et al.*, 1999). Additionally, viruses may influence bacterial diversity at the genetic level since they can mediate genetic exchange via transduction (Miller and Sayler, 1992; Paul, 1999).

Although the proportion of bacteriophages in the total virioplankton is not known, many authors have speculated that bacteriophages comprise the majority of the virioplankton population. In fact, in aquatic systems viruses are, in general, strongly correlated with bacterioplankton density (Heldal and Bratback, 1991; Maranger and Bird, 1995, Steward *et al.*, 1996) and the virioplankton density has been found to be higher when bacterial productivity is also higher (Steward *et al.*, 1996; Weinbauer and Hofle, 1998).

In aquatic systems, predation has been reported as a major mortality factor in bacterial communities (Weisse, 1997; Weisse and Muller, 1998). Heterotrophic nanoflagellates and ciliates are the most important grazers of bacteria in both marine (Sherr *et al.*, 1989; Wikner *et al.*, 1990) and freshwater systems (Bloem *et al.*, 1989; Sanders *et al., 1989).* Several studies have shown that grazing by protists influences both cell size distribution (Simek *et al.*, 1997; Hahn and Hofle, 1998) and the taxonomic structure (Simek *et al.*, 1997; Hanhn and Hofle, 1998, Suzuki, 1999) of the bacterial community.

Grazing rates have been observed to be higher on active than on inactive bacteria (Sherr *et al.*, 1992; del Giorgio *et al.*, 1996) and on motile bacteria than on non-motile bacteria (González *et al.*, 1993). Furthermore, protists selected their prey based on size (González *et al.*, 1990; Pernthalen *et al.*, 1996; González, 1999) controlling, in this way, the size distribution of the bacterioplankton. Size selective grazing has been shown to result in an increased dominance of small cells that escape predation (Anderson *et al.*, 1986) or, after periods of intensive grazing, in an increased dominance of larger cells, since these cells are difficult to ingest by a small flagelattes (Pernthaler *et al.*, 1996; Hahn *et al.*, 1999; Jurgens *et al.*, 1999). Estimates of bacterial mortality due to predation in aquatic systems have been reported to range from 10 to 80% (Solic and Krstulovic, 1994; Fuhrman and Noble, 1995; González, 1999; Ferrier-Pagès and Furla, 2001) depending on environmental and biological variables (Vaqué *et al.*, 1994).

3. Thesis outline

This work is focused on the trophic processes of the water column of Ria de Aveiro and on the factors underlying the shape of the spatial and temporal profiles of bacterioplankton activity in this ecosystem.

In order to interpret the functioning of an ecosystem one has to approach the interplay of physical, chemical and biological processes and the interaction of the different compartments of the system. It is essential, in particular, to evaluate the different factors that may control the fluxes of materials and energy.

It is well recognized that bacteria play an important role in driving the fluxes of carbon and nutrients and that they exert a relevant control on microbial food webs (Azam, 1998; Pomeroy and Weibe, 1998).

The characterisation of longitudinal profiles of estuarine waters is fundamental in order to establish a general outline for the rational underlying the imports and exports between the estuary and the sea, putting in evidence some of the factors that control these profiles.

In estuarine systems, strong tidal currents may act as a major determinant of bacterioplankton distribution. Tidal currents may increase seston concentration and, consequently, decrease light penetration restricting primary production and, in turn, bacterial production.

In the case of Ria de Aveiro, a shallow system, the stratification of the water column is mostly unnoticeable even in the deeper zone. In fact, the difference in salinity and temperature values between surface and bottom, when existing, is very small (Moreira *et al.*, 1993). The Ria de Aveiro is characterised as a well-mixed estuary (Pritchard, 1967). The bacterioplankton may, however, follow or not the general pattern of the system.

Dissolved organic carbon released by the phytoplankton, mainly at the senescence phase, has been reported as the best source of organic matter for bacterial growth (Wetzel, 1984; Hobbie, 1988; Malinsky-Rushansky and Legrand, 1996). In some estuaries, however, other carbon sources, for example allochthonous inputs, may be equally available for the bacterioplankton. Allochthonous inputs will tend, in this case, to uncouple bacterial abundance and production from phytoplankton abundance and primary production, affecting the natural pattern of the two communities. Bacterioplankton abundance and distribution follows then the occurrence of natural and allochthonous sources of carbon and nutrients. In estuarine systems it is relevant to take into account not only river discharges but also the diffuse inputs and runoff from the banks surrounding the system.

In shallow systems, as the case of Ria de Aveiro, the benthic compartment may also be an important source of organic matter and inorganic nutrients by simple diffusion. In fact, estuarine sediments are considered a privileged compartment for the deposition, accumulation and mineralisation of locally produced and deposited organic matter. On the other hand, transfer by simple advection or after sediment resuspension linked to erosion by tidal currents or to bioturbation may enrich the water column with bacteria, particulate materials and nutrients.

Experimental research is frequently required in order to clarify hypothesis on the biological processes underlying the trophic processes observed in aquatic systems. For instance, the fraction of active bacteria in marine communities seems to vary widely in different systems (del Giorgio and Scarborough, 1995) with important and foreseeable effects on organic matter recycling but the reasons for this variability is not yet clear. On the other hand, a variable fraction of the non-active bacteria are still viable, and may be activated at different times through metabolic adaptation or through suitable stimulation. The experimental approach to the environmental conditioning of bacterial viability may explain not only the different status of the bacterioplankton in different communities and also clarify the ranking of factors inducing positive or negative responses in a particular community.

The effect of long residence time of estuarine water on the differentiation and adaptation of the mixed marine and freshwater bacterial populations has not been translated into the question of

the presence or not of true brackish water communities. This may possibly be addressed under a well-controlled experimental set up.

The viral impact on bacterial mortality in sea- and brackish water, a subject that deserves attention since 1989, is mostly dependent on manipulation of the two communities under experimental conditions (Peduzzi and Weinbauer, 1993; Weinbauer and Peduzzi 1995a).

The knowledge of the functional responses of an ecosystem to natural or anthropogenic factors may allow the prediction of natural tendencies linked to the alterations in its trophic status as well as the possible effects of specific interventions. In the case of Ria de Aveiro, a system with considerable anthropogenic influence, a database of relevant parameters defining the functioning and the reactivity of the ecosystem to physical and chemical perturbations may be important in predicting and evaluating the range of possible effects and in avoiding undesired impacts. Natural or managing problems like massive release of nutrients during storms, increased eutrophication, and dredging may be equated at the light of experimental findings. It is known that dredging operations are frequently carried out in the estuary of Ria de Aveiro and that these activities interfere with the quality of the water column (Almeida and Alcântara, 1992). It has also been observed that the bacterial community in the bottom sediment is two to three orders of magnitude denser than in the water column (Almeida and Alcântara, 1992; Alcântara *et al.*, 1996). Although bacterial productivity in the sediment is not yet determined in this system, it was observed that, in a per volume basis, the rate of oxygen consumption in the sediment was 9 to 70 times higher than in the water column (Cunha *et al.*, 1999). It is foreseeable that sediment resuspension during dredging, besides affecting the zoobenthos, will increase not only water turbidity but also bacterioplankton activity in the water column and organic carbon turnover. This will interfere with the natural pattern of bacterioplankton variation. The expected positive effects of the sanitary outfall under construction, on the quality of the Ria de Aveiro system may be followed in order to evaluate the capacity of the system for auto-depuration and as a test for present predictions. The sanitary outfall will discharge high numbers of freshwater bacteria and high levels of organic matter in the nearshore waters. In an ecological perspective, it will be interesting to investigate if freshwater bacteria, when suddenly discharged in the nearshore will be able to degrade the particulate and dissolved organic matter of the effluent, driving these materials through the microbial food web to higher trophic levels.

Objectives of the work:

1. To characterize the variability of the bacterial density (total and active bacteria) and productivity in the water column of the estuary.

2. To determine the relative importance of autochthonous primary production and allochthonous organic inputs as sources of organic matter to bacterioplankton growth.
3. To investigate the response of the bacterial community to periodic changes in salinity and nutrient availability.
4. To evaluate the effect of viral infection as a factor of bacterioplankton loss and to determine how it compares with the predation pressure.
5. To investigate the trophic effects of sediment resuspension and runoff on bacterioplankton activity.

To achieve these objectives several field and experimental studies were undertaken.

Field-work

Field research in the marine and in the brackish water zones of the estuary was driven in order to determine longitudinal, vertical, transversal, and tidal profiles of bacterioplankton and phytoplankton abundance and production, virioplankton abundance as well as accompanying physical and chemical parameters.

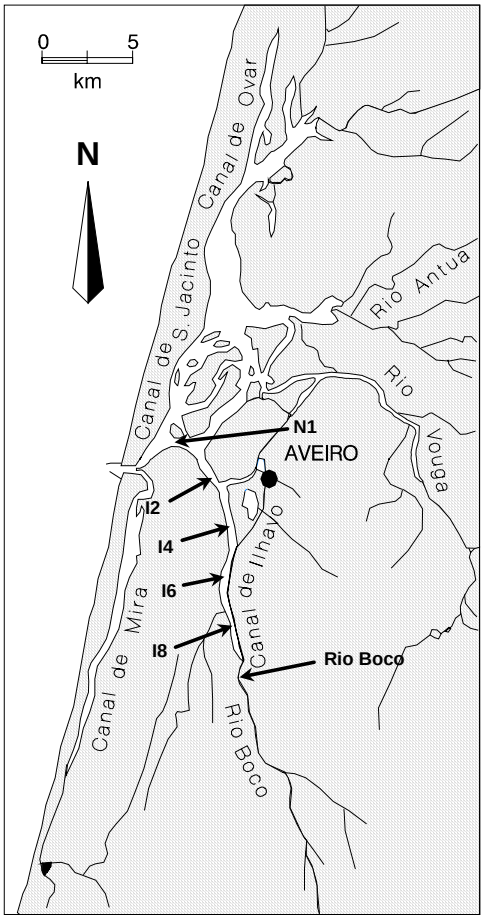


Figure 1: Ria de Aveiro with sampling stations indicated by arrows. Station N1, in Canal de Navegação, is in the marine zone of the ecosystem. I2, I4, I6 and I8 are brackish water stations along the salinity gradient of Canal de Ílhavo. Rio Boco is a river station.

The marine zone encompasses a water volume of 31.2 Mm^3 (26% of the total water volume) and has a maximum depth of 20 m at the mouth of the estuary. In this work, the marine zone was represented by station N1 (figure 1), located in the Canal de Navegação. The chosen location - 2 Km from the mouth - avoids most of the water turbulence and traffic that exists closer to the mouth of the estuary and the strong currents that would risk the instrumental readings and sample collection. On the other hand, this location allows the better detection of the effects of transport of particulate and dissolved materials coming from the brackish sections. In order to put in evidence eventual stratification and local resuspension of sediments in the marine zone, measurements were performed during the warm season at four different depths (0.2 m below surface, 4.5, 2.5 and 0.5 m above sediment surface) along six spring and neap tidal cycles. During the cold season the sampling effort was reduced. Water sampling at 0.2 m below

surface was performed only at slack water (high tide and low tide).

The brackish water zone encompasses a water volume of 88.6 Mm^3 (74% of the total water volume). The mid- and inner estuary is flat, shallow (average depth of 1 m) and generally flanked by salt marshes and mud flats. In order to assess the relative impact of flooding and runoff as well as of sediment resuspension in this zone, vertical and transversal profiles of bacterioplankton abundance and activity were studied and characterised. The brackish water zone was represented by station I6 (figure 1) located in the Canal de Ílhavo. This Channel is one of the five main channels in the Ria. Its location is very convenient as it follows the direction of the water current passing station N1. It has an easy access from the margins, is relatively calm, is not too deep and it is not far from the laboratory. The sediments along this channel vary from sandy to silty what makes possible to study resuspension and runoff in different geological conditions. Measurements were performed at two depths (0.2 m below surface and 0.5 m above sediment) along a transect established between the east margin (close to a salt marsh) and the center of the channel. The study involved four tidal cycles during spring and neap tides in the warm season. In the cold months, this station was also sampled at slack water but only at the center of the channel and at 0.2 m below surface.

During the warm season, bacterio-, virio- and phytoplankton abundance and production were studied along a longitudinal gradient of salinity, extending across the outer- mid and upper sections of the estuary. Six sampling stations, including stations N1 and I6, spaced regularly at 3 km were established along the longitudinal gradient (figure 1). Station N1 and station I2 in the Canal de Navegação, stations I4, I6 and I8 in the Canal de Ílhavo and also Rio Boco. Samples were collected only at low tide and high tide, at the center of the channels, and close to the surface (0.2 m below surface).

Experimental approaches

The interpretation of some of the results obtained in fieldwork was subjected to experimental verification.

Laboratory experiments were performed to characterise the viability state of the bacterioplankton through stimulation with organic and inorganic nutrients. The number of active bacteria and their proportion in the total bacterial community were determined after brief (10 minutes) or long (up to 3 hours) pre-exposure to low concentrations of organic and inorganic compounds followed by staining with CTC.

Also, the effects of the changes in salinity and other water properties on bacterial activity during tidal transport were studied in laboratory assays, in diffusion chambers. One same population (marine population from station N1 or estuarine population from station I6) was exposed to changed environmental conditions in the presence of the contrasting water (water from the other station). In each case, bacterial abundance (total and active bacteria) and productivity

were measured after 2, 4 and 6 hours of exposure and compared with the initial characteristics of the water and with the unchanged control. In order to test the effects of reversion exposure, an extra sample of each zone was incubated in the contrasting water for the first 2 hours, and then transferred to its natural environment. Incubation for an additional 4 hours period revealed the response to the reversed conditions. To evaluate the effects of increased salinity on bacteria, samples from stations N1 and I6 were exposed to natural brackish water conditions (18 psu), as well as to the same water after adding of NaCl to a final salinity of 34 psu, similar to that of the marine zone.

The potential contributions of viral infection and predation to bacterial mortality was assayed in an artificial microcosm set-up on six combinations of plankton variables affecting the presence/absence of predators, viruses-to-bacteria ratio, viruses-to-bacteria distance and bacterial growth rate. The number of bacteria was determined after 6, 12 and 24 hours of incubation and compared with the initial values of bacterial density.

Vertical and tidal profiles obtained at stations N1 and I6 were compared and the factors influencing bacterial abundance and productivity were identified (chapter 2).

The contribution of primary production versus allochthonous carbon to bacterioplankton growth was studied along the longitudinal gradient of salinity during the warm season. In the cold season the study was restricted to stations N1 and I6 (chapter 3).

Fieldwork data of bacterial abundance and activity obtained during the tidal cycles in both zones were compared with experimental work of bacterial stimulation assays and of diffusion chambers experiments in order to test whether a single, largely adaptable community exist in the estuary, or several distinct communities can be recognized (chapter 4).

Vertical, longitudinal and tidal profiles of virioplankton abundance were determined and compared with those of bacterioplankton. The impact of the virioplankton on the bacterial community was assayed in a laboratory microcosm set-up and compared with that of predation (chapter 5).

The vertical profiles of bacteriological, chemical and physical parameters were analysed for evidence of sediment resuspension during tidal cycles at stations N1 and I6. Runoff from salt marshes was also investigated but only in the brackish water area. In this area, transversal profiles of bacterioplankton abundance (total and particle-attached bacteria) and productivity were compared with the profiles of seston, particulate organic carbon, chlorophyll and dissolved organic carbon in order to evaluate the impact of runoff from salt marshes (chapter 6).

CHAPTER 2

FACTORS INFLUENCING BACTERIAL PRODUCTION IN A SHALLOW ESTUARINE SYSTEM

Almeida MA, Cunha MA and Alcântara F

Departamento de Biologia - Universidade de Aveiro
Campus Universitário de Santiago, 3810-193 AVEIRO, PORTUGAL

ABSTRACT: The bacterioplankton of the marine and brackish water zones of the complex system Ria de Aveiro was characterised as profiles of bacterial abundance and biomass productivity. During the warm season, total bacteria ranged from 0.2 to 8.5 x 10^9 cells l^{-1} and active bacteria number from 0.1 to 3.1 x 10^9 cells l^{-1}. Total and active bacterial numbers were, on average, 3 times higher in brackish than in marine water. Bacterial productivity on different dates and different tides in the marine zone varied from 0.05 to 4.5 µg C l^{-1} h^{-1}. Here the average productivity (1.1 µg C l^{-1} h^{-1}) was 3.5 times less than in brackish water (average 3.8 µg C l^{-1} h^{-1}; range 0.7-14.2 µg C l^{-1} h^{-1}). Specific productivity per cell varied from 0.05 to 2.61 fg C $cell^{-1}$ h^{-1}, a range that was similar throughout the ecosystem. However, specific productivity per active cell was 19% higher in brackish water. Bacterial production variation was best explained by the number of active bacteria, which, in turn, was highly associated with total bacterial number, temperature and particulate organic carbon. In the marine zone, bacterial production was also influenced by depth and salinity. In the brackish zone, the set of independent variables explained a smaller percentage of bacterial production variation than in marine zone, suggesting greater importance of other variables. In the marine zone, and mainly near low tide, productivity was significantly higher (average 3.3 times) at the surface (down to 0.5 m) than in the deeper layers of the water column. This stratification of bacterial productivity was linked to the increased specific productivity per active cell, as no modification in the proportion of active cells in the population could be detected. The vertical profile of bacterial production in the deeper zone of this estuarine ecosystem, in which no clear salinity or thermal stratification occurs throughout the tidal cycle, seemed to reflect a biochemical stratification generated by increased phytoplankton exudation and/or by photochemical transformation of semi-labile or recalcitrant organic compounds. Shallower water masses tend to blur this surface effect. The relative importance of photochemical transformation in the pattern of estuarine bacterial production will therefore tend to vary with the bathymetry of the system.

INTRODUCTION

It is well known that the distribution and intensity of bacterial biomass production in estuaries depend on the location and size of carbon and nutrient sources, and on profiles of physical and chemical characteristics of the environment associated with water currents (15, 16, 22, 23, 28, 34). Autochthonous organic matter is liberated by a variety of processes including phytoplankton excretion and mortality (14, 17), zooplankton feeding (30, 37, 39), bacterial mortality (21, 31, 44) and detrital hydrolysis (7). Several studies indicate that the organic matter excreted by phytoplankton is very labile and subjected to high turnover rates by heterotrophic bacteria (20, 24). Cole *et al.* (13) suggested that approximately half the carbon released by phytoplankton is processed by bacteria. Even so, labile organic matter is frequently accumulated in aquatic systems (41). This may happen, for instance, when bacteria compete with phytoplankton for inorganic nutrients (38). On the other hand, organic matter produced in marine ecosystems may exhibit low biological lability when it includes chemical structures that require complex ectoenzymatic hydrolysis (2) or physico-chemical transformation. Photochemical transformation has been

identified as an important process in substrate modification (3, 9, 11). Bano *et al.* (3) observed that exposure of water samples to natural or simulated daylight, increased bacterial use of organic matter by 300%, and that humic substances were the principal carbon source for the additional bacterial growth. Bushaw-Newton & Moran (9) also demonstrated that humic substances irradiated with natural sunlight supported enhanced bacterial growth due to the increased availability of both carbon and nitrogen. In comparative studies of bioavailability, natural humic substances from near-surface water showed the same quality as carbon sources for bacteria as DOC (10). It has also been observed that humic substances in surface water are significantly better sources of carbon and nitrogen for bacteria than those from deep water (10).

Allochthonous organic matter, particularly in coastal systems, is an important substrate supply for bacterioplankton (5, 27), its importance increasing, in general, when the volume of the water body decreases (40). We have determined (unpublished data) for the shallow estuary of Ria de Aveiro, that phytoplankton was potentially sufficient to support the whole bacterial production during the warm season but not during the cold months. Allochthonous carbon seemed, however, to contribute a significant fraction of the bacterial production even during the warm season.

In order to understand the variation of bacterial production within a shallow estuarine system we compared information derived from longitudinal and vertical profiles in different tidal phases.

MATERIALS AND METHODS

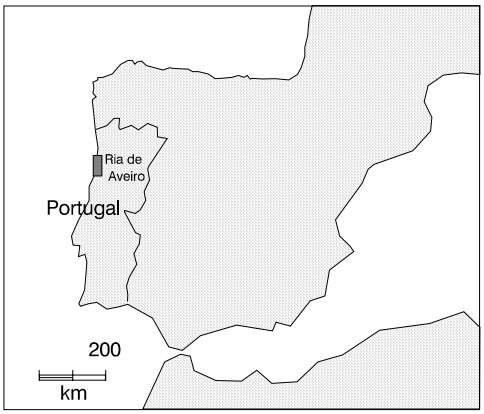
Study site

Ria de Aveiro (Figure 1) is a tidal lagoon on the western coast of Portugal, connected to the Atlantic by a narrow opening. The lagoon covers an area of 66 and 83 km^2 at low and high tide, respectively (35). It exchanges with the sea a volume of water of 89 Mm^3 in tides of 1 to 3 m amplitude (35). Several rivers carry fresh water into the lagoon with an average water input of 1.8 Mm^3 during a tidal cycle (4). The Ria has a complex topography, with different channels spreading from the mouth towards the different streams, forming a complex estuarine ecosystem. In this study, we compared one station in the main channel — Canal de Navegação (station N1) — representing the marine zone of the system (MZ), with station I6, a brackish water station in the Canal de Ílhavo (a secondary channel), which represents the brackish water zone (BZ).

Sampling

All sampling took place during the warm season (early May to early September). Station N1 was studied over six different tidal cycles (two in June 1996, two in August 1996 and two in

September 1997). Station I6 was sampled over four tidal cycles (one in April and three in May 1997). Samples were collected from near-water surface (0.2 m depth) and deep water (0.5 m above sediment floor), at high tide (HT) and low tide (LT) and intermediate time intervals of 2 hours (HT+2h, HT-2h, LT-2h, LT+2h). In the marine zone water samples were also collected in 1996 at 2.5 and 4.5 m above the bottom. Water samples were transported and processed within 2-3 hours of collection.



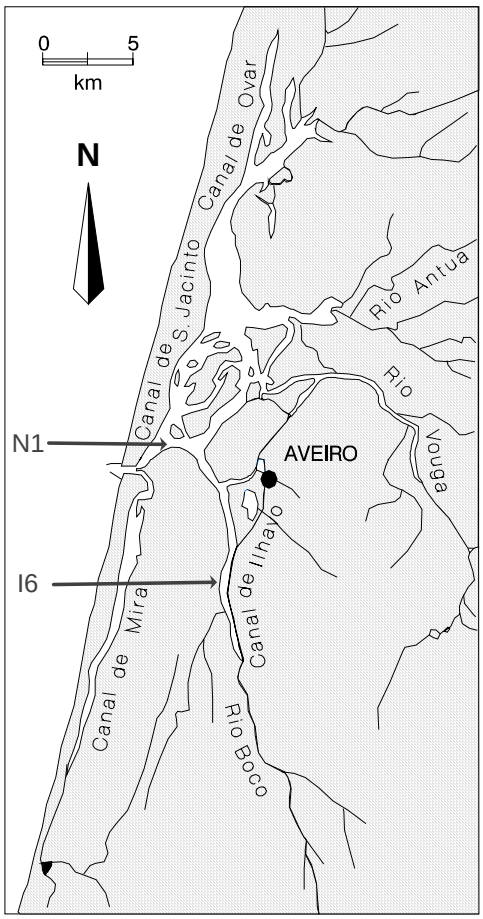


Figure 1: The estuarine ecosystem of the Ria de Aveiro with sampling stations indicated by arrows. Station N1 in Canal de Navegação, represents the marine zone and station I6, in Canal de Ílhavo, represents the brackish water zone.

Physical, chemical and phytoplankton characteristics. Temperature and salinity were obtained with a conductivity meter (WTW — Wissenschaftlich Technische Werkstätten, Model LF 196). Dissolved oxygen concentration, expressed as percentage of saturation, was determined with a oxygen meter (WTW, Model OXI 96) equipped with a stirrer (WTW, Model BR 190). The concentration of suspended solids was determined by filtration of triplicate 0.5 l water sample aliquots through pre-weighed and pre-combusted Whatman GF/C filters. The filters were washed with 100 ml of ultra-pure water, dried at 60ºC for 24 hours and suspended solids calculated as the increase in dry weight. Particulate organic matter was determined as the loss of weight after 4 hours incineration at 525 ºC (29). Particulate organic carbon (POC) was calculated as 50% of the particulate organic matter (32). Samples for nutrient assay (PO_4^{3-}, $NO_3^- + NO_2^-$) were filtered through 0.45 µm acetate membranes (MSI - Micron Separation Inc.). Phosphate was determined by the molybdate method and NO_2^- plus NO_3^- by the sulfanilic acid method after reduction of NO_3^- to NO_2^- in a cadmium column. Analyses were performed using a Segmented Flow Injection Automatic Analyser Alliance Instruments – Evolution II (1). Chlorophyll *a* (CHLO) was estimated fluorimetrically (42) after filtration of 0.5 l triplicate sub-samples through Whatman GF/C filters and overnight cold extraction in 90% (v/v) acetone.

Total bacterial number (TBN). Bacterial cells were counted by epifluorescence microscopy using a Leitz Laborlux K microscope. The samples were fixed with 2% (v/v) formaldehyde (final concentration), filtered through 0.2 µm pore black polycarbonate membranes (Poretics) and stained with 0.03% (w/v) acridine orange (25). At least 200 cells or 20 microscope fields were counted in each of three replicate preparations.

Microautoradiography (MAR). Active bacterial numbers (ABN) were determined by microautoradiography (12) after amendment of samples to 30 nM ^{3}H-leucine (Amersham, specific activity 15.9-24.8 GBq mmol^{-1}). After 5 hours incubation at *in situ* temperature, samples were fixed with 2% (v/v) formaldehyde and triplicate sub-samples (2-3 ml) of each sample (10 ml) filtered through 0.2 µm black polycarbonate membranes. The filters were placed face down on slides coated with the autoradiographic emulsion NTB-2 (Kodak) and exposed in total darkness, at 4°C, for 7 days. The slides were developed with Kodak Detkol (one-to-one dilution in ultrapure water) and fixed in Kodak fixer. The developed autoradiograms were stained with acridine orange solution (0.04%) and hydrated in citrate buffer (0.004 M). Microautoradiographs were examined using a combination of epifluorescence (as above) and bright-field illumination in a Leitz Laborlux microscope. Cells were counted as active if associated with three or more silver grains.

Bacterial biomass productivity (BBP) and specific biomass productivity (BBP_{cell} and $BBP_{active\ cell}$). BBP was determined in 10-ml triplicate sub-samples plus a control that was fixed by addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (30 nM) of ^{3}H-leucine (Amersham, specific activity 15.9-24.8 GBq mmol^{-1}) for 1 hour, at *in situ* temperature, in the dark. After incubation, sub-samples were fixed at 2% (v/v) formaldehyde. Protein precipitation was performed through the addition of 1 ml of 20% (w/v) ice-cold TCA followed by incubation for 15 minutes on ice. Sub-samples were then filtered through 0.2 µm polycarbonate membranes (Poretics), rinsed with 2 ml of 5% (w/v) ice-cold TCA and 5 ml of 90% (v/v) ice-cold ethanol (8). After standing for 3 days in scintillation cocktail UniverSol (ICN Biomedicals, USA) the radioactivity was measured in a liquid scintillation counter (Beckman LS 6000 IC). BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (36). BBP_{cell} was calculated as the quotient BBP/TBN (BBP_{cell}) and $BBP_{active\ cell}$ was obtained from BBP/ABN.

Statistical methods. SPSSWIN 7.1 was used for data analysis. As an attempt to explain the variation of bacterial abundance and production, stepwise multiple regression analysis was used. Temperature, salinity, depth, POC and chlorophyll were used as independent variables. ABN and TBN were also included as independent variables for the regression analysis of BBP and ABN,

respectively. The significance of difference between surface and bottom values in bacterial abundance, bacterial production and physico-chemical characteristics was assessed using one-way ANOVA. Three sub-samples were used for each variable at the six tidal phases in different dates. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) were used. The significance of both depth and tide on bacterial production variation was assessed using two-way ANOVA.

RESULTS

Physical, chemical and phytoplankton characteristics

Salinity varied from 23.7 to 36.1 PSU (average 34.4 PSU) in the marine zone (MZ) and from 11.4 to 33.7 PSU (average 26.0 PSU) in the brackish water zone (BZ). The average water temperature was 16.8°C (range 15.3 - 20.6 °C) in the MZ and 20.2 °C (range 17.0 - 23.5° C) in the BZ. Water depth varied from 0.5 m and 9.5 m (average 7.4 m) in MZ and between 1.3 m and 3.3 m (average 2.3 m) in BZ. The mean value of dissolved oxygen was 76.7 % in the MZ and 69.8 % in the BZ.

Table 1: Bacterial abundance and production in the marine (MZ) and brackish water (BZ) zones of the Ria de Aveiro. Total bacterial number (TBN), active bacterial number (ABN), proportion of active bacteria (%AB), bacterial productivity (BBP), specific BBP per cell (BBP_{cell}), specific BBP per active cell ($BBP_{active\ cell}$). Near surface water was collected 0.2 m the surface (S), deep water was collected 0.5 m above the sediment (D). The values shown are the average and range (in parentheses) of six (MZ) and four (BZ) tidal cycles on different sampling dates in the warm season.

ZONE		TBN ($x10^9$cells l^{-1})	ABN ($x10^9$cells l^{-1})	%AB	BBP (µg $Cl^{-1}h^{-1}$)	BBP_{cell} (fg C cell $l^{-1}h^{-1}$)	$BBP_{active\ cell}$ (fg C cell $^{-1}h^{-1}$)
	S	2.3	0.5	27.7	1.6	0.9	3.1
		(0.2-8.1)	(0.1-1.3)	(10.0-53.0)	(0.3-4.5)	(0.2-2.3)	(0.6-7.2)
MZ	D	1.8	0.5	28.6	0.6	0.5	1.5
		(0.2-5.9)	(0.1-1.7)	(5.9-55.0)	(0.05-2.1)	(0.05-2.6)	(0.2-6.1)
	S+D	2.0	0.5	27.3	1.1	0.7	2.2
		(0.2-8.1)	(0.1-1.7)	(5.9-55.0)	(0.05-4.5)	(0.05-2.6)	(0.2-7.2)
	S	5.9	1.5	26.2	3.8	0.7	2.8
		(3.1-8.1)	(0.3-3.0)	(10.0-44.0)	(1.2-7.4)	(0.2-1.7)	(0.9-9.6)
BZ	D	5.8	1.4	25.1	3.7	0.6	2.6
		(2.6-8.5)	(0.2-3.1)	(8.0-42.0)	(0.8-14.2)	(0.3-2.5)	(0.7-4.6)
	S+D	5.9	1.5	25.1	3.8	0.7	2.7
		(2.6-8.5)	(0.2-3.1)	(8.0-44.0)	(0.7-14.2)	(0.2-2.5)	(0.7-9.6)

Particulate organic carbon (POC) varied from 3.0 to 15.5 mg l^{-1} (average 7.2 mg l^{-1}) in the MZ and between 3.0 and 8.5 mg l^{-1} (average 5.5 mg l^{-1}) in BZ. In the MZ the concentration of NO_2^- plus NO_3^- varied from 1.1 to 3.8 µM and the concentration of $PO4^{3-}$ from 0.9 to 3.2 µM. In the BZ the concentration of these nutrients was about twice that in the marine zone. The concentration of nutrients was higher at LT in both the marine and brackish water zones. Chlorophyll *a*

concentration varied in the different phases of the tidal cycle from 1.2 to 5.5 µg l^{-1} (average 2.6 µg l^{-1}) in the MZ, and from 0.2 to 23.0 µg l^{-1} (average 4.8 µg l^{-1}) in the BZ. The values were lower at HT in both zones. No stratification pattern was shown by any of these variables in either of the two zones of the lagoon (ANOVA, $p>0.05$).

Geographical distribution of bacterial number and productivity

Total bacterial number at station N1 (MZ) varied from 0.2 to 8.1 x 10^9 cells l^{-1} and at station I6 (BZ) from 2.6 to 8.5 x 10^9 cells l^{-1}. The active bacterial number ranged from 0.1 to 1.7 x 10^9 cells l^{-1} at N1 and from 0.2 to 3.1 x 10^9 cells l^{-1} at I6. The average numbers were three times higher in the BZ than in the MZ. The fraction of active bacteria (%AB) varied from 5.9% to 55% and the average value was similar in both zones (26.6%) (Table 1). Bacterial biomass productivity (BBP) ranged from 0.05 to 4.5 µg C l^{-1} h^{-1} at station N1 and from 0.7 to 14.2 µg C l^{-1} h^{-1} at I6. The average was 3.5 times higher in the BZ (Table 1). Specific productivity per cell (BBP_{cell}) ranged from 0.05 to 2.61 fg C l^{-1} h^{-1} and the average value was similar at both stations (0.7 fg C $cell^{-1}$ h^{-1}) (Table 1). Productivity per active cell ($BBP_{active\ cell}$) was, however, 19% higher at station I6 (range 0.7-9.6 fg C $cell^{-1}$ h^{-1}) than at station N1 (0.2-7.2 fg C $cell^{-1}$ h^{-1}) (Table 1).

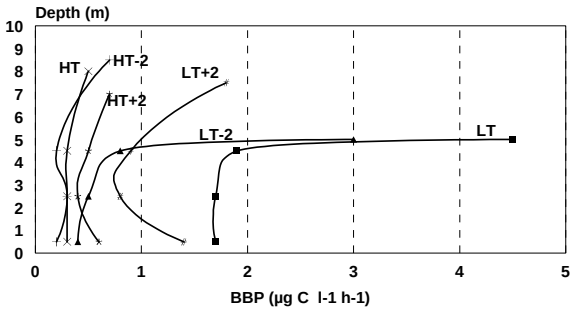
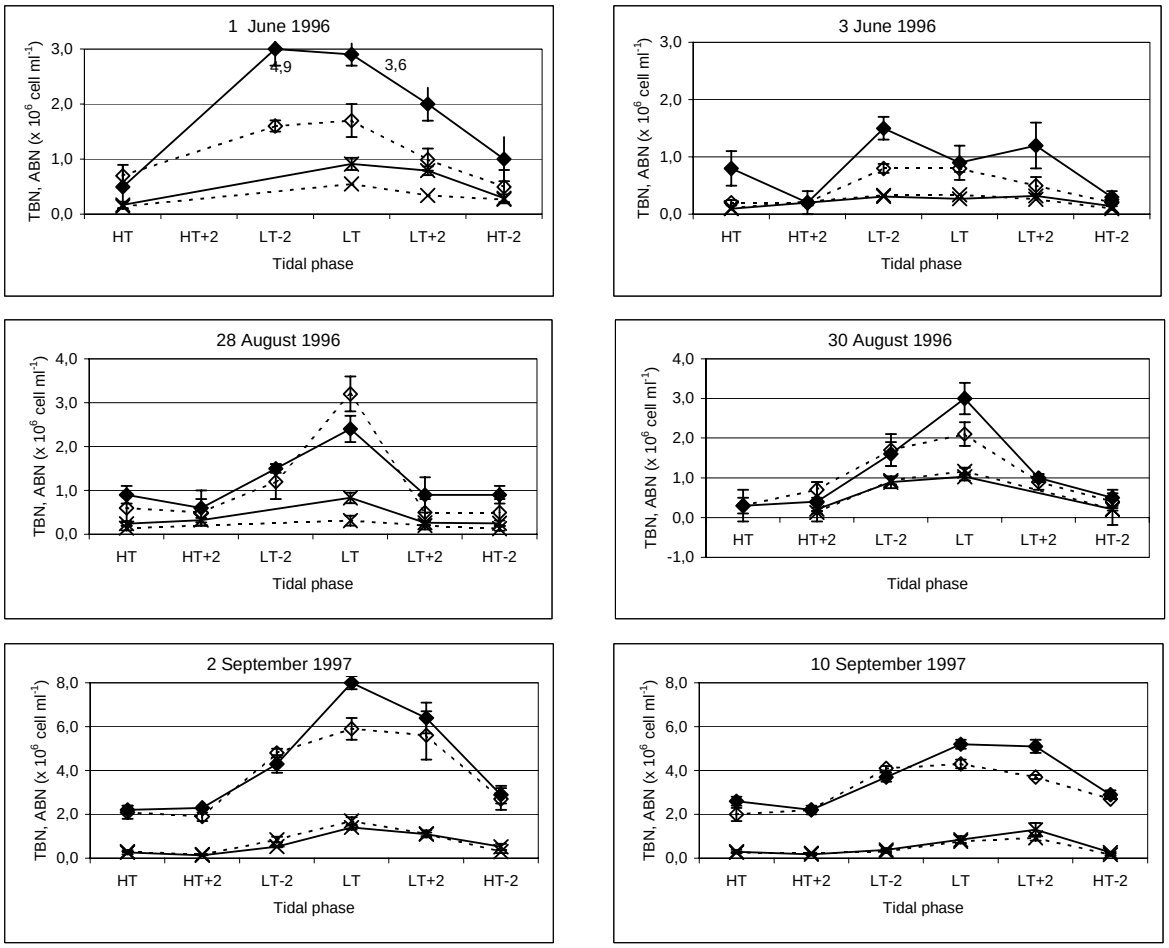


Figure 2: Vertical profiles of bacterial biomass production (BBP) in the marine zone (30 August 1996). Determinations at 0.2 m below surface, and at 4.5 m, 2.5 m and 0.5 m above the sediment at high tide (HT) and low tide (LT) and at intervals of 2 hours as indicated.

Vertical profiles of bacterial number and productivity

Bacterial production in the MZ was similar at 4.5 m, 2.5 m and 0.5 m above the sediment but it was less than in surface water (Figure 2). Further data compared only surface (0.2 m) and deep (0.5 m above the sediment) water layers. TBN and ABN were more abundant at the top of the water column (Figure 3), but the difference between surface and deep water was not significant (ANOVA, $p>0.05$). The fraction of active bacteria was, however, similar at both depths (Table1). Bacterial productivity was significantly higher (ANOVA, $p<0.05$) near the surface (Figure 4) being on average 3.3 times greater than in deep water but reaching values up to 8.5 times greater. BBP_{cell} and $BBP_{active\ cell}$ were also higher near the surface, being on average 2.8 and 3.0 times greater than near the bottom (Figure 5). The larger BBP registered in near-surface water was circumscribed to a maximum depth of 0.5 m (Figure 1). The contribution of the surface water layer to the total BBP in

the marine water column was 35%. The stratification of BBP, BBP_{cell} and $BBP_{active\ cell}$ was particularly noticeable near low tide (Figures 4 and 5). No stratification of bacterial number, fraction of active bacteria or bacterial productivity was observed in the brackish water zone (Figures 6, 7, Table 1). The difference between surface and deep water was not significant (ANOVA, $p>0.05$). Specific productivity per cell was, however, frequently higher near surface



(Figure 8) but the average was similar in surface and bottom water (Table 1).

Figure 3: Total bacterial number (TBN) at 0.2 m below the surface and at 0.5 m above the sediment in the marine zone (station N1) at high tide (HT), low tide (LT) and at intervals of 2 hours as indicated. —◆— TBN at surface water, ---◇--- TBN at deep water, —×— ABN at surface water, ---×--- ABN at deep water.

Fluctuation of bacterial number and productivity during the tidal cycle

The abundance and productivity of the bacterioplankton reacted to tidal fluctuation in both the marine and brackish water zones (Figures 3-8). The maximum and minimum values occurred around LT and HT slack water, respectively. The fluctuation in abundance of total bacteria and active bacteria was greater at the marine site. The percentage of active cells fluctuated randomly. The tidal effects on bacterial production were also greater in the marine zone.

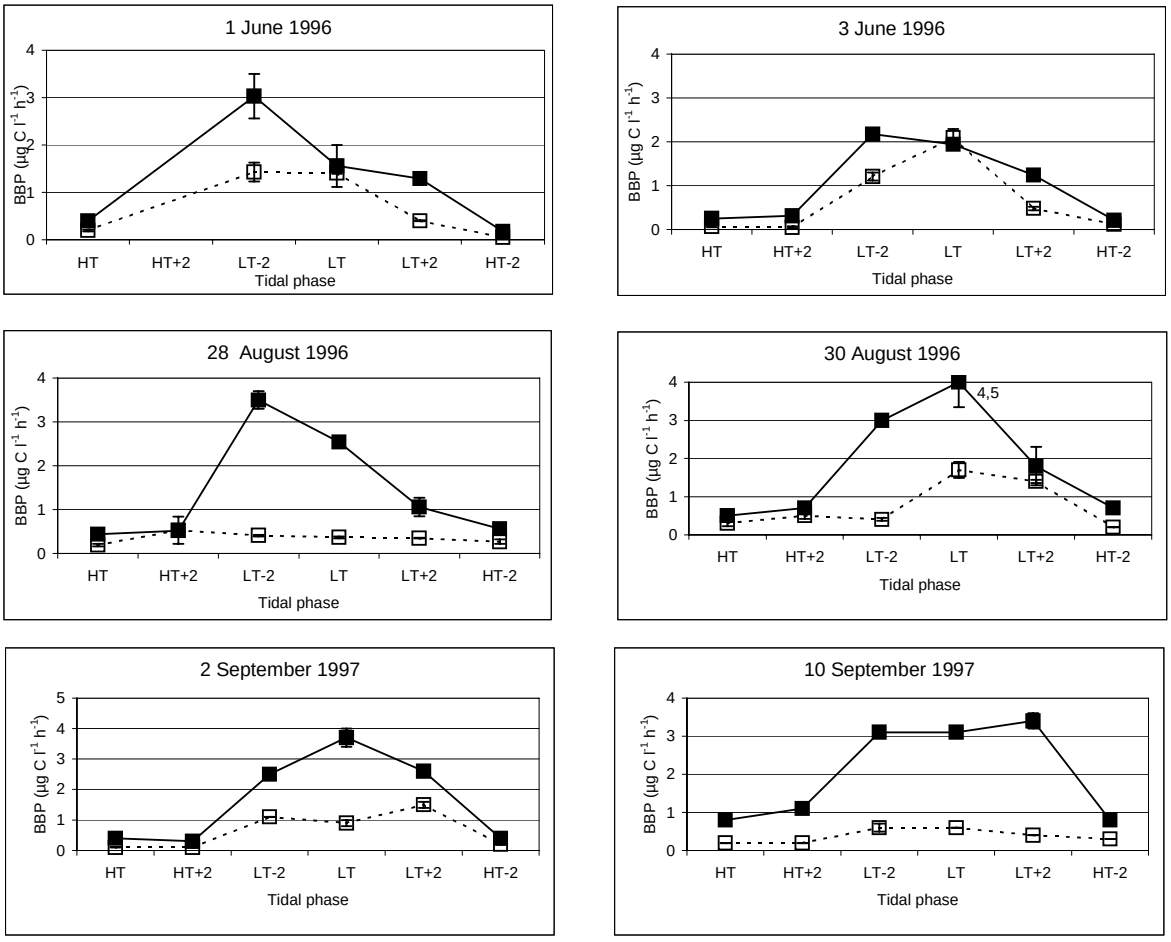


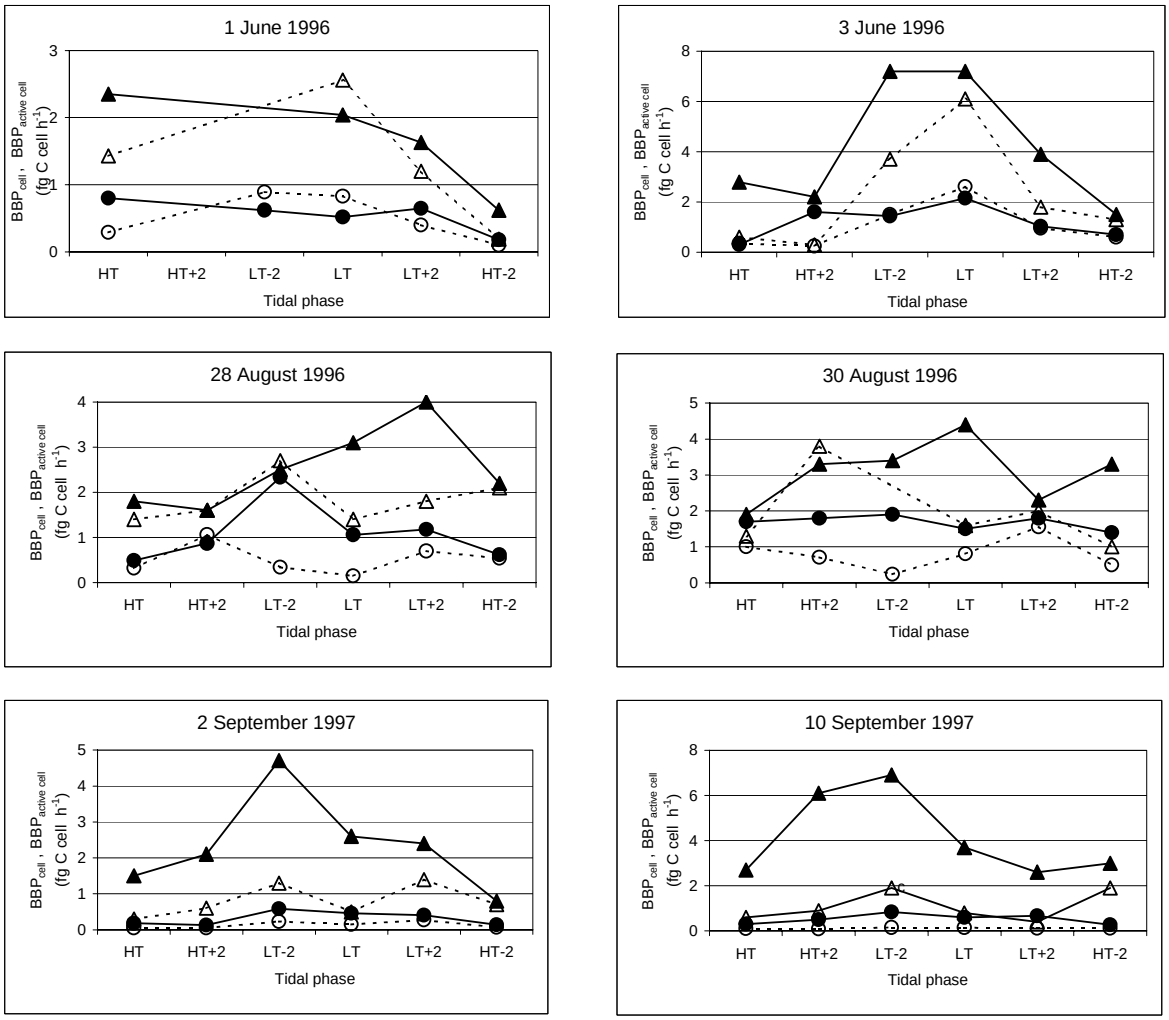
Figure 4: Tidal variation in bacterial biomass productivity (BBP) in the marine zone (station N1). Legends as in figure 3. —■— BBP at surface water, ---□--- BBP at deep water.

Statistical analysis

Stepwise multiple regression showed ABN as the variable that better explained the BBP (Table II). In the MZ, depth and salinity explained also the BBP variation. In this zone, ABN, depth and salinity explained 69% of BBP variation. In BZ only 12% of the BBP variation was explained by ABN. TBN variation was better explained by temperature in MZ (66%) and by percentage of dissolved oxygen and chlorophyll in BZ (33%) (Table III). TBN was the variable that better explained ABN variation (Table IV). In the MZ, TBN, POC, temperature and percentage of dissolved oxygen explained 64% of ABN variation. In the BZ, only 26% of ABN variation was explained by total bacterial number.

One-way ANOVA indicated a significant difference between surface and bottom values of BBP only in MZ ($p<0.05$). Bacterial abundance (TBN and ABN), chlorophyll concentration and physico-chemical characteristics were not significantly different between near-surface and bottom

water in both zones ($p>0.05$). In the MZ, the results of two-way ANOVA showed the significance of the depth and tide on bacterial production variation. The interaction between these two factors was also significant indicating that depth and tide may act synergistically (Table V). However, the homogeneity of variances in this test was not observed ($p<0.05$). In the BZ, the effect of depth and tide on bacterial production variation was not significant ($p>0.05$). In this case the homogeneity of



variances was observed ($p=0.106$).

Figure 5: Tidal variation in bacterial specific productivity in the marine zone (station N1). Legends as in figure 3. —●— BBP_{cell} at surface water, ---○--- BBP_{cell} at deep water, —▲— $BBP_{active\ cell}$ at surface water, ----△---- $BBP_{active\ cell}$ at deep water.

DISCUSSION

In this estuary, the fluctuation of bacterial production during the warm season was dependent on the absolute size of the active bacteria sub-population and on the intensity of their activity rather than on increment of the active fraction. It depended also on the variable intensity of the bacterial activity in response to the changing water properties during transport. It was observed

that the fluctuation in bacterial production during the tidal cycle resulted not only from the fluctuation in TBN (and ABN), but also from the rapid response of the active cell productivity to

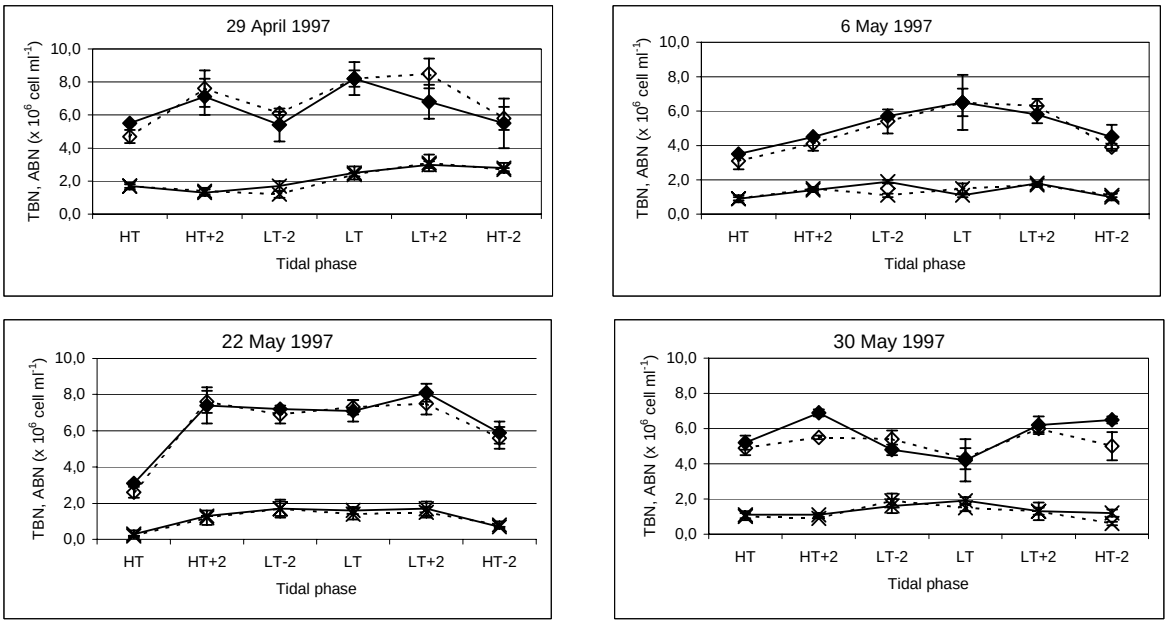


Figure 6: Tidal variation in total bacterial number (TBN) in the brackish water zone (station I6). Legends as in figure 3. —◆— TBN at surface water, ---◇--- TBN at deep water, —×— ABN at surface water, ---×--- ABN at deep water.

the changing water conditions. In marine zone, bacterial production variation was also influenced by depth and salinity. In the shallower brackish water zone, the set of independent variables explained a smaller percentage of bacterial production variation than in the marine zone, suggesting the greater importance of other variables, namely diffusion of nutrients from sediment and allochthonous organic matter inputs. Allochthonous organic matter is most abundant in the inner- and mid-estuary of Ria de Aveiro (Silva, personal communication). Additionally, it has been

Table II: Multiple stepwise regression between LogBBP and physico-chemical and biological variables. Results are present for data of the two zones (n = 118) and separately for marine zone (n = 70) and for brackish water zone (n = 48). β: standardized coefficient of regression; p: level of significance of the slopes in the regression equations. Variables as in Table I.

	Independent variables	Regression equation	Adjusted r^2
MZ and BZ	LogABN (β=0.518; p=0.000)	LogBBP=-7.073+0.668LogABN-	0.712
	Depth (β=-0.347; p=0.000)	0.137Depth-0.040Salinity	
	Salinity (β=-0.179; p=0.004)		
MZ	LogABN (β=0.502; p=0.000)	LogBBP=-2.784+0.670LogABN-	0.694
	Depth (β=-0.452; p=0.000)	0.133Depth-0.16404Salinity	
	Salinity (β=-0.276; p=0.001)		
BZ	LogABN (β=0.375; p=0.017)	LogBBP=-6.711+0.555LogABN	0.118

reported (18, 33) that these compounds determine the major composition of the humic fraction of dissolved organic compounds in the surface water of this estuary. The combined effects of these factors give rise to larger fluctuations in bacterial production in the marine zone than in the brackish water zone (average tidal fluctuation in production of 12.5 and 4.9 in the MZ and BZ, respectively).

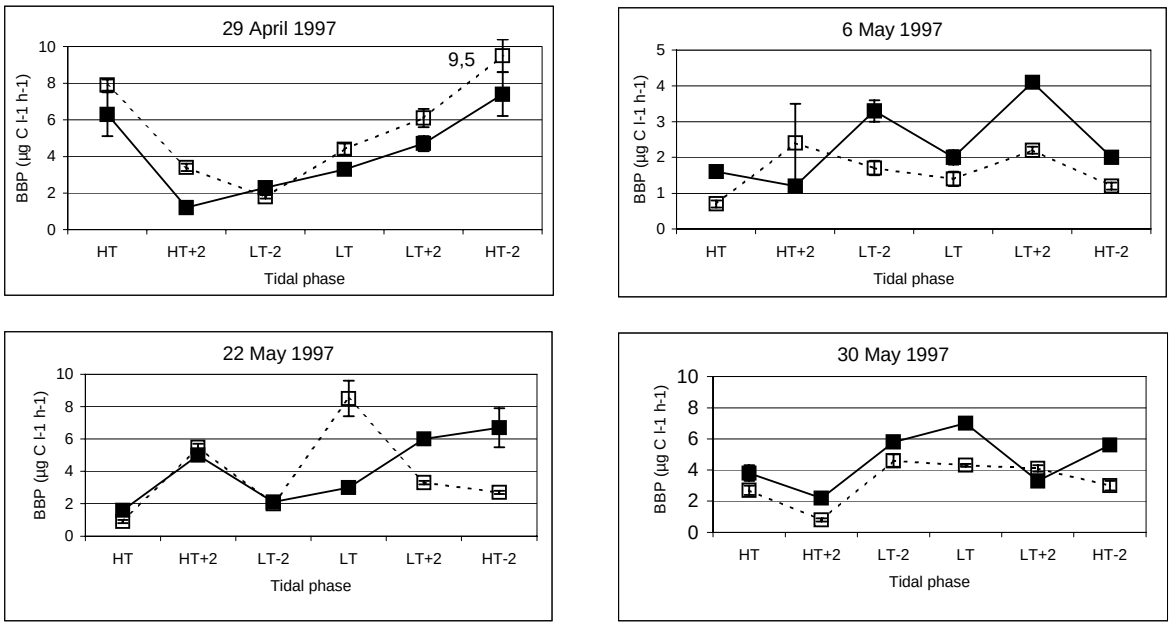


Figure 7: Tidal variation in bacterial biomass productivity (BBP) in the brackish water zone (station I6). Legends as in figure 3. —■— BBP at surface water, ---□--- BBP at deep water.

The stratification of bacterial production and bacterial cell productivity in the deeper area of the estuary asks for an additional factor influencing the spatial pattern of productivity. The enhancement of bacterial productivity in near-surface water by a factor of 3.3, against the expected

Table III: Multiple stepwise regression between LogTBN and physico-chemical and biological variables. Results are present for data of the two zones (n = 118) and separately for marine zone (n = 70) and for brackish water zone (n = 48). β: standardized coefficient of regression; p: level of significance of the slopes in the regression equations. Variables as in Table I.

	Independent variables	Regression equation	Adjusted r^2
MZ and BZ	Temperature (β=0.660; p=0.000)	LogTBN=8.719+0.313Temperature	0.568
	LogChlor (β=0.179; p=0.023)	+0.345LogChlor	
MZ	Temperature (β=0.816; p=0.000)	LogTBN=4.259+0.587Temperature	0.658
	LogChlor (β=0.179; p=0.023)	+0.345LogChlor	
BZ	Log%O_2 (β=-0.419; p=0.005)	LogTBN=17.713-0.573 Log%O_2	0.263
	LogChlor (β=0.345; p=0.017)	+0.163 LogChlor	

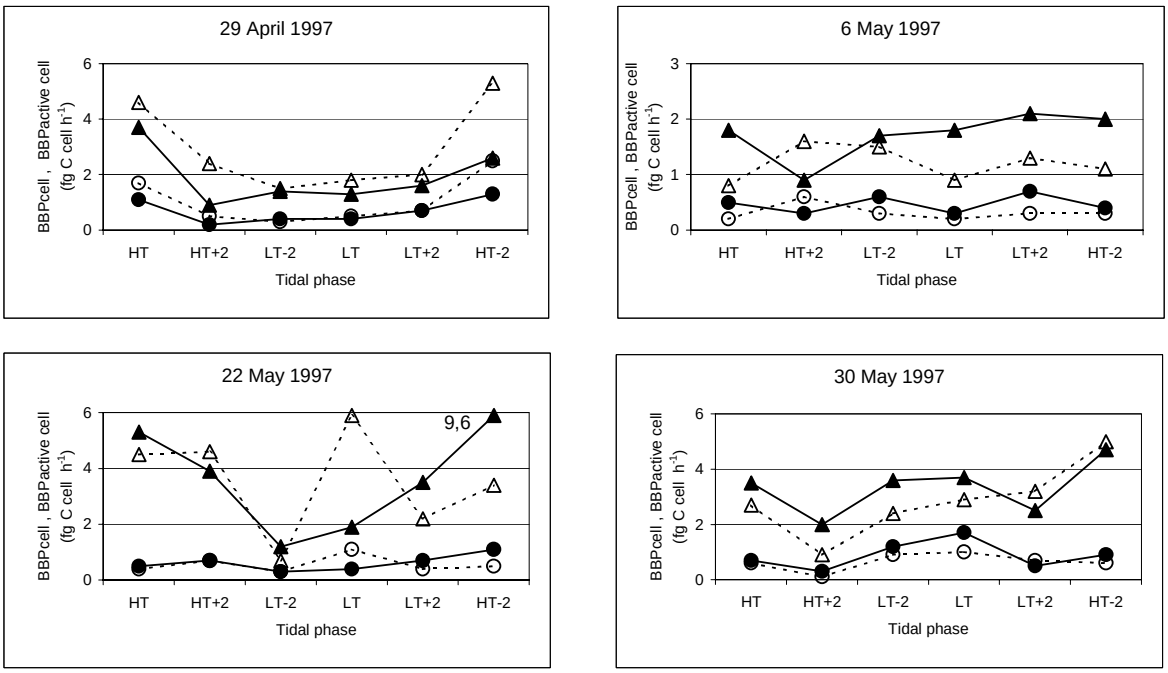


Figure 8: Tidal variation in bacterial specific productivity per cell (BBP_{cell}, $BBP_{active\ cell}$) in the brackish water zone (station I6). Legends as in figure 3. —●— BBP_{cell} at surface water, ---○--- BBP_{cell} at deep water, —▲— $BBP_{active\ cell}$ at surface water, ---△--- $BBP_{active\ cell}$ at deep water.

negative effect of solar irradiation, was not related to any of the studied physical and chemical or biological variables, which were relatively constant along the water column. Although a first approach suggests that increased values of near-surface productivity could be associated with phytoplankton and bacterioplankton transport by surface water from the brackish water zone, other biological parameters (*i. e.* total and active bacterial numbers and chlorophyll concentration) were relatively constant along the water column. Even though, the phytoplankton distribution could not directly explain the higher bacterial productivity in the half-meter surface water layer, irradiation may enhance phytoplankton exudation (6, 19, 43) and consequently bacterial productivity in this water layer. In addition, higher bacterial productivity in near-surface water could be stimulated by photochemical transformation of recalcitrant dissolved organic matter into labile compounds that may stimulate locally and transiently the growth of active bacteria. Although chemical analysis showed that phosphate and nitrite plus nitrate levels were not significantly higher in near-surface water, it has been found that photodegradation generates low molecular weight organic compounds such as primary amines (9) amino acids, combined amino acids and urea (26) that are readily assimilated by bacterioplankton. The higher stratification of bacterial production near low tide suggests that part of the recalcitrant organic matter transformed by solar irradiation in the marine zone may be transported by tidal currents from the brackish water. It would be worthwhile to study vertical and longitudinal distribution of dissolved organic matter in the estuary and test the effects of irradiation on the degradation of recalcitrant DOM and on phytoplankton exudation.

Table IV: Multiple stepwise regression between LogABN and physico-chemical and biological variables. Results are present for data of the two zones (n = 118) and separately for marine zone (n = 70) and for brackish water zone (n = 48). β: standardized coefficient of regression; p: level of significance of the slopes in the regression equations. Variables as in Table I.

	Independent variables	Regression equation	Adjusted r^2
MZ and BZ	LogTBN (β=0.613; p=0.000) LogPOC (β=-0.304; p=0.000) Temperature (β=-0.257; p=0.004)	LogABN=6.171+0.552LogTBN- 1.012LogPOC+0.110Temperature	0.718
MZ	LogTBN (β=0.486; p=0.002) LogPOC (β=-0.348; p=0.000) Temperature (β=-0.488; p=0.002) Log%O_2 (β=-0.282; p=0.005)	LogABN=10.377+0.383LogTBN- 1.010LogPOC+0.270Temperature -1.095 Log%O_2	0.635
BZ	LogTBN (β=0.585; p=0.000)	LogABN=-1.643+1.010LogTBN	0.325

Table V: Two-way ANOVA for the bacterial production in the marine zone.

Source of variation	SS	df	MS	F	Significance
Tide	36.99	5	7.40	24.72	0.000
Depth	16.07	1	16.07	53.72	0.000
Tide * depth interaction	8.97	5	1.79	5.99	0.000
Residual	17.35	58	0.29		
Total	80.09	69	1.16		

The factors are tide (HT, LT and intermediate time intervals of 2 hours) and depth (m).

In the brackish water zone no stratification was detected. This may be caused by a much higher degree in turbidity that blocked light penetration and reduced photodegradation and/or phytoplankton exudation. On the other hand, substrate diffusion from the nearby sediment may increase bacterial production in deeper water, thereby obscuring the increase induced in near-surface water by photodegradation of recalcitrant organic compounds and/or by phytoplankton exudation.

The observed differences between surface and deep water bacterial production in the marine zone invite speculation about the relative importance of irradiation for the distribution of bacterial production within the system. It is worth noting that the vertical variation of bacterial production in the marine zone of the Ria de Aveiro was of the same magnitude as the longitudinal variation between brackish water and marine water. In the less eutrophic zones of estuaries, and in

coastal waters in general, solar irradiation may be instrumental in making available additional sources of nutrients for bacterial growth compensating for limitation of other sources

REFERENCES

1. Abreu S, Duarte A (1997) Biogest First Scientific Report - Environment & Climate Programme. Contract Nº ENV4-CT96-0213 (partner 6 participation)
2. Anderson TR & Williams PJ (1998) Modelling the seasonal cycle of dissolved organic carbon at Station E1 in the English Channel. Coastal Shelf Sci 46:93-109
3. Bano N, Moran MA, Hodson RE (1998) Photochemical formation of labile organic matter from two components of dissolved organic carbon in a freshwater wetland. Aquat Microb Ecol 16:95-102
4. Barrosa JA (1985) Breve caracterização da Ria de Aveiro. Jornadas da Ria de Aveiro. Volume II. Recursos da Ria de Aveiro. Edição da Câmara Municipal de Aveiro
5. Berger B, Hoch B, Kavka G, Herndl GJ (1995) Bacterial metabolism in the River Danube: parameters influencing bacterial production. Freshwater Biol 34:601-616
6. Berman T, Holm-Hansen, O (1974) Release of photoassimilated carbon as dissolved organic matter by marine phytoplankton. Marine Biology 28:305-310
7. Blackburn N, Ulla LZ, Hagstrom A (1996) Cycling of marine dissolved organic matter. II. A model analysis. Aquatic Microb Ecol 11:79-90
8. Blight SP, Bentley TL, Lefevre D, Robinson C, Rodrigues R, Rowlands J, Williams P (1995) Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128: 61-75
9. Bushaw-Newton Kl, Moran MA (1999) Photochemical formation of biologically available nitrogen from dissolved humic substances in coastal marine systems. Aquat Microb Ecol 18:285-292
10. Bussmann I (1999) Bacterial utilization of humic substances from the Arctic Ocean. Aquat Microb Ecol 19:37-45
11. Carlsson P, Granéli E, Tester P, Boni L (1995) Influences of riverine humic substances on bacteria, protozoa, phytoplankton, and copepods in a coastal plankton community. Mar Ecol Prog Ser 127:213-221
12. Carman K (1993) Microautoradiographic detection of Microbial Activity. *In*: Kemp PF, Sherr BF, Sherr EB and Cole JJ (eds.) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, New York. p 397-404
13. Cole JJ, Findlay S, Pace ML (1988) Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Mar Ecol Prog Ser 43:1-10.
14. Conan P, Turley C, Stuff E, Pujo-Pay M, Wambeke F (1999) Relationship between phytoplankton efficiency and the proportion of bacterial production to primary production in the Mediterranean Sea. Aquat Microb Ecol 17:131-144
15. Cunha MA, Almeida MA, Alcântara F (1999) Compartments of oxygen consumption in a tidal mesotrophic estuary (Ria de Aveiro, Portugal). Acta Oecologica 20 (4):227-235
16. Cunha M, Almeida MA, Alcântara F (2000) Patterns of variation of ectoenzimatic and heterotrophic bacterial activities along a salinity gradient in a shallow tidal estuarine. Mar Ecol Prog Ser 204:1-12.
17. Ducklow H, Carlson C, Smith W (1999) Bacterial growth in experimental plankton assemblages and seawater cultures from the *Phaeocystis antarctica* bloom in the Ross Sea, Antarctica. Aquat Microb Ecol 19:215-227
18. Esteves VI (1995) Extracção e caracterização de substâncias húmicas de diferentes ambientes aquáticos. Ph. D. Thesis. Universidade de Aveiro
19. Feuillade M, Feuillade J, Fiala V (1990) The effect of light on the release of organic compounds by the cyanobacterium *Oscillatoria rubescens*. Aquat Sci 52:348-359
20. Fuhrman JA & Azam F (1982) Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar Biol 66:109-120
21. Fuhrman J (1992) Bacterioplankton roles in cycling of organic matter: the microbial food web. In: Falkowski PG, Wood-head AD (eds.) Primary productivity and biogeochemical cycles in the sea. Plenum press, New York, pp 361-383
22. Fuks, D, Precali R, Devercovi M (1994) Bacterial production in the stratified Karstic estuary of the Krka River. Acta adriat 34 (1/2):21-28

23. Goosen NK, van Rijswijk, Kromkamp J, Peene J (1997) Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat Microb Ecol 12:223-232.
24. Hagstrom A, Larson U, Horstedt P, Normark S (1979) Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl Environ Microbiol 37:805-812
25. Hobbie JE, Daley R, Jasper S (1977) Use of Nuclepore Filters for Counting Bacteria by Fluorescence Microscopy. Appl Environ Microbiol 33:1225-1228
26. Jorgensen NO, Tranvik L, Edling H, Granéli W, Lindell M (1998) Effects of sunlight on occurrence and bacterial turnover of specific carbon and nitrogen compounds in lake water FEMS Microbiol Ecol 25:217-227
27. Mann CJ & Wetzel RG (1996) Dissolved organic carbon and its utilisation in a riverine Wetland ecosystem. Biogeochemistry 31:99-120.
28. Page HM, Petty RL, Maede DE (1995) Influence of watershed runoff on nutrient dynamics in a Southern California salt-marsh. Estuar Coast Shelf 41:163-180
29. Parsons TR, Maita Y, Lalli CM (1989) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford
30. Peduzzi P, Herndl GJ (1992) Zooplankton activity fueling the microbial loop: differential growth response of bacteria from oligo- and eutrophic waters. Limnol Oceanogr 37:1087-1092
31. Proctor LM, Fuhrman JA (1991) Roles of viral infection in organic particle flux. Mar Ecol Prog Ser 69:133-142
32. Rodier J (1996) L`analyse de l`eau: eaux naturelles, eaux résiduaires, eau de mer. 8 Edition, Dunod, Paris.
33. Santos EB, Duarte AC (1998) The influence of pulp and paper mill effluents on the composition of the humic fraction of aquatic organic matter. Wat Res 32:597-608
34. Shiah F, Ducklow HW (1994) Temperature and substrate regulation of bacterial abundance, production, and specific growth rate in Chesapeake Bay. Limnol Oceanogr 39:1243-1258
35. Silva JJ (1994) Circulação de Água na Ria de Aveiro: Contribuição para o Estudo da Qualidade da Água. Ph. D. Thesis. Universidade de Aveiro
36. Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic bacteria. Mar Ecol Prog Ser 51:201-213
37. Strom SL, Benner R, Ziegler S, Dagg MJ (1997) Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol Oceanogr 42:1364-1374
38. Thingstad TF & Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquat Microb Ecol 13:9-27
39. Tranvick LJ (1992) Allochthonous dissolved organic matter as an energy source for pelagic bacteria and the concept of the microbial loop. Hydrobiologia 229:107-114
40. Wetzel RG (1992) Gradient-dominated ecosystems: sources and regulatory functions of dissolved organic matter in freshwater ecosystems. Hydrobiologia 229:181-198
41. Williams PJ (1990) The importance of losses during microbial growth: commentary on the physiology, measurement and ecology of the release of dissolved organic material. Mar Microb Food Webs 4:175-206
42. Yentsch CS & Menzel DW (1963) A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res. 10:221-231
43. Zlotnik I & Dubinsky (1989) The effect of light and temperature on DOC excretion by phytoplankton. Limnol Oceanogr 34:831-839
44. Zweifel U & Hagstrom A (1995) Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). Appl Environ Microbiol 61 (6): 2180-2185

CHAPTER 3

SEASONAL CHANGE IN THE PROPORTION OF BACTERIAL AND PHYTOPLANKTON PRODUCTION ALONG A SALINITY GRADIENT IN A SHALLOW ESTUARY

Almeida MA, Cunha MA and Alcântara F

Departamento de Biologia - Universidade de Aveiro
Campus Universitário de Santiago, 3810-193 AVEIRO, PORTUGAL

ABSTRACT: We intended to evaluate the relative contribution of primary production versus allochthonous carbon in the production of bacterial biomass in a mesotrophic estuary. Different spatial and temporal ranges were observed in the values of bacterioplankton biomass (31-273 µg C l^{-1}) and production (0.1-16.0 µg C l^{-1} h^{-1}, 1.5-36.8 mg C m^{-2} h^{-1}) as well as in phytoplankton abundance (50-1700 µg C l^{-1}) and primary production (0.1-512.9 µg C l^{-1} h^{-1}, 1.5-512.9 mg C m^{-2} h^{-1}). Bacterial specific growth rate (0.10-1.68 d^{-1}) during the year did not fluctuate as much as phytoplankton specific growth rate (0.02-0.74 d^{-1}). Along the salinity gradient and towards the inner estuary, bacterio- and phytoplankton biomass and production increased steadily both in the warm and cold seasons. The maximum geographical increase observed in these variables was 12 times more for the bacterial community and 8 times more for the phytoplankton community. The warm to cold season ratios of the biological variables varied geographically and according to these variables. The increase at the warm season achieved its maximum in the biomass production, particularly in the marine zone and at high tide (20 and 112 times higher in bacterial and phytoplankton production, respectively). The seasonal variation in specific growth rate was most noticeable in phytoplankton, with seasonal ratios of 3 to 26. The bacterial community of the marine zone responded positively - generating seasonal ratios of 1-13 in bacterial specific growth rate - to the strong warm season increment in phytoplankton growth rate in this zone. In the brackish water zone where even during the warm season allochthonous carbon accounted for 41 % (on average) of the bacterial carbon demand, the seasonal ratio of bacterial specific growth rate varied from about 1 to 2. During the warm season, an average of 21% of the primary production was potentially sufficient to support the whole bacterial production. During the cold months, however, the total primary production would be either required or even insufficient to support bacterial production. The estuary turned then into a mostly heterotrophic system. However, the calculated annual production of biomass by bacterio- and phytoplankton in the whole ecosystem showed that auto- and heterotrophic production was balanced in this estuary.

Key Words: Bacterial production, specific growth rate, primary production, estuary

INTRODUCTION

The functioning and, in particular, the productivity of estuarine ecosystems is largely reflected in the levels of primary production and in the fate of autochthonous and allochthonous carbon. Changes in bacterial density and activity along trophic and salinity gradients affect differently the balance between carbon incorporation and carbon mineralisation in different parts of the estuary (Carlson & Ducklow, 1996; del Giorgio *et al*., 1997, del Giorgio & Cole, 1998). In the case of the mesotrophic ecosystem of Ria de Aveiro, most of the carbon which is uptaken by the water column bacteria is incorporated into biomass (about 90% for glucose) (Cunha *et al*., 2000). This is not the common conclusion derived from studies in other marine ecosystems where the bacterial growth efficiency in eutrophicated waters plateaux near 50% (reviewed by del Giorgio & Cole, 1998).

It is recognised that primary production plays a fundamental role on bacterial activity (Cole *et al*., 1988; Conan *et al*., 1999; Ducklow & Carlson, 1992) and that other factors, namely exogenous carbon (Cunha e*t al*., 1999; Goosen *et al*. 1997; Mann & Wetzel, 1996; Reitner *et al*., 1999) may affect the coupling of these activities in aquatic ecosystems. On the other hand, physical and other chemical factors such as temperature, salinity and the concentration of mineral nutrients (PO_4^{3-} and NO_3^-) are also involved in the modulation of the bacterioplankton metabolism (Almeida *et al*., 2001a; Cunha e*t al*., 2000; Riegsbee *et al*., 1996; Shiah & Ducklow, 1994; Tabor & Neihof, 1982).

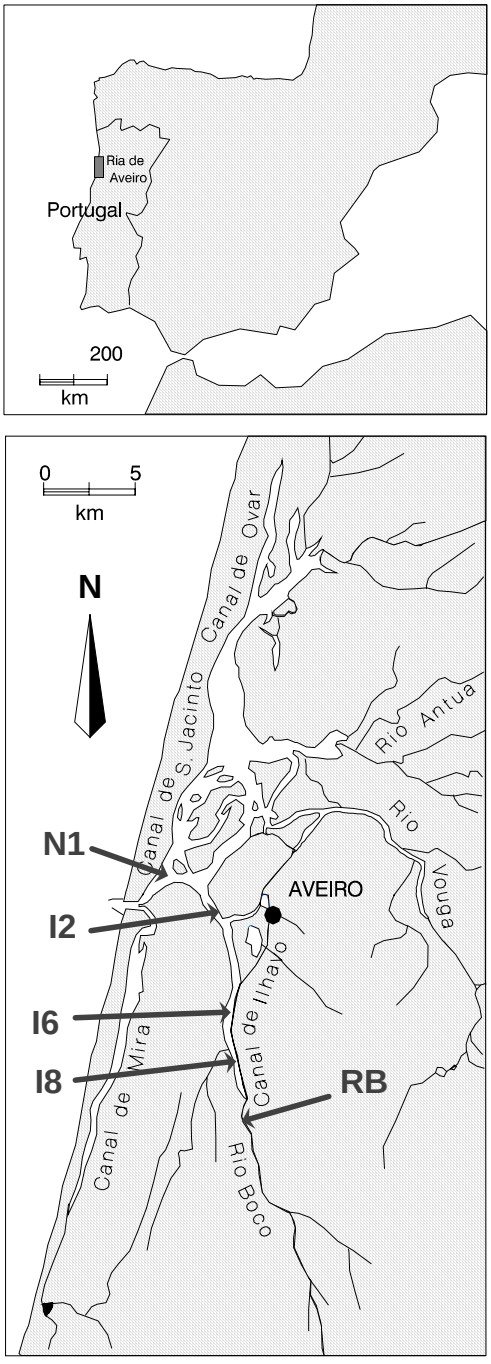


Figure 1: The estuarine system of Ria de Aveiro. Sampling stations are indicated by arrows along Canal de Ílhavo. Station N1 in Canal de Navegação represents the marine zone of the ecosystem. Stations I2, I6 and I8 represent the brackish water section of the salinity gradient along Canal de Ílhavo. Rio Boco (RB) is a river station although it is much affected by the variable strength of the tidal current.

It is also known that the heterotrophic bacterioplankton consumes a large, although variable, fraction of the primary production (up to 50%) in marine and freshwater ecosystems (Cole *et al*., 1988; Conan *et al*., 1999; Ducklow & Carlson, 1992; Lignell, 1990). The phytoplankton community, however, does not always supply the total amount of carbon required for the observed level of heterotrophic bacterial growth (Scavia & Laird, 1987). In this case other substrate sources have to be admitted (Shiah & Ducklow, 1994). In this study we intended to evaluate the importance of primary production in bacterial growth in a shallow estuary during the warm and cold seasons.

MATERIALS AND METHODS

Description of study site

Ria de Aveiro (see Fig. 1) is a mesotrophic shallow estuarine system on the western coast of Portugal connected to the Atlantic by a narrow opening. It is subjected to considerable inputs of industrial and domestic discharges occurring mainly at the periphery.

The lagoon covers an area of 66 to 83 Km^2, respectively at low and high tide (Silva, 1994). It

exchanges with the sea 89 Mm^3 of water in tides of 1 to 3 m amplitude (Silva, 1994). Several rivers carry an average of 1.8 Mm^3 of fresh water into the lagoon during a tidal cycle (Barrosa, 1985). The Ria has a complex topography, with three channels spreading from the mouth towards the different streams forming a multi-estuarine ecosystem. Secondary branches originate from the three main channels to form a tight net of channels, islands and mudflats. In this study, we chose the Canal de Ílhavo, the smallest of the second-order channels, with a water volume of 2.8 Mm^3 at low tide and 9.3 Mm^3 at high tide. This channel offers the advantages of a straighter water circulation and the absence of meaningful agriculture activity along the banks that might affect irregularly its trophic condition.

The marine zone (MZ) of the ecosystem is represented by station N1 in Canal de Navegação. Stations I2, I6 and I8 represent the salinity gradient in the brackish water section (BZ). Rio Boco (RB) is the river station that is periodically affected by the variable strength of the tidal current.

Sampling. Water samples were collected along the longitudinal profile of salinity (see Fig. 1) in summer of 1997 (12^{th} and 20^{th} July). In the cold season, samples were collected only at two sampling sites (station N1, 10^{th} December 1997 and 9^{th} January 1998; station I6, 28^{th} October and 7^{th} November 1997). Surface water (0.2 m below surface) was collected at HT and LT at the centre of the channels. The diel variation in bacterial production was studied in the marine zone during the warm season (16 and 17 of June 1997). In this case surface water samples were collected at intervals of 1 hour during a total of 24 hours. Water samples were transported to the laboratory and processed within 2-3 hours after collection.

Physical and chemical variables. Samples for nutrient (PO_4^{3-}, $NO_3^- + NO_2^-$) analysis were filtered through MSI acetate membranes with a 0.45 µm pore size. PO_4^{3-} was determined by the molibdate method and NO_2^- plus NO_3^- were quantified by the sulfanilic acid method after reduction of NO_3^- to NO_2^- in a cadmium column. The analyses were performed in a Segmented Flow Injection Automatic Analyser Alliance Instrument – Evolution II (Abreu & Duarte, 1997). The concentration of suspended solids was determined after filtration of triplicate 0.5 l water aliquots through pre-weighted and pre-combusted Whatman GF/C filters. The filters were dried at 60ºC for 24 hours and suspended solids (seston) calculated as the increase in dry weight. The organic matter fraction in the seston (POM) was determined as the loss of weight after 4 hours combustion at 525 ºC (Parsons *et al.*, 1989). Particulate organic carbon (POC) was assumed to be 50% of POM (Rodier, 1996). The depth of the water column was measured with a Sonar probe (Hondex PS-7 LCD Digital Sounder). The turbidity of the water column was assessed as Secchi depth values (SD). Temperature and salinity values were read in a WTW LF 196 Conductivity Meter. Dissolved

oxygen concentration, expressed as percentage of saturation, was determined with a WTW OXI 96 oxygen meter equipped with a WTW BR 190 stirrer.

Total Bacterial number (TBN) and bacterial biomass (BB). Bacterial cells were counted by epifluorescence microscopy with a Leitz Laborlux K microscope equipped with a I 2/3 filter for blue light. The samples were fixed with 2% formaldehyde (final concentration), filtered through black polycarbonate membranes of 0.2 µm (Poretics) and stained with 0.03% acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted in each of three replicate preparations. Bacterial biovolume was determined after measurement of 50 cells in each of triplicate samples. Cell volume was converted to cell carbon after assuming a conversion factor of 350 fg C μm^{-3} (Bjornsen, 1986).

Bacterial production (BP). Bacterial production was determined in 10 ml triplicate sub-samples plus a control that was immediately fixed by addition of formaldehyde (2% final concentration). The samples were incubated in the presence of a saturating concentration (30 nM) of ^{3}H-leucine (Amersham, specific activity of 58-92 Ci $mmol^{-1}$). Incubation was carried out for 1 hour at *in situ* temperature. After incubation, samples were fixed with formaldehyde. Protein precipitation was performed through the addition of 1 ml 20% ice-cold TCA followed by 15 minutes incubation in ice. These samples were filtered through 0.2 µm polycarbonate membranes (Poretics), rinsed twice with 2 ml of 5% ice-cold TCA and 5 ml of 90% ice-cold ethanol (Blight *et al*., 1995). After standing for 3 days in scintillation cocktail (Universol) the radioactivity was read in a liquid scintillation counter (Beckman LS 6000 IC). Bacterial production was calculated on a per litre basis (µg C l^{-1} h^{-1}) and integrated per square meter (mg C m^{-2} h^{-1}) according to water depth. The total BP for the ecosystem during the cold or the warm season was calculated from the averaged BP at the two tides (HT, LT) expressed on a per litre basis. These values were multiplied by 24 hours not taking into account the eventual difference in production during night and day periods. The semester production in each zone resulted from diel production multiplied by 183 days and the average volume of water in the respective zone - 32.1 Mm^3 in the MZ and 88.6 Mm^3 in the BZ - (Silva, 1994). The annual BP in the lagoon resulted from the sum of the two semester values.

Bacterial specific growth rate (BGR). BGR was calculated as the ratio of bacterial production (BP), expressed in µg C l^{-1} h^{-1}, and bacterial biomass (BB) expressed in µg C l^{-1}. BGR per day respected a 24 hour period.

Chlorophyll *a* (CHLO). The concentration of chlorophyll *a* was determined fluorimetrically (Yentsch & Menzel, 1963) after filtration of 0.5 l triplicate samples through Whatman GF/C filters

and overnight extraction in 90% acetone, at 4°C. Phytoplankton biomass was calculated from the concentration of chlorophyll *a* assuming a conversion factor of 50 (Eppley *et al.*, 1977).

Primary production (PP). Primary production was determined by the ^{14}C-bicarbonate uptake method (Steeman-Nielsen, 1952). Duplicate samples (50 ml) were incubated with 8 µCi ^{14}C-bicarbonate (Amersham, specific activity of 50-60 mCi mmol^{-1}) at simulated *in situ* light conditions adjusted with neutral light filters to 100%, 50%, 25%, 12,5%, 1% and 0,1% of incident light intensity. After 4 hours of incubation, 10 ml triplicate samples were filtered through a 0.2 µm pore-size membranes (Poretics polycarbonate membranes). The filters were treated with fuming HCl for 15 minutes and placed in picovials containing 5 ml of scintillation cocktail (Universol). The radioactivity was read immediately in a Beckman LS 6000 IC scintillation counter. Secchi depth was used for the calculation of the depths corresponding to 50, 25, 10, 1 and 0.1% of incident light intensity. Secchi depth was multiplied by an empirical factor, 0.40, 0.81, 1.35, 2.70 and 5.40 respectively for 50, 25, 10, 1 and 0.1% of incident light intensity (K. Gocke, pers. com.). The integrated value, expressed in mg C m^{-2} h^{-1}, was calculated taking into account the production and the thickness of each photic layer. PP was then averaged according to the total volume of the water column (µg C l^{-1} h^{-1}). The total PP in the cold and warm seasons and the annual PP in the lagoon was calculated as described above for bacterial production but taking into account 12 hour period of insulation.

Table 1: Physical and chemical variables. Salinity (SAL), temperature (TEMP), percentage of dissolved oxygen (%SAT O_2), depth, Secchi depth (SD), particulate organic carbon (POC), dissolved PO_4^{3-} (PO_4^{3-}), dissolved NO_2^- plus NO_3^- (NO_2^-+NO_3^-) and chlorophyll *a* (CHLO) in the warm and cold seasons at high (HT) and low tide (LT).

			SAL (UPS)	TEMP (°C)	% SAT O_2	DEPTH	SD (m)	POC (mg l^{-1})	PO_4^{3-} (µM)	NO_2^-+NO_3^- (µM)	CHLO (µg l^{-1})
Warm Season	HT (12th Jul 1997)	N1	35.6	16.6	78	7.5	4.2	6.0	0.26	1.42	6.9
		I2	34.7	18.5	78	7.7	3.5	6.0	0.38	1.41	8.2
		I6	32.3	21.3	78	2.4	1.8	6.5	1.20	2.37	12.1
		I8	29.0	23.3	78	2.3	1.0	6.5	1.01	2.84	33.5
		RB	7.3	24.1	116	1.0	0.5	6.5	0.40	204.60	22.8
	LT (20th Jul 1997)	N1	33.5	20.8	96	5.8	2.0	6.5	0.89	0.87	11.2
		I2	33.3	22.1	92	4.2	1.2	6.0	1.45	2.21	9.6
		I6	28.9	24.9	76	1.5	0.8	6.0	1.66	1.96	19.2
		I8	25.2	25.9	ND	2.0	0.7	7.0	2.14	5.53	18.6
		RB	4.5	26.6	ND	0.5	0.5	5.5	1.48	111.60	13.0
Cold Season	HT (9th Jan 1998 (7th Nov 1997)	N1	33.1	14.9	90	13.0	8.8	9.5	ND	ND	1.6
		I6	27.2	16.0	85	3.0	8.4	7.8	ND	ND	5.0
	LT (16th Dec 1997 (28th Oct 1997)	N1	21.8	11.2	95	12.3	95	8.6	ND	ND	0.9
		I6	19.2	18.6	51	1.8	51	6.7	ND	ND	7.5

ND – not determined

Phytoplankton specific growth rate (PGR). The capacity of the autotrophic organisms to fix inorganic carbon was calculated as the ratio between PP expressed in µg C l^{-1} h^{-1} and the

phytoplankton biomass expressed in µg C l^{-1} (Parsons *et al*., 1984). Specific growth rate per day was calculated multiplying that ratio by the averaged period of insulation (12 hours).

RESULTS

Physical and chemical variables

A clear longitudinal gradient of salinity was observed between station N1, in the marine zone, (33.5-35.6 PSU) and Rio Boco (4.5-7.3 PSU) (as shown in Table 1). In winter the salinity values were 6-8 PSU lower when compared to summer values. Water temperature in the warm season varied between 16.6 and 26.6 °C increasing upstream in daylight. In the cold season the temperature declined to 11.2-18.5°C, not as much as usual for winter. The level of oxygen saturation in the water column fluctuated from 76 to 116%. POC ranged from 5.5 to 7.0 mg l^{-1} in the warm season, and from 6.7 to 9.5 mg l^{-1} in winter. The longitudinal profile of water depth varied from 0.5 to 7.7 m during the warm season and from 1.8 to 13.0 m in the cold months. Secchi depth was greater in the marine zone, ranging along the salinity gradient from 0.5 to 4.5 meters in the warm season and from 0.8 to 2.0 meters in winter. The concentration of NO_2^- plus NO_3^- in the warm season showed a longitudinal pattern of variation characterised by an enormous peak (111.6-204.6 µM) in the inner estuary. This peak did not reach the mid estuary and only a small variation in this concentration (0.9-5.5 µM) could be noticed from the mid-estuary to the mouth. The variation in the concentration of PO_4^{3-} (0.3-3.3 µM) was not so clearly patterned, but the lowest concentrations were always found at the outer estuary and the peaks at the mid-estuary (as shown in Table 1). The nutrients levels were usually higher at low tide throughout the longitudinal profile producing LT/HT ratios of 2.3 for NO_2^- plus NO_3^- and 4.4 for PO_4^{3-}. In winter, the nutrient levels in Ria de Aveiro, are usually up to 20 times higher for NO_2^- plus NO_3^- and up to 2 times higher for PO_4^{3-} (T. Vinhas, M Valença, unpublished data).

Table 2: Warm to cold season ratios of bacterioplankton and phytoplankton variables measured at HT and LT in the marine (MZ) and brackish water zones (BZ). Bacterial biomass (BB), bacterial production (BP), bacterial specific growth rate (BGR), phytoplankton biomass (PB), phytoplankton production (PP) and phytoplankton specific growth rate (PGR).

VARIABLE	MZ (Station N1)		BZ (Station I6)	
	HT	LT	HT	LT
BB	1.4	3.0	2.2	1.7
BP	20.0	2.1	2.0	3.2
BGR	13.3	1.0	1.0	1.9
PB	5.0	12.0	2.4	2.6
PP	112.3	34.7	20.7	8.6
PGR	26.3	2.6	9.3	5.7

Biological variables

Bacterial biomass (BB) varied from 45 to 273 µg C l^{-1} in the warm season (see Fig. 2) establishing a sharp pattern of enrichment from the outer to the mid-inner estuary. In the cold

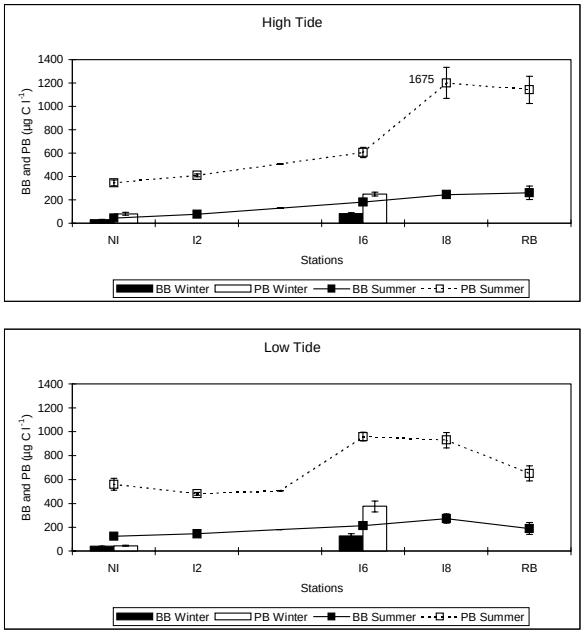


Figure 2: Bacterio- and phytoplankton biomass in the warm season (HT 12th July 1997, LT 20th July 1997) and cold season (Station N1, HT 9th January 1998 and LT 16th December 1997; Station I6, HT 7th November 1997 and LT 28th October 1997). Bacterial biomass (BB), phytoplankton biomass (PB).

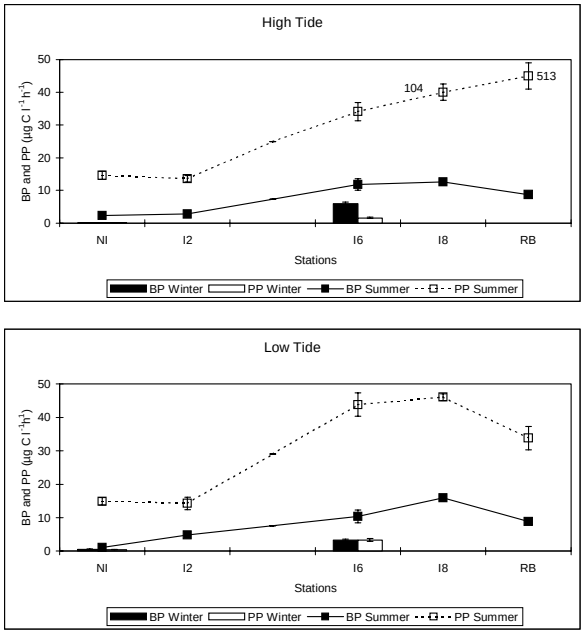


Figure 3: Bacterio- and phytoplankton production in the warm season (HT 12th July 1997, LT 20th July 1997) and cold season (Station N1, HT 9th January 1998 and LT 16th December 1997; Station I6, HT 7th November 1997 and LT 28th October 1997). Bacterial production (BP), phytoplankton production (PP).

season, BB decreased, in general, by a factor of 2 (as shown in Table 2). BB was higher at low tide (LT) in the marine and brackish water zones and in both seasons. Phytoplankton biomass (PB) showed also a clear longitudinal pattern of variation with a maximum value of 1675 µg C l^{-1} at station I8, in summer and at high tide (HT). In the cold months the levels of phytoplankton biomass decreased 8.5 times at station N1 and 2.5 at station I6 (as shown in Table 2). At station I6, in the brackish water zone, phytoplankton biomass was higher at LT (1.5 x) in opposition to station N1, in the marine zone, where phytoplankton was 1.8 times more concentrated at HT. Bacterial production (BP) was more intense in the brackish water stations of the lagoon. In the warm season BP ranged from 1.1 to 16.0 µg C l^{-1} h^{-1} (6.3 to 36.8 mg C m^{-2} h^{-1}) along the longitudinal profile (see Fig. 3). In a 24 hour tidal cycle in the marine zone during the warm season, BP decreased to around 50% during the dark period when compared to the diel average (see Fig. 4). At noon, BP was around twice the diel average while at mid-night, in the same tidal phase, BP was 1.6 times lower than the diel average and 2.7 times lower than the noon value. In winter, BP values (0.1 to 6.0 µg C l^{-1} h^{-1} or 1.5 to 17.7 mg C m^{-2} h^{-1}) decreased 2 times at LT and 20 times at HT in the outer estuary (station N1) and within a range of 2-3 times in the mid estuary (station I6) as shown in

Table 2. However, the pattern of BP variation with tide was not very clear in any of the two stations.

Phytoplankton production (PP) in summer varied along the longitudinal profile from 13.6 to 512.9 µg C l^{-1} h^{-1} (60.1 to 512.9 mg C m^{-2}·h^{-1}) increasing from the outer to the inner estuary (see Fig. 3). The sharpness of the increasing profile was attenuated when PP was integrated according to water column depth. In fact, although phytoplankton activity was lower in the MZ, the greater water depth of this zone increased the integrated value. In the cold season, PP varied from 0.11 to 3.3 µg C l^{-1} h^{-1} (1.5 to 6.0 mg C m^{-2}·h^{-1}). Compared to summer values it decreased 35-112 times at station N1 and 9-21 at station I6 (as shown in Table 2). The maximum values of PP per unit of volume were generally observed at LT.

Table 3: Estimated values of the annual production of bacterioplankton and phytoplankton biomass in the marine zone (MZ), in the brackish water zone (BZ) and in the whole lagoon of Ria de Aveiro (MZ+BZ). Bacterial production (BP), primary production (PP).

		Warm Season	Cold season	Total Annual
		ton C 6 $month^{-1}$ (µg C l^{-1}6 $month^{-1}$)	ton C 6 $month^{-1}$ (µg C l^{-1}6 $month^{-1}$)	ton C y^{-1} (µg C l^{-1}6 y^{-1})
MZ ($31.2\ Mm^3$)	BP	243 (7776)	45 (1425)	288 (9201)
	PP	999 (31968)	18 (606)	1017 (32574)
BZ ($88.6\ Mm^3$)	BP	4248 (47952)	1773 (20001)	6021 (67953)
	PP	5991 (67608)	474 (5358)	6465 (72966)
MZ + BZ ($119.8\ Mm^3$)	BP	4491 (55728)	1818 (21426)	6309 (77154)
	PP	6990 (99576)	492 (5964)	7482 (105540)

In the marine zone, BP expressed on an annual basis and on a per unit water volume was lower (9201 µg C l^{-1} y^{-1}) than PP (32574 µg C l^{-1} y^{-1}). In the cold season, however, BP (1425 µg C l^{-1} h^{-1}) amounted to a higher value than PP (606 µg C l^{-1} h^{-1}). On the other hand, in the BZ, BP (67953 µg C l^{-1} y^{-1}) and PP (72966 µg C l^{-1} y^{-1}) were similar on an annual basis (as shown in Table 3). Considering the total water volume of the lagoon (119.8 Mm^3 being 31.2 Mm^3 in the MZ and 88.6 Mm^3 in the BZ) (Silva, 1994), BP summed to 6309 ton C y^{-1} and PP 7482 ton C y^{-1} fractionated as shown in table 3.

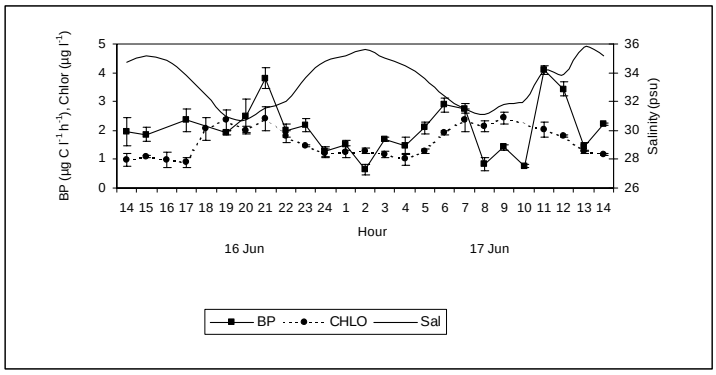


Figure 4: Diel fluctuation of bacterial production (BP) and chlorophyll a (CHLOR) in response to tidal current in the marine zone (warm season). Salinity (Sal).

In the cold and warm seasons, bacterial specific growth rate (BGR) generally increased in less saline waters, showing a range of 0.10 to 1.68 d^{-1} (see Fig. 5). In the marine zone, BGR at HT was 13 times lower in cold water than in summer. At LT BGR was similar in both seasons. In the brackish water zone, BGR at HT was similar in the warm and cold weather but at LT was about 2 times higher in warm water. Phytoplankton specific growth rate ranged from 0.32 to 0.74 d^{-1} in the warm season and from 0.02 to 0.12 d^{-1} in winter (see Fig. 5). In summer PGR could increase up to 26 times at HT in the marine zone. PGR maxima were observed at HT and coincided with PP maxima in both seasons.

In the warm season, BP/PP varied from 0.02 to 0.35 along the longitudinal profile of Canal de Ílhavo (see Fig. 6) with maxima at LT. The proportion of bacterioplankton biomass to phytoplankton biomass (BB/PB) in this season ranged from 0.13 to 0.30. Both ratios tended to increase from the outer- to the mid-inner estuary. In the cold season, however, BP/PP and BB/PB ratios increased to values of 1.00 to 3.61 and 0.34 to 0.91, respectively (see Fig. 6).

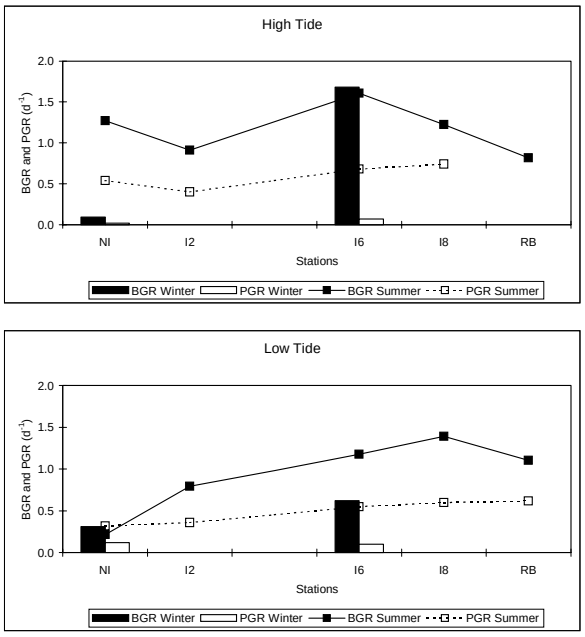


Figure 5: Bacterio- and phytoplankton specific growth rates in the warm season (HT 12th July 1997, LT 20th July 1997) and cold season (Station N1, HT 9th January 1998 and LT 16th December 1997; Station I6, HT 7th November 1997 and LT 28th October 1997). Bacterial specific growth rate (BGR), phytoplankton specific growth rate (PGR).

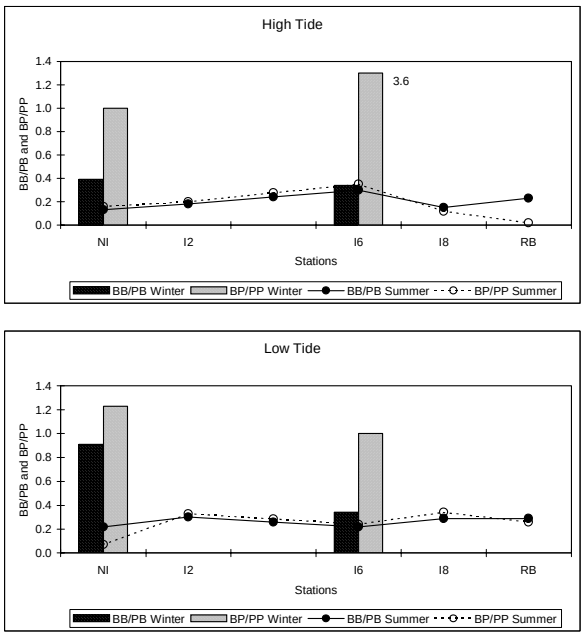


Figure 6: Bacterio- to phytoplankton ratios of biomass (BB/PB) and production (BP/PP) in the warm season (HT 12th July 1997, LT 20th July 1997) and cold season (Station N1, HT 9th January 1998 and LT 16th December 1997; Station I6, HT 7th November 1997 and LT 28th October 1997).

DISCUSSION

Different seasonal, geographical and tidal conditions, involving temperature, salinity and nutrient supply, originated a wide range of bacterioplankton specific growth rates (0.14 – 1.68 d^{-1}). Warm weather enhanced the specific bacterial growth rate by factors of up to 2 in brackish water

and of up to 13 in marine water. The factor of increase in BGR fluctuated considerably during the tidal cycle. Previous laboratory experiments have demonstrated the strong response of bacterial activity to exposure to contrasting water properties (Cunha *et al.*, 2001). Taking these results into consideration, the tidal fluctuation of BGR was interpreted as the result of poor growth of brackish water bacteria when transported to a high salinity environment as well as of the stimulation of marine water bacteria when transported to brackish water. The positive response of marine bacteria to brackish water is, however, counterbalanced to some extent by the low salinity. Preliminary results showed that the stimulation could be enhanced if salinity of brackish water was increased by the addition of NaCl (M. A. Almeida, M. A. Cunha, F. Alcântara, accepted for publication). These facts resulted in an absence of a significant increase of BGR at LT in the marine zone as well as at HT in the brackish water zone during the warm season as might be expected. For this reason, the general increase in BGR from the mouth to the mid-inner estuary was clearer at LT. These facts taken together help to explain the observed spatial variation in bacterial production and reinforce our prior conclusion on the existence of bacterial communities with distinct salinity optima in the estuary (M. A. Almeida, M. A. Cunha, F. Alcântara, accepted for publication). The seasonal and geographical variation of phytoplankton specific growth rate followed the same pattern as BGR but was affected by higher factors. Phytoplankton growth rate in the cold season decreased by factor of 3 to 26, probably due more to reduction of insulation than the small decrease in temperature, whereas BGR decreased by factors of 1-13. The greater (2-4 times) PGR values observed in the brackish water zone followed the increase in nutrients concentrations.

In the cold months and throughout the longitudinal profile of salinity, the bacterio- to phytoplankton biomass ratio was considerably high (0.34-0.91) suggesting that primary production may not be enough to support secondary production. Indeed, the production ratio (BP/PP) along the salinity gradient varied, during this season, from 1.00 to 3.61. We infer that in the cold season, the potential contribution of allochthonous carbon to bacterial biomass production was very high (close to 100%) in both zones of the lagoon.

In the warm season, the scenery in the lagoon changed. The bacterio- to phytoplankton biomass ratio along the longitudinal profile of salinity decreased to much less than 1 (0.13 to 0.30) both at HT and LT. These ratios suggest that bacterial production, in the warm season, might be essentially supported by phytoplankton carbon. In fact, the results showed that BP/PP ranged from 0.02 to 0.35. The carbon incorporated in bacterial biomass at the different sampling stations corresponded, on average, to about 21% of primary production, an acceptable value as observed in other environments where phytoplankton could support the whole bacterial production (Cole *et al.*, 1988; Fuks *et al.*, 1994; Kuosa & Kivi, 1989; Lignell, 1990). Assuming that the contribution of primary production to bacterial production in winter is negligible, that the sources of allochthonous carbon are seasonally stable and that the water temperature did not vary drastically from one season to the other, the bacterial production in winter could be taken as a measure of allochthonous carbon

inputs and used to more accurately estimate the contribution of phytoplankton to bacterial production in warm weather. The fraction of primary production diverted to the bacterial biomass production in the warm season (BP_{summer}–BP_{winter}/PP_{summer}) would decrease to 13%. These figures are within the range of the estimated 6-16% by Conan *et al.* (1999) for aquatic systems in general and of the experimental estimated 5-15% obtained by Fernandez *et al.* (1994) in the Mediterranean Sea. Neither is it far from the 20% value reported by Rheinheimer (1981) for the Kiel Bight. Furthermore, the observation of diel fluctuation in bacterial productivity, characterised by a decreased average during the dark period, suggests coupling between phytoplankton production and consumption by bacteria as reported by Gasol *et al. (*1998). Although during the warm season primary production could be enough to support the whole bacterial production in the estuary, in the brackish water zone allochthonous carbon could contribute, nevertheless, to the production of a significant fraction of bacterial biomass. Taking into consideration the previous estimate of allochthonous organic carbon inputs it is possible to estimate the contribution of primary production to bacterial production during the warm season by subtracting the fraction sustained by allochthonous sources from the total bacterial production in summer. The fraction of bacterial production sustained by primary production was estimated in this way as 59% of the total bacterial production in the brackish water zone and as 74% in the marine zone. This confirms the results of other authors who found that in mid-inner estuary the coupling between BP and PP was lower than in the outer estuary (Shiah & Duklow, 1995).

CONCLUSION

The specific growth rate of the bacterioplankton within the estuary was patterned according to space and season along the estuarine gradient. The presence of bacterial communities with distinct salinity optima, adapted to marine or brackish water conditions, seemed to affect the pattern at different tides.

The geographical, seasonal and diel variations in biomass production bring about the conclusion that on an annual basis and on a per water volume unit, the ratio bacterial production to primary production was less than 1 in the marine zone and almost 1 in the brackish water zone. This defines two broad subsystems where bacterioplankton and phytoplankton are in different balance in relation to biomass production.

REFERENCES

- Abreu, S. & A. Duarte, 1997. Biogest First Scientific Report Environment & Climate. Programme Contract nº ENV4-CT96-0213 (partner 6 participation).
- Almeida, M. A., M. A. Cunha & F. Alcântara, 2001a. Factors influencing bacterial production in a shallow estuarine system. Microb Ecol (in press).
- Barrosa, J. A. 1985. Breve caracterização da Ria de Aveiro. *In*: Jornadas da Ria de Aveiro. Volume II. Recursos da Ria de Aveiro. Edição da Câmara Municipal de Aveiro.
- Bjornsen, P. K., 1986. Automatic determination of bacterioplankton biomass by image analysis. Appl Environ Microbiol 51 (6):1199-1204.

- Blight, S. P., T. L. Bentley, D. Lefevre, C. Robinson, R. Rodrigues, J. Rowlands, & P. Williams, 1995. Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar. Ecol. Prog. Ser. 128:61-75.
- Carlson, C .A. & H., W. Ducklow, 1996. Growth of bacterioplankton and consumption of dissolved organic carbon in the Sargasso Sea. Aquat. Microb. Ecol. 10:69-85.
- Cole, J. J., S. Findlay & M. L. Pace, 1988. Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Mar. Ecol. Prog. Ser. 43:1-10.
- Conan, P. C. Turley, E. Stuff, M. Pujo-Pay, F. & Van Wambeke, 1999. Relationship between phytoplankton efficiency and the proportion of bacterial production to primary production in the Mediterranean Sea. Aquat. Microb. Ecol. 17:131-144.
- Cunha, M. A., M. A. Almeida & F. Alcântara, 1999. Compartments of oxygen consumption in a tidal mesotrophic estuary. Acta Oecologica 20 (4):227-235.
- Cunha, M. A., M. A. Almeida & F. Alcântara, 2000. Patterns of variation of ectoenzimatic and heterotrophic bacterial activities along a salinity gradient in a shallow tidal estuarine. Mar. Ecol. Prog. Ser. 204:1-12.
- Cunha, M. A., M. A. Almeida & F. Alcântara, 2001. Short-time responses of the natural planktonic bacterial community of the changing water properties in an estuarine environment: ectoenzimatic activity, glucose incorporation and biomass production. Microb Ecol (in press).
- del Giorgio P., Y. T. Prairi, & D. F. Bird, 1997. Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. Microb. Ecol. 34:144-154
- del Giorgio, P. & J. Cole, 1998. Bacterial growth efficiency in natural aquatic systems. Annual Rev. Ecol. Syst. 29:503-541.
- Ducklow, H. W & C. A. Carlson, 1992. Oceanic bacterial production. Adv. Microb. Ecol. 12:113-181.
- Eppley, R. W., W. G. Harrison, S. W. Chisholm & E. Stewart, 1977. Particulate organic matter in surface waters off Southern California and its relationships to phytoplankton. J. Mar. Res. 35:671-696.
- Fernandez, M., M. Binai & F. An Wambeke, 1994. Bacterial biomass, heterotrophic production and utilisation of dissolved organic matter photosynthetically-produced in the Almeria-Oran front. J. Mar. Sys. 5:313-325.
- Fuks, D., R. Precali & M. Devercovi, 1994. Bacterial production in the stratified Karstic estuary of the Krka River. Acta Adriat. 34 (1/2):21-28
- Gasol, J. M., M. D. Doval, J. Pinhassi, J. I. Caldéron-Paz, N. Guixa-Boixareu, D. Vaqué, C. Pedrós-Alió, 1998. Diel variations in bacterial heterotrophic activity and growth in the northwestern Mediterranean Sea. Mar. Ecol. Prog. Ser. 164:107-124
- Goosen, N. K., P. van Rijswijk, J. Kromkamp & J. Peene, 1997. Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat. Microb. Ecol. 12:223-232.
- Hobbie, J. E., R. Daley & S. Jasper, 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. environ. Microbiol. 33:1225-1228
- Kuosa, H. & K. Kivi, 1989. Bacteria and heterotrophic flagellates in the pelagic carbon cycle in the northern Baltic Sea. Mar. Ecol. Prog. Ser. 53:93-100.
- Lignell, R., 1990. Excretion of organic carbon by phytoplankton: its relation to algal biomass, primary productivity and bacterial secondary productivity in the Baltic Sea. Mar. Ecol. Prog. Ser. 68:85-99.
- Mann, C. J. & R. G. Wetzel, 1996. Dissolved organic carbon and its utilisation in a riverine Wetland ecosystem. Biogeochemistry 31:99-120.
- Parsons, T. R., Y. Maita & C. M. Lalli, 1989. A manual of chemical and biological methods for seawater analysis. Pergamon Press, Oxford.
- Parsons, T. R., M. Takahashi & B. Hargrave, 1984. Biological oceanic processes, 3rd ed. Pergamon Press, Oxford.
- Reitner, B., A. Herzig & G. Herndl, 1999. Dynamics in bacterioplankton production in a shallow, temperate lake (Lake Neusiedl, Austria): evidence for dependence on macrophyte production rather than on phytoplankton. Aquat. Microb. Ecol. 19:245-254.
- Rheinheimer, G., 1981. Investigation on the role of bacteria in the food web of the Western Baltic. Kieler Meeresforsch., Sonderh 5:284-290.
- Riegsbee, W., L. Simpson & J. Oliver, 1996. Detection of the viable but nonculturable state in *Escherichia coli* 0157:h7. Journal of Food Safety 16:255-262

- Rodier, J., 1996. L`analyse de l`eau: eaux naturelles, eaux résiduaires, eau de mer. 8 Edition, Dunod, Paris.
- Scavia, D. & G. A. Laird, 1987. Bacterioplankton in Lake Michigan: dynamics, controls, and significance to carbon flux. Limnol. Oceanogr. 32: 1017-1033.
- Shiah, F. & H. W. Ducklow, 1994. Temperature and substrate regulation of bacterial abundance, production, and specific growth rate in Chesapeake Bay. Limnol. Oceanogr. 39:1243-1258.
- Shiah, F.& H. W. Ducklow, 1995. Multiscale variability in bacterioplankton abundance, production, and specific growth rate in a temperate salt-marsh tidal creek. Limnol. Oceanogr. 40(1):55-56.
- Silva, J., 1994. Circulação de água na Ria de Aveiro: contribuição para o estudo da qualidade da água. Ph.D. Thesis. Universidade de Aveiro.
- Steemann-Nielsen, E., 1952. The use of radioactive carbon (^{14}C) for measuring organic production in the sea. J. Cons. Int. Expl. Mer. 18:117-140.
- Tabor, P. S. & R. A. Neihof, 1982. Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl. Environ. Microbiol. 44 (4):945-953.
- Yentsch, C. S. & D. W. Menzel, 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep Sea Res. 10:221-231.

CHAPTER 4

PHYSIOLOGICAL RESPONSES OF MARINE AND BRACKISH WATER BACTERIAL ASSEMBLAGES IN A TIDAL ESTUARY (RIA DE AVEIRO, PORTUGAL)

Almeida MA, Cunha MA and Alcântara F

Departamento de Biologia - Universidade de Aveiro
Campus Universitário de Santiago, 3810-193 AVEIRO, PORTUGAL

ABSTRACT: The reactivity of spatially distinct bacterial communities within an estuarine gradient to contrasting water properties was evaluated in the field and experimentally tested in diffusion chambers. In field conditions, it was observed that total and active bacterial numbers were, on average, 3 times higher in brackish water than in marine water. The fraction of active bacteria was, however, similar in both zones (26.6% on average). Total leucine incorporation, as a measure of biomass productivity, was 3.5 times higher in the brackish water zone following the increase in bacterial population size. Productivity seemed also to depend on the level of activity of individual cells since specific leucine incorporation per active cell was 19% higher in the brackish water zone. Experimental assays in diffusion chambers showed that when the marine bacterial community was exposed for 6 hours to brackish water, the fraction of active bacteria and leucine incorporation increased (20 to 60% and 50 to 220%, respectively). The opposite was observed when the brackish water community was exposed to marine water (20% decrease in the fraction of active bacteria and 50 to 80% in leucine incorporation). The positive response of marine bacteria to the brackish water conditions was higher when the salinity of the brackish water was artificially increased to 34 psu. Brackish water bacteria, however, responded negatively to this increase in salinity. When the marine community was exposed for only 2 h to brackish water it promptly showed increased activity. The immediate transfer of this community to marine water for an extra 4 h period induced a return to the initial low activity level. In contrast, the negative response of the brackish water community after 2 h of exposure to marine water was irreversible when transferred back to brackish water. Bacteria remained at a low activity level for the extra 4 hours. The high bacterial abundance and production in mid-estuary and the similar patterns of variation of total and active bacteria throughout the system, seemed to indicate conservative transport of an euryhaline bacterial community from its main source in the mid estuary. However, the experimental assays with diffusion chambers invalidated this hypothesis. They suggest the presence of 2 communities: a nutrient-limited marine zone community that grows optimally at high salinity, and a nutrient-replete brackish water community requiring salinities bellow 25 psu.

Key Words: Active bacteria, Leucine incorporation, Bacterial stimulation, Estuary

INTRODUCTION

Bacterioplankton communities in estuarine systems are frequently exposed to varying environmental conditions. The mixing of seawater and freshwater, the different residence time, the transportation of materials from diverse sources, including salt marshes, mudflats, rivers and bottom sediments and different climatic conditions, may induce different patterns of bacterial abundance and activity in different estuaries. Estuarine gradients of bacterial abundance are generally characterised by a steady increase in density (factors up to 3) from the outer- to the upper-estuary (Palumbo & Fergunson 1978, Goosen *et al.* 1997) or to the mid-estuary (Wright & Coffin 1983, Bordalo *et al.* 1998, Cunha *et al.* 2000). Although the metabolically active cells in these populations correspond generally to a small fraction, rarely reaching 50 % (Rodriguez *et al*. 1992, Button *et al*. 1993, del Giorgio & Scarborough 1995, Zweifel & Hagstrom 1995, Ullrich *et al*. 1996, Sherr *et al.* 1999, Choi *et al.* 1999), the shifts in water characteristics have been reported

to induce stronger responses in the size of the fraction of active bacteria than in the total bacterial number (del Giorgio & Scarborough 1995). The relative size of this fraction is minimal in oligotrophic ocean water (<5%) and increases towards the estuaries up to 50% (Rodriguez *et al*. 1992, Button *et al*. 1993, Gasol *et al*. 1995, del Giorgio & Scarborough 1995). The reasons underlying this variation are not well understood. Substrate availability has been extensively studied as a potentially important factor controlling cell activity. A number of studies showed positive correlation between the number of active bacteria and the concentration of inorganic nutrients and of dissolved organic carbon in highly productive lakes and estuaries (del Giorgio & Scarborough 1995). The systematic differences in the percentage of active cells in systems with comparable trophic conditions suggest at other factors combine to control bacterial activity (del Giorgio & Scarborough 1995). Temperature (Quinn 1984, Wright & Coffin 1984, Tabor & Neihof 1984, Shiah & Ducklow 1994), salinity (Rigsbee *et al*. 1996), viral infection (Proctor & Fuhrman 1990) and bacterial predation (Gonzalez *et al*. 1990, Sherr *et al.* 1992, Bird & Kalf 1993, Cole & Caraco 1993) may interact and produce different final results.

Bacterial responses to changing water properties are generally higher in terms of activity rates than in terms of abundance (del Giorgio *et al*. 1996). Leucine incorporation, different enzymatic activities and glucose turnover, although maximal in the mid-estuary, may show, however, different environmental reactivities (Cunha *et al.* 2000). These differences in metabolic properties along the estuarine gradients may be related to the spatial distribution of distinct bacterial communities. In fact, Bidle & Fletcher (1995) have already found, in Chesapeake Bay, genetic differences in free-living bacteria populations from the upper- and mid- bay regions. Murray *et al.* (1996) analysed the phylogenetic diversity of bacterial assemblages in two California estuaries and concluded that bacterial similarity in adjacent stations was greater than in distant stations. These differences in genetic similarity may, according to the authors, be interpreted to result from the mixing of different communities or to the influence of the organic matter gradient in the physiology of the same microbial assemblage.

Previous studies on the dynamics of bacterioplankton in the lagunar system of Ria de Aveiro found a curvilinear profile of bacterial abundance and bacterial activities with peaks at the 20-30 psu section of the estuary (Almeida *et al.* in press, Cunha *et al.* 2000). Nutrients, organic matter and phytoplankton biomass concentrations were maximal in the mid- upper- estuary (Cunha *et al.* 1999, 2000, in press). In this study we attempt to distinguish 2 hypotheses in experimental conditions: the patterned gradient of bacterial activity results from flexible physiological responses of a single bacterial community to a changing environment or either from different reactivities of bacterial communities with distinct activity profiles.

MATERIALS AND METHODS

Study site

Ria de Aveiro (Figure 1) is a tidal lagoon on the western coast of Portugal, which is connected to the Atlantic Ocean by a narrow opening. The lagoon covers an area of 66 to 83 km^2 at low (LT) and high tide (HT), respectively (Silva, 1994). It exchanges with the sea 89 millions of cubic meters of water over tides amplitude ranging from 1 to 3 m (Silva 1994). Several rivers carry fresh water into the lagoon with an average water input of 1.8 millions of cubic meters during a tidal cycle (Barrosa, 1985). The Ria has a complex topography, with different channels spreading from the mouth towards the different streams forming, in fact, a multi-estuarine ecosystem. Secondary branches originate from the 3 main channels forming a tight network of smaller channels, islands and mudflats. For this study, two sampling stations were selected to represent contrasting water characteristics: Station N1 in Canal de Navegação, representing the marine zone, and station I6 in Canal de Ílhavo, representing the brackish water zone.

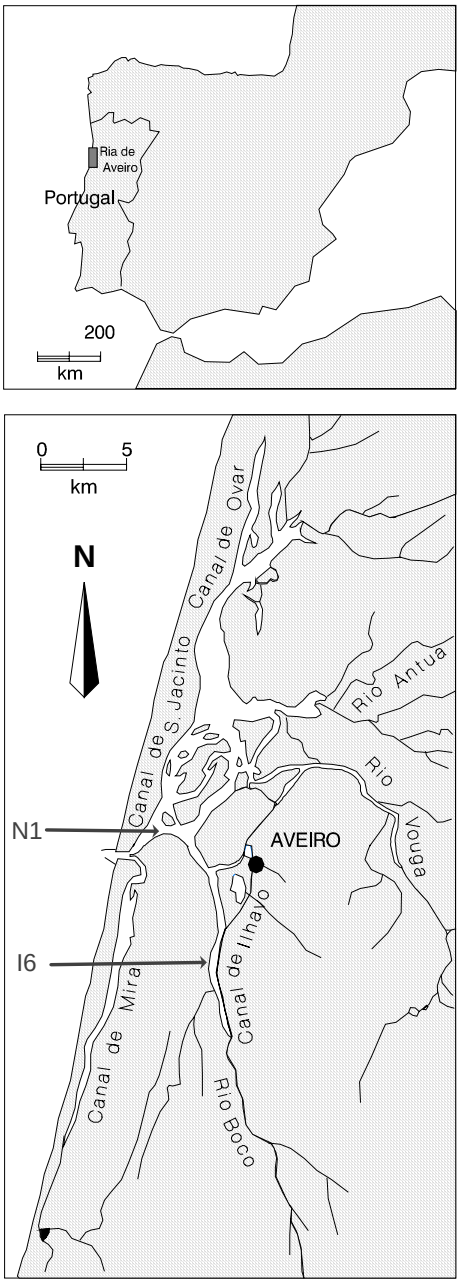


Figure 1:Ria de Aveiro with sampling stations indicated by the arrows. Station N1 in Canal de Navegação, representing the marine zone and station I6, in Canal de Ílhavo, representing the brackish water zone.

Sampling

The marine zone of the Ria was studied during 6 different tidal cycles (1 and 3 June 1996, 28 and 30 August 1996, 2 and 10 September 1997), 5 of them in spring tide and 1 in neap tide. The brackish water zone was sampled during 4 tidal cycles (29 April and 5, 22 and 30 May 1997), 2 in spring tide and 2 in neap tide. Samples were collected at surface (0.2 m from the surface) and deep water (0.5 m above sediment floor) at HT and LT and at intervals of 2 h (HT-2h, HT+2h, LT-2h, LT+2h). Water samples were transported and processed within 2-3 h after collection.

Samples for experimental assays were collected at the same 2 zones. For better contrast of the environmental

conditions at the 2 zones, station N1 was sampled at flood tide, 2 h before HT, and station I6 at ebb tide, 2 hours before LT. Water samples collected during the flood tide in the marine zone represented marine environmental conditions and the corresponding bacterial community. Samples collected during ebb tide at station I6 represented the characteristics and the bacterial community of the brackish water zone of the estuary. For assays in diffusion chambers, surface water was sampled in February and June 1998 and in November 1999. For studying the response of bacteria to the amendment of nutrient several surface samples were collected at the 2 stations from January 1998 to November 1999.

Physical and chemical characteristics. Temperature and salinity were read in a conductivity meter (millions of cubic meters, WTW — Wissenschaftlich Technische Werkstätten). Dissolved oxygen concentration, expressed as percentage of saturation, was determined with an oxygen meter (Model OXI 96, WTW — Wissenschaftlich Technische Werkstätten) equipped with a stirrer (Model BR 190, WTW — Wissenschaftlich Technische Werkstätten). The concentration of suspended solids was determined after filtration of triplicate 0.5 l water aliquots through pre-weighted and pre-combusted Whatman GF/C filters. The filters were dried at 60ºC for 24 hours and suspended solids (seston) were calculated as the increase in dry weight. The organic matter fraction in the seston was determined as the loss of weight after 4 hours combustion at 525 ºC (Parsons *et al.* 1989).

Total bacterial number. Bacterial cells were counted by epifluorescence microscopy with a Leitz Laborlux K microscope equipped with a I 2/3 filter for blue light. The samples were fixed with 2% formaldehyde (final concentration), filtered through black polycarbonate membranes of 0.2 µm (Poretics) and stained with 0.03% acridine orange (Hobbie *et al.* 1977). At least 200 cells or 20 microscope fields were counted in each of 3 replicate preparations.

Active bacterial number

Microautoradiography. The number of active bacteria was determined by microautoradiography (Carman, 1993) after amendment with a 30 nM ^{3}H-leucine solution (Amersham specific activity of 58-92 Ci mmol^{-1}). After 5 hours incubation at *in situ* temperature, triplicate sub-samples (2-3 ml) of each 10 ml sample were filtered through black polycarbonate membranes. The filters were placed face down on slides coated with the autoradiographic emulsion NTB-2 (Kodak). The exposure, in total darkness and at 4°C, was carried out for 7 days. The slides were developed with Kodak Detkol (1:1 dilution in ultrapure water) and fixed in Kodak fixer. The developed autoradiograms were stained with acridine orange solution (0.04%) and hydrated in citrate buffer (0.004 M). Microautoradiographs were examined using a combination of

epifluorescence (as above) and bright-field illumination (in a Leitz Laborlux microscope). Cells were counted as active if associated with 3 or more silver grains.

CTC reduction. Five millilitre triplicate water sub-samples were placed in sterile plastic tubes and supplemented with freshly prepared 5-cyano-2,3-ditolyltetrazolium chloride (CTC) solution to a final concentration of 5 mM (Ullrich *et al*. 1996). A fixed control was prepared by first adding filtered formaldehyde (2% final concentration) to an otherwise similarly prepared water sample, which, after 30 min, was further supplied with the CTC solution. Controls and test samples were incubated in the dark for 4 hours at room temperature with slow agitation (100 rpm). Samples were filtered through 0.2 µm black polycarbonate membranes (Poretics) and observed for red fluorescence. At least 200 CTC positive cells, or 20 microscope fields, were enumerated in each of the 3 replicate samples.

Leucine incorporation. Leucine incorporation was determined in 10-ml triplicate sub-samples plus a control that was fixed by the addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (30 nM) of ^{3}H-leucine (Amersham, specific activity of 58 to 92 Ci mmol^{-1}) for 1 h at *in situ* temperature. Sub-samples were fixed in 2% formaldehyde. Protein was precipitate through the addition of 1 ml of 20% ice-cold TCA, followed by incubation for 15 min in ice. Sub-samples were filtered through 0.2 µm polycarbonate membranes (Poretics), and rinsed with 2 ml of 5% ice-cold TCA and 5 ml of 90% ice-cold ethanol (Blight *et al*. 1995). After 3 days of being in scintillation cocktail (Universol) the radioactivity was read in a liquid scintillation counter (LS 6000 IC, Beckman). Leucine incorporation was calculated using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (Simon & Azam, 1989). Cell-specific leucine incorporation was calculated as the quotient between leucine incorporation and the total bacterial number, whereas leucine incorporation per active cell was the ratio between leucine incorporation and the active bacterial number.

Bacterial activity in diffusion chambers assays. These assays were devised to measure rapid (up to 6 h) responses of the bacterial community of a tidal estuarine environment to the conditions prevailing in different phases of the tidal cycles. The idea behind this was to expose the same population to changing environmental conditions. Two experimental incubation systems were prepared, each consisting of a stirred, temperature controlled, water bath filled with 15 l of water, either from station N1 (representing the marine zone) or from station I6 (representing the brackish water zone). Diffusion chambers with a capacity 170 ml were prepared as described by Pereira & Alcântara (1993). Two 12.5 cm diameter of 0.2 µm pore size polycarbonate membranes formed the sides of each chamber, and allowed ample percolation of water and dissolved substances. One set of 7 diffusion chambers was filled with water from the marine zone and another set with water

from the brackish zone. Four chambers from each set were incubated in the bath filled with the same type of water (1 blank and 3 controls) and the other 3 in the contrasting bath (test chambers). The closed chambers were incubated completely immersed in the water bath at 20°C for 6 hours, corresponding approximately to the duration of half of a tidal cycle. Samples were taken after 2, 4 and 6 h of incubation without removing the chambers from the bath. Each time a volume of 40 ml of water was collected through an opening at the top of the chamber. Dilution due to permeation after sampling (entrance of water due to the reduction of pressure inside the chambers) was taken into account and corrected using the blank chambers, which were also sampled after 2, 4 and 6 h. In the blank chambers the remaining volume of water was always measured and again inserted into the chamber. At the end of the exposure period, the volume of water inside the test, control and blank chambers was also measured. The initial characteristics of the marine and brackish water samples were determined immediately after field sampling.

Table 1: Combinations of supplements added to marine and brackish waters. * Arginine, histhidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine (Sigma LAA-10);** Thiamine (B_1), Riboflavine (B_2), Pyridoxine (B_6), vitamin B_{12}, pantothenic acid, inositol, choline, biotin and folic acid. These components were added to the water samples in a concentration similar to that obtained if a yeast extract at 0.1% final concentration would have been used; *** Filtered (0.2 µm pore size membranes) extract of diatoms (*Amphora coffeaeformis, Melosira nummuloides, Bacillaria paxillifera, Coscinodiscus granii, Navicula directa, Cymbella sp)* suspended in their culture medium; **** Coast Seafood Algae – Diet C, *Thalassiosira pseudonana* (clone 3H), *Skeletonema sp.*, *Isochrysis galbana*, *Chaetoceros calcitrans.*

Substrate (concentration)	Marine water	Brackish water
Glucose (0.1%)		x
Sodium phosphate (0.05)		x
Sodium phosphate (0.1%)		x
Fructose (0.1%)		x
Sacarose (0.1%)		x
Mixture of 10 amino acids (20 µM)*	x	x
Complex B vitamins**	x	
Yeast extract (0.025, 0.05, 0.1, 0.2%)	x	x
Yeast extract base without amino acid and nitrogen (0.1%)		x
Yeast extract base without amino acids (0.1%)		x
Yeast extract base without amino acids (0.1%) + amino acids (20 µM)		x
Yeast extract base without amino acids (0.1%) + amino acids (20 µM) + complex B vitamins		x
Yeast extract base without amino acids (0.1%) + amino acids (20 µM) + glucose (0.1%) + sodium phosphate (0.1%)	x	
Exudate of diatoms isolated in the Ria de Aveiro (0.1%)***		x
Diatoms growth medium (f/2) (0.1%)		x
Suspension of filtered commercial exudate of algae (0.1%)****	x	

In June 1998, to test the effects of reversion exposure, an extra chamber of each set was incubated in the contrasting water for the first 2 hours, sampled and transferred to the natural environment (bath with similar water) before being refilled and incubated for an additional 4 h. Further samples were taken after 4 and 6 h of incubation in the now reversed conditions.

In November 1999, to evaluate the effects of increased salinity on bacteria, samples from stations N1 and I6 were exposed to natural brackish water conditions (salinity 18 psu) and to the same water to which NaCl was added to get a salinity of 34 psu, which was similar to that of the marine zone and processed as above.

These samples were analysed for total bacterial number, active bacterial number and leucine incorporation. The active bacterial numbers were determinated in February and June 1998 by the microautoradiography method, while in November 1999 the CTC reduction method was used. The values obtained for total bacterial number, active bacterial number and leucine incorporation were multiplied by the inverse of the factor of dilution determined in the blank chamber.

Bacterial activity after supplement amendment. Surface water samples were complemented with different combinations of inorganic and organic substrates to induce recovery from occasional dormancy in the bacterial communities. Marine and brackish water samples were separately enriched with different substrates as described in table 1.

To avoid significant dilution of the samples, 10 to 100 times concentrated substrate solutions were used. Samples for active cells counting were taken 10 min after substrate addition, except in November 1999, when the pre-stimulation period was extended to 1, 2 or 3 h. Active bacterial numbers were determined using the CTC technique (see above). It is assumed that during the short incubation period the total number of bacterial cells did not increase significantly. To avoid interference of CTC fluorescence in total bacterial counts, parallel sub-samples were incubated, after the supplement addition but without CTC, for determination of total counts.

RESULTS

Physical, chemical and biological properties of marine and estuarine water

During the tidal cycle, the salinity varied from 23.7 to 36.1 psu (average 34.4 psu) in the marine zone and from 11.4 to 33.7 psu (average 26.0 psu) in the brackish water zone. The average water temperature was 16.8°C (range 15.3 to 20.6 °C) in the marine zone and 20.2 °C (range 17.0 to 23.5° C) in the brackish water zone. Oxygen concentration was usually below saturation in both zones. The mean value was 76.7 % in the marine zone and 69.8 % in the brackish water zone. Particulate organic matter varied from 6.0 to 31.0 mg l^{-1} (average 14.4) in the marine zone and

from 6.0 to 17.0 mg l^{-1} (average 11.0) in the brackish water zone. No stratification could be assigned, in either of the 2 lagoon zones, to any of the physical and chemical variables studied.

Table 2: Bacterial abundance and leucine incorporation in the marine and brackish water zones of the Ria de Aveiro. Determinations at surface (0.2 m below surface) and deep water (0.5 m above floor), at high tide and low tide and at intervals of 2 h. The value showen for each tidal phase was calculated as the average of six (marine zone) and four (brackish water zone) determinations in different sampling dates of the warm season. TBN: total bacteria number, ABN: active bacteria number, %AB: proportion of active bacteria, LI: leucine incorporation, LI_{cell}: specific LI per cell, $LI_{act\ cell}$: specific LI per active cell, S: surface water, D: deep water. Ranges of values are shown in parentheses.

ZONE		TBN ($x10^9$cells l^{-1})	ABN ($x10^8$cells l^{-1})	%AB	LI ($\mu g\ Cl^{-1}h^{-1}$)	LI_{cell} (fg $Ccell^{-1}h^{-1}$)	$LI_{act\ cell}$ (fg C $cell^{-1}h^{-1}$)
Marine zone	S	2.3	5.1	27.7	1.6	0.9	3.1
		(0.2-8.1)	(1.0-13.0)	(10.0-53.0)	(0.3-4.5)	(0.2-2.3)	(0.6-7.2)
	D	1.8	4.6	28.6	0.6	0.5	1.5
		(0.2-5.9)	(1.4-17.0)	(5.9-55.0)	(0.05-2.1)	(0.05-2.6)	(0.2-6.1)
	S+D	2.0	4.9	27.3	1.1	0.7	2.2
		(0.2-8.1)	(1.0-17.0)	(5.9-55.0)	(0.05-4.5)	(0.05-2.6)	(0.2-7.2)
Brackish water zone	S	5.9	15.3	26.2	3.8	0.7	2.8
		(3.1-8.1)	(3.0-30.0)	(10.0-44.0)	(1.2-7.4)	(0.2-1.7)	(0.9-9.6)
	D	5.8	14.3	25.1	3.7	0.6	2.6
		(2.6-8.5)	(2.0-31.0)	(8.0-42.0)	(0.8-14.2)	(0.3-2.5)	(0.7-4.6)
	S+D	5.9	14.7	25.1	3.8	0.7	2.7
		(2.6-8.5)	(2.0-31.0)	(8.0-44.0)	(0.7-14.2)	(0.2-2.5)	(0.7-9.6)

Total bacterial number varied, in different phases of the tidal cycles from 0.2 x 10^9 to 8.5 x 10^9 cells l^{-1} (Table 2). The active bacteria number (determined by microautoradiography) varied from 0.9 x 10^8 to 31.0 x 10^8 cells l^{-1} (Table 2). Bacterial abundance (total and active) was, on average, 3 times higher in the brackish water zone than in the marine zone. In both zones the highest values of total and active bacteria were observed near LT (Fig. 2). Although total and active bacterial densities were frequently higher at the surface in the marine zone, the values were not significantly different from those observed near the bottom. In the shallower brackish water zone, the values were rather similar throughout the water column. The average fraction of active bacteria was similar in both zones (26.6%), down the water column and over the tidal cycle (Table 2).

Leucine incorporation varied from 0.05 to 14.2 µg C l^{-1} h^{-1} (Table 2) and the average value was 3.5 times larger in the brackish water zone. The values were higher near LT mainly in the marine zone. In the marine zone, leucine incorporation was higher at surface water, reaching values of up to 8.5 times higher than that near-bottom water. In the brackish water zone the leucine incorporation did not vary along the water column (Fig. 3). Leucine incorporation per cell (from total bacterial number) was similar in both zones (0.7 fg C $cell^{-1}h^{-1}$, on average) and ranged from 0.05 to 2.6 fg C $cell^{-1}$ h^{-1} (Table 2). However, leucine incorporation per active cell was, on average, 19 % higher in the brackish water zone than in the marine zone (range from 0.2 to 9.6 fg C active $cell^{-1}$ h^{-1}). In the marine zone leucine incorporation per cell and per active cell were also higher near

LT and the maximum values were observed at the surface. In the brackish water zone, these parameters were similar at LT and HT, and were constant down the water column (Fig. 3).

Bacterial activity in diffusion chambers assays

Salinity in diffusion chambers filled with water from the brackish water zone was 9-23 psu lower than in chambers filled with marine water (Fig. 4). The time course of the salinity values indicated that the permeation through the membranes allowed a rapid (2-3 h) equilibrium in the concentration of dissolved materials between the chamber and the bath compartments (Fig. 4).

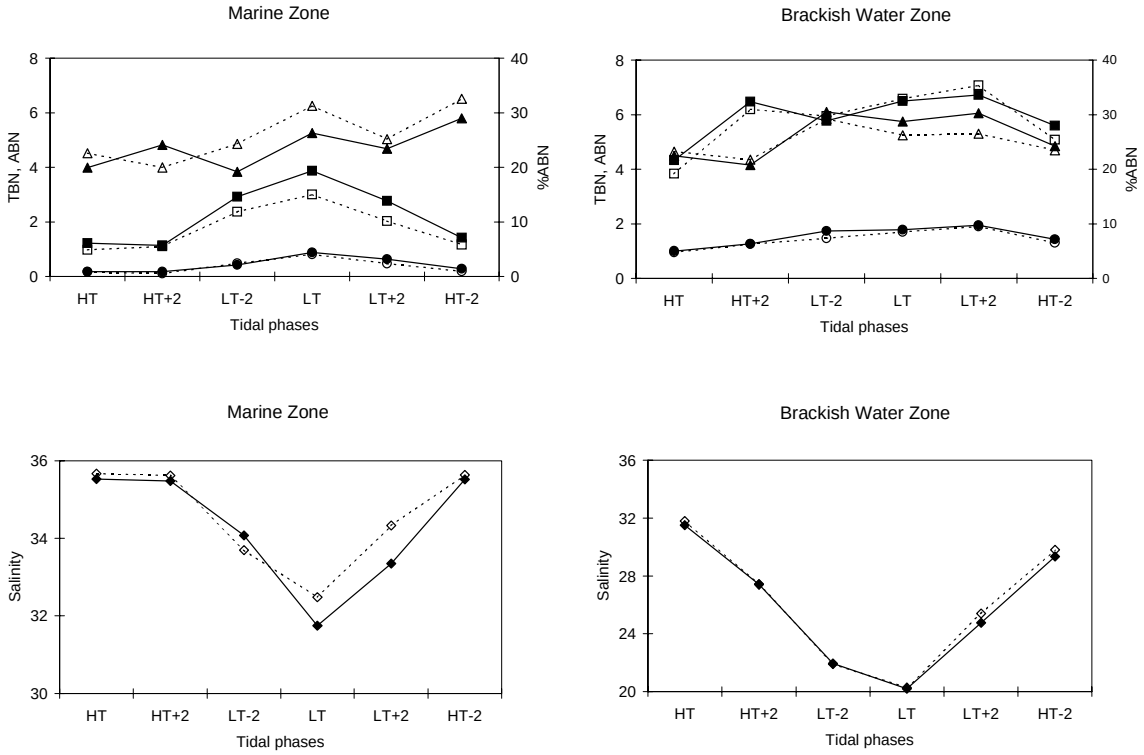


Fig. 2: Bacterial abundance in the marine zone and brackish water zones of the Ria de Aveiro. Determinations at surface (0.2 m below surface) and deep (0.5 m above sediment floor) water, at high tide (HT) and low tide (LT) and at intervals of 2 hours as indicated. The values shown for each tidal phase were calculated as the average of six (marine zone) and four (brackish water zone) determinations at different sampling dates of the warm season. Total bacterial number (x 10^9 l^{-1}) at surface water (TBN —■—) and deep water (TBN ---□---); active bacterial number (x 10^8 l^{-1}) at surface water (ABN —●—) and deep water (ABN ---○---); percentage of active bacteria at surface water (%ABN —▲—) and at deep water (%ABN ---Δ---); salinity (psu) at surface water (—◆—); salinity (psu) at deep water (---◇---).

At time zero of the incubations, the total bacterial number was 1.3 to 2.6 times higher in the brackish water. The active bacterial number, determined by microautoradiography, (February and June 1998) was 2.3 to 2.8 times higher in the brackish water, but when determined through CTC reduction (November 1999) was 5.6 times higher in the brackish water zone. The percentage of active bacterial cells varied from 14.2 to 32.1 % as determined by microautoradiography (February and June 1998), and between 2.9 and 6.4% when determined by the CTC reduction

method (November 1999). Leucine incorporation was also higher in the brackish water (2.0 to 10.8 times).

The bacterial community responded quickly to exposure to the contrasting water. Changes in leucine incorporation and in the proportion of active bacteria were detectable after 2 h of exposure to contrasting conditions (Figs. 5 and 6). When the marine bacterial community was exposed to the brackish water environment, the fraction of active bacteria and leucine incorporation increased. The opposite was observed when the brackish water community was exposed to marine water.

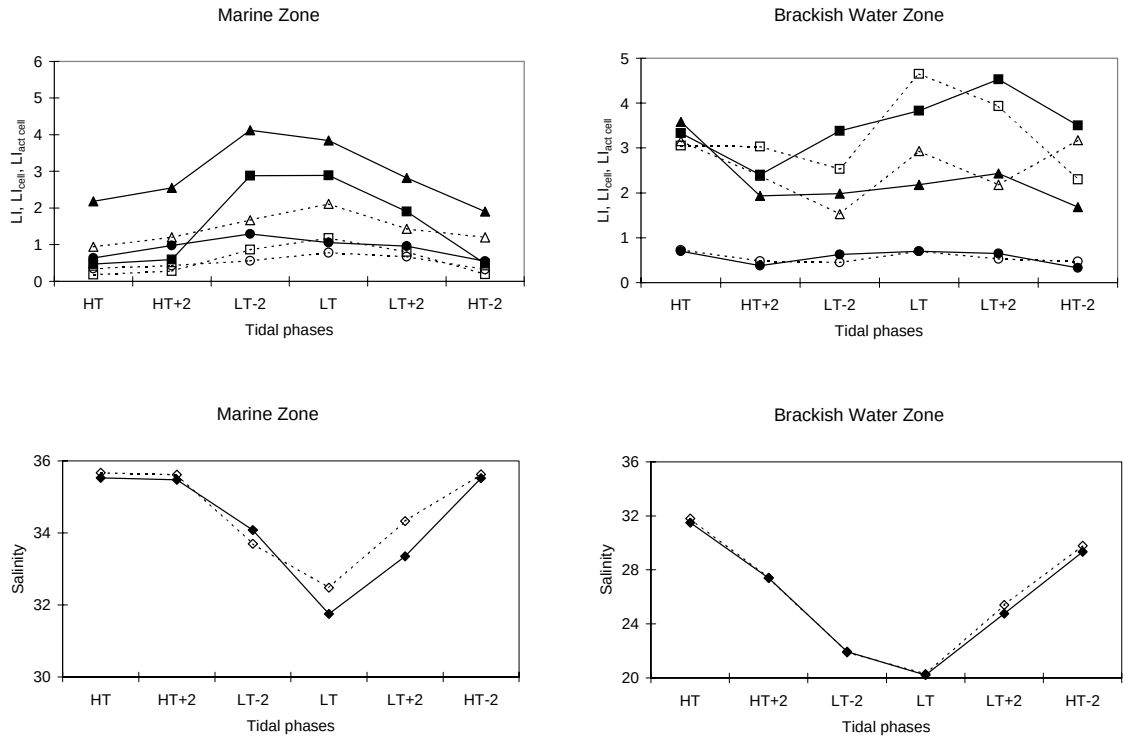


Fig. 3: Bacterial productivity in the marine zone (marine zone) and brackish water zones (brackish water zone). Determinations as indicated in figure 2. Leucine incorporation (μg C l^{-1} h^{-1}) at surface water (LI —■—) and deep water (LI ---□---); leucine incorporation per cell (fg C $cell^{-1}$ h^{-1}) at surface water (LI_{cell} —●—) and deep water (LI_{cell} ---○---); leucine incorporation per active cell (fg C active $cell^{-1}$ h^{-1}) at surface water ($LI_{act\ cell}$ —▲—) and at deep water ($LI_{act\ cell}$ ---Δ---); salinity (psu) at surface water (—◆—); salinity (psu) at deep water (---◇---).

The amplitude of positive and negative effects was different between variables and between experiments. The variation in the fraction of active bacteria during exposure was lower than that observed in leucine incorporation. The fraction of active bacteria and bacterial productivity in the marine zone increased 20 to 60% and 50 to 220%, respectively, in relation to the control when exposed to brackish water conditions. The fraction of active bacteria and leucine

incorporation in the brackish water decreased 20% and 50 to 80%, respectively, after 2-6 h of incubation in marine water (Figs. 5 and 6).

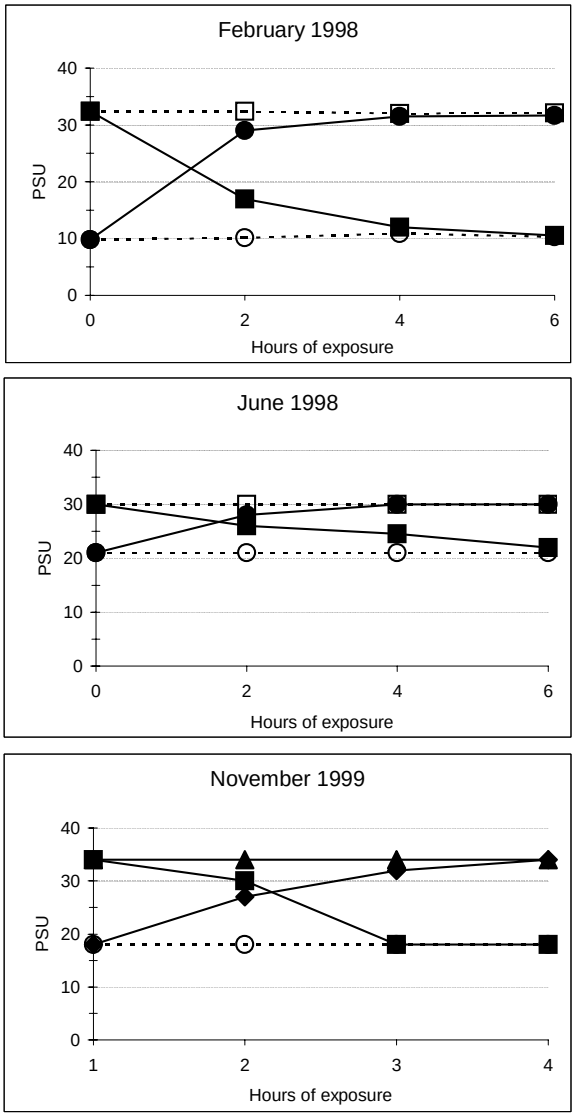


Figure 4: Salinity evolution within the diffusion chambers when exposed during 6 hours to water of the contrasting quality. ---□-- marine water chamber in marine water bath and similarly, —■— marine water in brackish water, ---○--- brackish water in brackish water, —●— brackish water in marine water, —▲— marine water in brackish water increased to 34 psu, —◆— brackish water in brackish water increased to 34 psu

The results of the reversion assays performed in June 1998 indicated, as mentioned before, that 2 h exposure of the bacterial community for 2 h to the contrasting water was sufficient to activate marine bacteria or to inactivate brackish water bacteria (Fig. 7). The fraction of active bacteria increased by 25% in the marine community and decreased by 22% in the brackish water community after 6 h of exposure. Leucine incorporation increased by 30% in the marine zone and decreased by 56% in the brackish water zone. The positive response of marine bacteria to brackish water was reversible: when marine bacteria returned to marine water the fraction of active bacteria and leucine incorporation decreased, reaching values similar to those of the control. However, the negative effect of marine water on the brackish water community was irreversible. After 2 h, when the brackish water bacterial community returned to brackish water, the fraction of active bacteria and leucine incorporation remained stable during the 4 h of incubation.

The positive response of marine bacteria to brackish water conditions was higher when the salinity of the brackish water was artificially increased to 34 psu (Fig. 8). After 6 hours, the fraction of active bacteria and the rate of leucine incorporation were approximately 50% higher than that observed in the 18 psu natural salinity of the parallel experiment. Brackish water bacteria, however, responded negatively to the increase in salinity. After 2 h of incubation, the fraction of active bacteria and the rate of leucine incorporation were, respectively, 13 and 23% lower those that registered in natural salinity.

Table 3: Fraction of active cells after exposure to various combinations of supplements during different intervals of time. Marine water was collected between March 1998 and November 1999. * Arginine, histhidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine (Sigma LAA-10); ** Thiamine (B_1), Riboflavine (B_2), Pyridoxine (B_6), vitamin B_{12}, pantothenic acid, inositol, choline, biotin and folic acid. These components were added to the water samples in a concentration similar to that obtained if a yeast extract at 0.1% final concentration were used; *** Coast Seafood Algae – Diet C, *Thalassiosira pseudonana* (clone 3H), *Skeletonema sp*., *Isochrysis galbana*, *Chaetoceros calcitrans.* ND: not determined.

	Fraction of active cells (%)				
	No amendment	Exposure			
Supplements	(control)	10 minutes	1 hour	2 hours	3 hours
Mixture of 10 aminoacids (20 μM)*	2.9	1.7	2.0	2.0	2.5
Complex B vitamins**	3.8	4.5	ND	ND	ND
Yeast extract base without amino acid (0.1%) + amino acids + glucose + sodium phosphate (see materials)	2.9	2.1	2.2	2.5	2.4
Suspension of filtered commercial exudate of algae (0.1%)***	2.9	1.5	1.6	1.6	1.5

Bacterial activity after supplement amendment

The active bacterial number (determined by the CTC reduction method) did not increase in marine and brackish water communities after the addition of different combinations of inorganic and organic substrates (Tables 3 and 4). The initial fraction of active bacteria determined with this technique was very small in both zones (average 5.0 %) and did not change significantly after substrate additions. The fraction of active bacteria determined in these assays by the CTC technique was much lower than that obtained by microautoradiography. Comparative studies had already showed that the CTC reduction technique gave a lower percentage of active bacteria than given by the microautoradiography technique

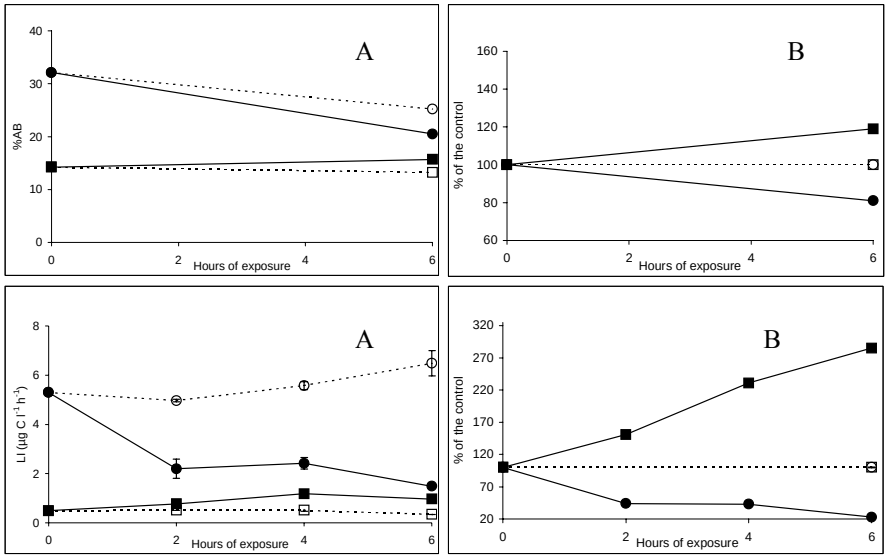


Fig. 5: (A) Variation in the fraction of active bacteria (%AB) and in leucine incorporation (LI) during 6 h of exposure of bacterial communities to contrasting water. (B) Percentage of variation in relation to the control of %AB and LI during the 6 h of exposure. February 1998. ---□-- marine water chamber in marine water bath and, similarly, —■— marine water in brackish water , ---○--- brackish water in brackish water, —●— brackish water in marine water

Table 4: Fraction of active cells after exposure to various combinations of supplements. Brackish water of was collected between January 1988 and January 1999. * See Table 3 for amino acid composition; ** Filtered (0.2 µm pore size membranes) extract of diatoms (*Amphora coffeaeformis, Melosira nummuloides, Bacillaria paxillifera, Coscinodiscus granii, Navicula directa, Cymbella sp)* suspended in their culture medium.

Substrate (concentration)	Non-amended control (%AB)	Amended (%AB)
Glucose (0.1%)	9.9	11.0
Sodium phosphate (0.05), 0.1%)	9.9	11.1
Sodium phosphate (0.1%)	4.3	4.3
Fructose (0.1%)	5.7	8.3
Sacarose (0.1%)	3.9	4.0
Mixture of 10 aminoacids (20 µM)*	9.9	10.1
Yeast extract base without amino acids and nitrogen (0.1%)	3.8	3.8
Yeast extract base without amino acids (0.1%)	3.8	3.8
Yeast extract base without amino acids (0.1%) + amino acids (20 µM)	3.8	3.8
Yeast extract base without amino acids (0.1%) + amino acids (20 µM) + complex B vitamins	3.8	3.8
Exudate of diatoms isolated in the Ria de Aveiro (0.1%)**	4.3	7.5
Diatoms growth medium (f/2) (0.1%)	4.3	4.7

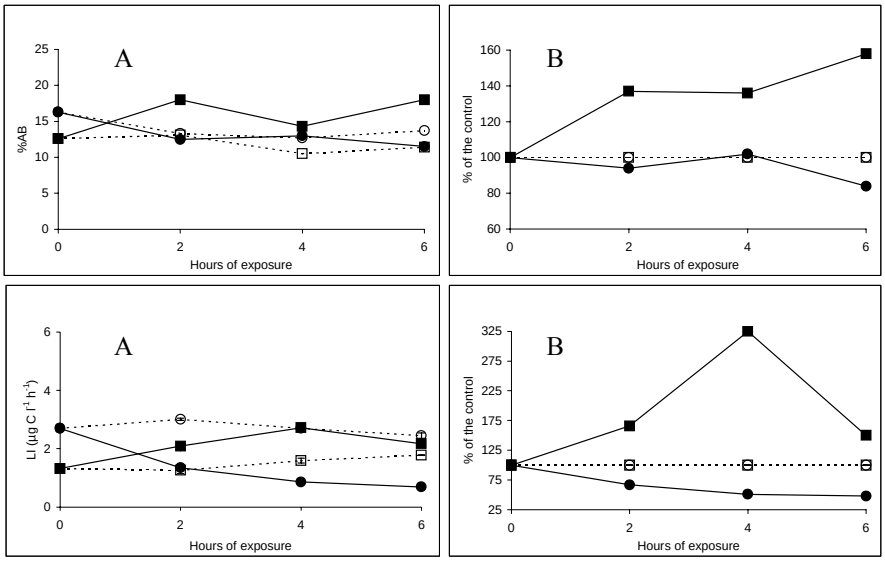


Fig. 6: (A) Variation in the fraction of active bacteria (%AB) and in leucine incorporation (LI) during 6 h of exposure of bacterial communities to contrasting water. (B) Percentage of variation in relation to the control of %AB and LI during the 6 h of exposure. June 1998. ---□-- marine water chamber in marine water bath and, similarly —■— marine water in brackish water, ---○--- brackish water in brackish water , —●— brackish water in marine water.

(54 %, on average; data not shown). It is important to note, in disagreement with other authors (Choi *et al.* 1999), that it was not possible to detect activity effects by yeast extract, TSB (Tryptic Soy Broth) medium, peptone and triptone as these substrates formed fluorescent precipitates after CTC addition that might be confused with bacteria. For this reason the results concerning these substrates could not be considered.

DISCUSSION

Previous work in this estuary (Cunha *et al.* 2000) has shown that bacterioplankton abundance and activity (leucine incorporation, ectoenzymatic activities and glucose turnover) follow a curvilinear longitudinal profile. These variables reach maximal values generally in mid-estuary (20 to 30 psu) in response to a set of favourable conditions including organic matter and nutrients availability. For this reason, it may be expected that in this estuary section not only would the number of active bacteria be greater than in any other region of the salinity gradient but also their proportion in the total bacterial community would increase. The percentages of active bacteria in marine and brackish water were, however, very close (average 27.9 % in the marine zone and 25.1 % in the brackish water zone) in similar sampling occasions although they could vary within a considerable range (6 to 55 % in the marine zone; 8 to 44 % in brackish water). This variation was erratic, with no relation to tidal water circulation. The constancy in the fraction of the active bacteria may be interpreted as denoting the conservative transport of bacterial cells along the estuarine salinity gradient. The alteration in the average level of activity per active cell (19% increase in brackish water) would be explained by the observed nutrient gradient. This hypothesis of conservative transport is weakened by the relatively long residence time of freshwater in the mid-estuary (10 to 97 days) and in the outer-estuary (1 to 45 days) (Silva 1994).

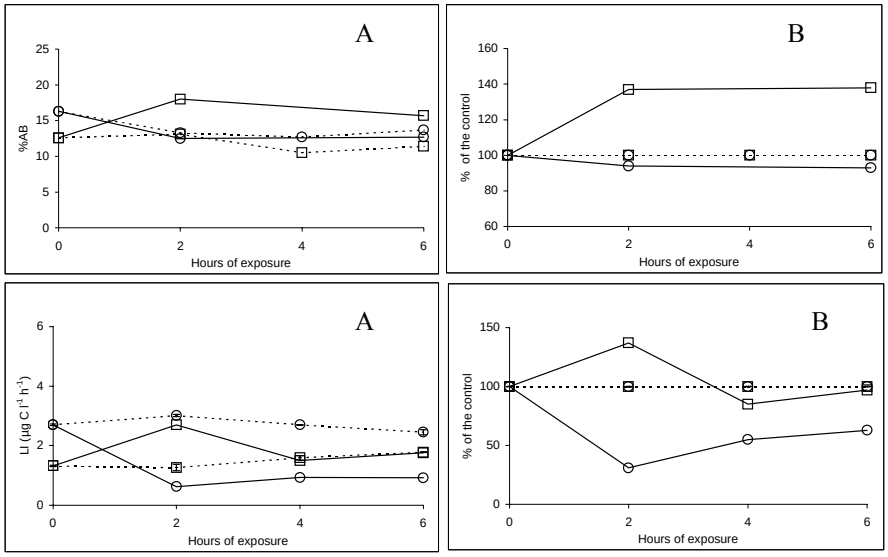


Figure 7: (A) Variation in the fraction of active bacteria (%AB) and in leucine incorporation (LI) during 2 hours of exposure of bacterial communities to the contrasting water followed by reversion to water of their natural environment for 4 extra hours. (B) Percentage of variation in relation to the control of %AB and BBP during the 6 hours of exposure. June 1998. —□— marine water in brackish water and reversion to marine water, —○— brackish water in marine water and reversion to brackish water, ---□---- marine water in marine water, ---○--- brackish water in brackish water.

The long exposure of the bacterial community during transport to a less favourable environment than upstream may interfere with the spatial patterns of abundance and activity. When residence time is long, distinct bacterial assemblages may occur in different zones of the estuary.

To clarify these two interpretations and in order to evaluate the importance of the salinity gradient in the eventual differentiation of estuarine bacterial communities, we looked for experimental evidence in diffusion chambers experiments. When the marine and brackish water communities were exposed to reciprocally exchanged water they responded promptly (< 6 h) and inversely to the new environment. Leucine incorporation and the fraction of active bacteria increased in the marine community incubated in brackish water. On the contrary, the brackish water community challenged with marine water responded negatively reducing leucine incorporation and the fraction of active bacteria. Again, this may be associated solely with the higher concentrations of organic carbon and nutrients in the microcosms exposed to brackish water. It is underlined that the decrease in salinity (from 32 to 16 psu, on average) did not preclude the positive response of the marine community to brackish water although the artificial increase of the brackish water salinity to 34 psu produced a more intense reaction in the marine bacteria.

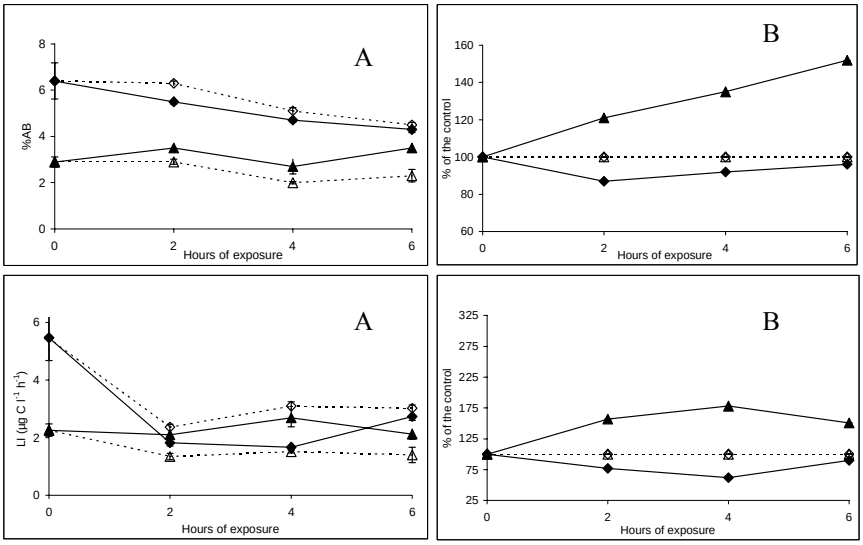


Fig. 8: (A) Variation in the fraction of active bacteria (%AB) and in leucine incorporation (LI) during 6 h of exposure of marine and brackish water bacterial communities to brackish water with natural salinity (18 PSU) or with increased salinity (34 psu). (B) Percentage of variation in relation to the control of %AB and LI during the 6 h of exposure. November 1999. ---Δ-- marine water in brackish water with natural salinity (18 psu), ---◇--- brackish water in brackish water with natural salinity (18 psu), —▲— marine water in brackish water with increased salinity (34 psu), —◆— brackish water in brackish water with increased salinity (34 psu).

The attempted discrimination between several simple and complex substrates that could be more or less involved in the promotion of bacterial activity in the conditions of this estuary was not successful. The size of the active community was insensitive to 3 h exposure to any of the chosen substrates. These results, confronted with the positive reactions obtained with natural water in diffusion chambers, may indicate that a combination of factors may be required to induce bacterial activation. The awakening cell activity after experimental stimulation may not, however, be detectable through CTC-reduction due to the toxicity of the chemical (Davey & Kell 1996, Ullrich *et al*. 1996, Karner & Fuhrman 1997).

The reversion experiments in microcosms produced results that were more informative. They showed a distinct contrast between brackish water and marine bacteria. After the prompt and

positive response during the initial 2 h incubation in nutrient rich brackish water, the marine bacteria, when transferred back to marine water, reverted rapidly to the activity profiles shown by the control. In similar experiments with brackish water bacteria exposed to marine water and then returned to brackish water, the response was negative, rapid and irreversible in the 6 h course of the experiment. This suggests that the high percentage of non-active bacterial cells observed in this estuary may result, at least partially, from the impact of the flooding current on brackish water bacteria. In fact, at HT, this current brings into the lagoon enough water to increase salinity above 28 psu in 99% of the total water volume (compared with 35% in LT) (Silva 1994). Alternatively, the irreversible negative responses of brackish water bacteria to marine water in contrast with the positive and reversible reactions of marine bacteria to brackish water suggest the presence of distinct bacterial assemblages adapted to the environmental conditions prevailing in different sections of the estuary as also found in other estuaries through genetic approaches by denaturing gradient gel electrophoresis of 16S rDNA fragments and by low molecular weight RNA analysis **(**Bidle & Fletcher 1995, Murray *et al.* 1996). The bacterial reactivity speaks against the hypothesis of conservative transport in this estuary.

CONCLUSION

Most (ca 70%) of the bacterioplankton from this estuary was inactive as revealed by microautoradiography. Amendment assays with different nutrients could not stimulate hypothetical inactive cells, indicating that this fraction is composed mostly of dead cells. The CTC-reduction technique used in these assays may not, however, be the best method to detect incipient activity. The small size of the fraction of active cells in the mid-estuary can result from the negative and mostly irreversible impact of flooding water on brackish water bacteria as observed experimentally.

The hypothesis of conservative transport of the upper- and mid-estuary bacteria suggested by field data on bacterial abundance and activity in Ria de Aveiro is not sustained by the experimental results obtained in diffusion chambers. These results suggest the presence of 2 different communities: (1) a marine zone community, limited by a short supply of nutrients, reacting positively to nutrient-rich brackish water (down to 16 psu) and to high salinities (ca 34 psu) and (2) a brackish water community, growing in rich water, that reacts negatively and irreversibly to salinities above 25 psu. Salinity and nutrient availability seem to control the activity of these 2 communities generating an apparently conservative longitudinal continuum.

LITERATURE CITED

- Almeida MA, Cunha MA, Alcântara F (in press) Factors influencing bacterial production in a shallow estuarine system. Microb Ecol
- Barrosa JA (1985) Breve caracterização da Ria de Aveiro. Jornadas da Ria de Aveiro. Volume II. Recursos da Ria de Aveiro. Edição da Câmara Municipal de Aveiro, Aveiro
- Bidle KD, Fletcher M (1995) Comparison of free-living and particle associated bacterial communities in the Chesapeake Bay by stable low-molecular-weight RNA analysis. Appl Environ Microbiol 61:944-952
- Bird DF, Kalff J (1993) Protozoan grazing and size-activity structure in limnetic bacterial communities. Can J Fish Aquat 50:370-380
- Blight SP, Bentley TL, Lefevre D, Robinson C, Rodrigues R, Rowlands J, Williams P (1995) Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128:61-75
- Bordalo AA, Pinto MM, Carvalho LM (1998) Dinâmica sazonal e espacial de variáveis abióticas e bióticas no estuário do Rio Douro. Actas do 1º Simpósio Interdisciplinar sobre processos estuarinos, Universidade do Algarve, Faro, p 47-48
- Button DK, Robertson BR (1993) Use of high-resolution flow cytometry to determine the activity and distribution of aquatic bacteria. *In*: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, New York. p 163-173
- Carman K (1993) Microautoradiographic detection of Microbial Activity. *In*: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis Publishers, New York. p 397-404
- Choi JW, Sherr BF, Sherr EB (1999) Dead or alive? A large fraction of ETS-inactive marine bacterioplankton cells, as assessed by reduction of CTC, can become ETS-active with incubation and substrate addition. Aquat Microb Ecol 18:105-115
- Cole JJ, Caraco NF (1993) The pelagic food web of oligotrophic lakes. *In*: Ford TE (ed.). Aquatic microbiology. An ecological approach. Blackwell Scientific Publication. Boston. p 101-111
- Cunha MA, Almeida MA, Alcântara F (1999) Compartments of oxygen consumption in a tidal mesotrophic estuary (Ria de Aveiro, Portugal). Acta Oecol 20 (4):227-235
- Cunha MA, Almeida MA, Alcântara F (2000) Patterns of variation of ectoenzimatic and heterotrophic bacterial activities along a salinity gradient in a shallow tidal estuarine. Mar Ecol Prog Ser 204:1-12
- Cunha MA, Almeida MA, Alcântara F (in press) Short-time responses of the natural planktonic bacterial community of the changing water properties in an estuarine environment: ectoenzimatic activity, glucose incorporation and biomass production. Microb Ecol
- Davey HM, Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analysis. Microbiol Rev 60:641-696
- del Giorgio PA, Scarborough G (1995) Increase in the proportion of metabolically active bacteria along gradients of enrichment in freshwater and marine plankton: implications for estimates of bacterial growth and production rates. Journal of Plankton Research 17:1905-1924
- del Giorgio PA, Gasol JM, Vaqué D, Mura F, Agustí S, Duarte CM (1996) Bacterioplankton community structure: protists control net production and the proportion of active bacteria in a coastal marine community. Limnol Oceanogr 41:1169-1179
- Gasol JM, del Giorgio PA, Massana R, Duarte CM (1995) Active versus inactive bacteria: size-dependence in a coastal marine plankton community. Mar Ecol Prog Ser 128:91-97
- González JM, Sherr EB, Sherr BF (1990) Size selective grazing on bacteria by natural assemblage of estuarine flagellates and ciliates. Appl Environ Microbiol 40:156-160
- Goosen NK, van Rijswijk P, Kromkamp J, Peene J (1997) Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat Microb Ecol 12:223-232
- Hobbie JE, Daley R, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228
- Karner M, Fuhrman JA (1997) Determination of active marine bacterioplankton: a comparison of universal 16s rRNA probes, autoradiography, and nucleoid staining. Appl Environ Microbiol 63:1208-1213

- Murray AE, Hollibaugh JT, Orrego C (1996) Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676-2680
- Palumbo AV, Fergunson RL (1978) Distribution of suspended bacteria in the Newport River estuary, North Carolina. Est Coast Mar Sci 7:521-529
- Parsons TR, Maita Y, Lalli CM (1989) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford
- Pereira G, Alcântara F (1993) Culturability of *Escherichia coli* and *Stretrococcus faecalis* in batch culture and *in situ* in estuarine water (Portugal). Wat Res 27:1351-1360
- Proctor LM, Fuhrman JA (1990) Viral mortality of marine cyanobacteria and bacteria. Nature 343:60-62
- Quinn JP (1984) The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in the aquatic environment. J Appl Bacteriol 57:51-57
- Riegsbee W, Simpson L, Oliver J (1996) Detection of the viable but nonculturable state in *Escherichia coli* 0157:h7. J Food Saf. 16:255-262
- Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF (1992) Use a fluorescent redox probe for direct visualisation of actively respiring bacteria. Appl Environ Microbiol 58 (6):1801-1810
- Sherr BF, Sherr EB, McDaniel J (1992) Effects of protistan grazing on the frequency of dividing cells in bacterioplankton. Appl Environ Microbiol 58:2381-2385
- Sherr BF, del Giorgio P, Sherr EB (1999) Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. Aquatic Microb Ecol 18:117-131
- Shiah F, Ducklow HW (1994) Temperature and substrate regulation of bacterial abundance, production, and specific growth rate in Chesapeake Bay. Limnol Oceanogr 39:1243-1258
- Silva JJ (1994) Circulação de Água na Ria de Aveiro: Contribuição para o Estudo da Qualidade da Água. Ph.D. thesis; Universidade de Aveiro
- Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic bacteria. Mar Ecol Prog Ser 51:201-213
- Tabor PS, Neihof RA (1984) Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl Environ Microbiol 48:1012-1019
- Ullrich S, Karrasch B, Hoppe H, Jeskulke K, Mehrens M (1996) Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3- ditolyl tetrazolium chloride. Appl Environ Microbiol 62:4587-4593
- Wright RT, Coffin RB (1983) Planktonic bacteria in estuaries and coastal waters of northern Massachusetts: spatial and temporal distribution. Mar Ecol Prog Ser 11:205-216
- Wright RT, Coffin RC (1984) Factors affecting bacterioplankton density and productivity in salt marsh estuaries. *In*: Klug MJ, Reddy CA (eds.) Current perspectives in microbial ecology. American Society for Microbiology, Washington. p 485-494
- Zweifel U, Hagstrom A (1995) Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). Appl Environ Microbiol 61:2180-2185

CHAPTER 5

LOSS OF ESTUARINE BACTERIA BY VIRAL INFECTION AND PREDATION IN MICROCOSM CONDITIONS

Almeida MA, Cunha MA and Alcântara F

Departamento de Biologia - Universidade de Aveiro
Campus Universitário de Santiago, 3810-193 AVEIRO, PORTUGAL

ABSTRACT: The bacterioplankton density in Ria de Aveiro, a shallow estuarine ecosystem, varied in the broad range of 1.9-10.6 x 10^9 cells l^{-1}. The range of values was about 2 times higher in brackish water than in marine water. At high tide bacterial abundance was 2-3 times lower than at low tide. The overall variation in virioplankton was in the range of 2.4-25.0 x 10^{10} particles l^{-1}. Brackish water was about 2 times richer in viral particles than the marine water. Near low tide the virioplankton was 2-3 times higher that at high tide. Viral density followed the pattern of bacterial abundance (it explained 40% of virioplankton variation). The viruses to bacterium ratio varied, throughout tidal cycles, by a factor of about 10 establishing the range 4.7-55.6 (average 17.6). This ratio was rather similar in the two estuarine zones. We compared the effects of infection and predation on the control of bacterioplankton size in the two zones of the estuary. The approach to this question was conducted in experimental microcosms, set-up in six combinations of plankton variables affecting the presence/absence of predators, virus-to-bacterium ratio (10-fold increase), virus-to-bacterium distance (2.2-fold increase), and bacterial growth rate. The results showed that predation was similar, in a percent basis, in marine (69%) and brackish water (73%). Viral infection was, however, higher in brackish water (59%) than in the marine water (36%). We conclude that the bacterioplankton along the salinity gradient evolves under biological pressures that are in different balance in the marine and brackish water zones. The effect of viral lysis on bacterial communities with enhanced growth (after yeast extract addition) was masked even when the initial ratio was 10-fold greater than in the natural samples. The high density of the virioplankton did not preclude the large and rapid increase in bacterial density. We suggest that the dynamics of the equilibrium between bacteria and viruses in the environment is driven to higher numerical levels during periods of intensive bacterial growth. On the contrary, at low bacterial growth rates the temporarily increased viruses–to–bacterium ratio may drive the equilibrium to its lowest levels.

INTRODUCTION

The growth of bacterioplankton in marine or estuarine water is affected by the environmental values of salinity, temperature, dissolved oxygen, competition for available nutrients as well as by predation (12, 18, 27). More recently a new element of the scenario became obvious when it was found that viral infection might greatly contribute to bacterial mortality in coastal ecosystems (10, 17, 33, 35). It was also found that bacterial mortality by viral infection might reach values similar to those of predation by flagellates (10, 34) in the community. Several authors reported impacts of viral lysis in the range of 1 to 100% (6, 7, 10, 22, 31). The contribution of viral lysis to the overall bacterioplankton mortality depends strongly on environmental conditions and on host community structure (36). The small number of the examined aquatic systems and the insufficient precision of *in situ* virus-mediated mortality determinations did not allow the confident understanding of the role of the virioplankton as an ecological mediator in the control of bacterioplankton density (36).

The reported densities of virioplankton in coastal ecosystems range from < 10^7 l^{-1} to > 10^{11} l^{-1} (36) but the proportion of the bacteriophage population in the total marine or estuarine

virioplankton is not established conclusively. It is widely believed (4, 9, 14), however, that they represent the major component of the picoplankton. Phage density and infectivity exert strong negative pressure on secondary production and respiration rate in bacterial communities (32, 35). However, the release of organic matter by viral lysis of different planktonic biota, namely bacteria, can be expected to induce the opposite effect when enhancing bacterial production.

The experimental approach developed in this work was intended to answer the question on the control of geographical and seasonal evolution of estuarine bacterioplankton density through predation and viral infection and effectiveness of this last factor in counteracting intensive bacterial growth.

MATERIALS AND METHODS

Study site

Ria de Aveiro (figure 1) is a tidal lagoon on the Northwest coast of Portugal separated from the Atlantic by a narrow opening. The lagoon covers an area of 66 to 83 Km^2 respectively at low and high tide (28). It exchanges with the sea a volume of water of 89 Mm^3 in tides of 1 to 3 m amplitude (28). Several rivers carry fresh water into the lagoon with an average water input of 1.8 Mm^3 during a tidal cycle (2). The Ria has a complex topography, with channels spreading from the mouth towards the streams, forming a complex estuarine ecosystem. Canal de Navegação is the main channel, and leads to the smaller channels. Here we study one of these smaller channels, Canal de Ílhavo (with a volume of 2.8 Mm^3 at LT and 9.3 Mm^3 at HT), that offers the advantages of a straighter water circulation and a diminished impact of agriculture. The freshwater supply to this channel is at the south end.

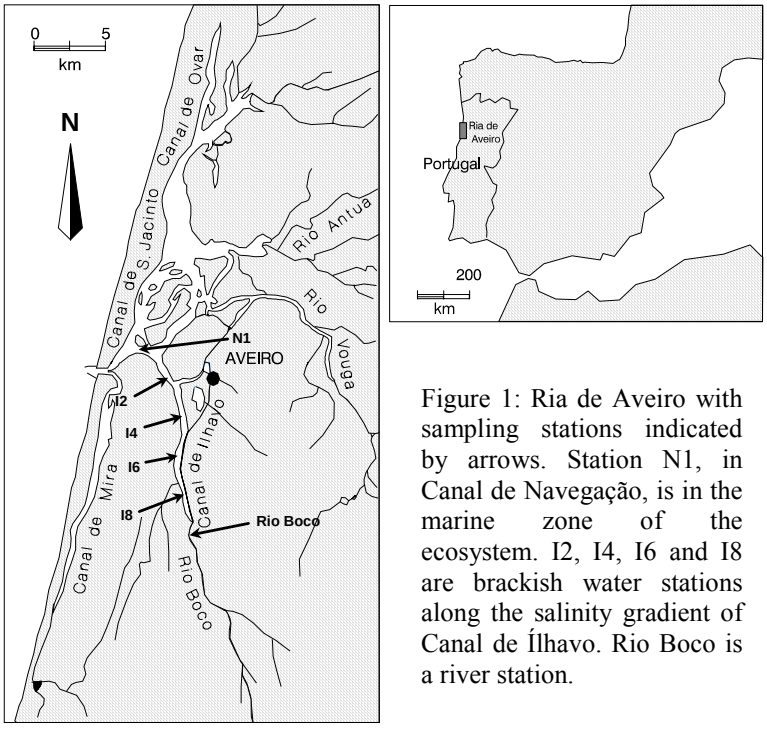


Figure 1: Ria de Aveiro with sampling stations indicated by arrows. Station N1, in Canal de Navegação, is in the marine zone of the ecosystem. I2, I4, I6 and I8 are brackish water stations along the salinity gradient of Canal de Ílhavo. Rio Boco is a river station.

Sampling

Samples were collected along a longitudinal profile of salinity (Figure 1) during the warm season (from early May to early September 1997). Six sampling sites spaced regularly at 3 Km

were established along the longitudinal profile of salinity. The stations were numbered, from north to south, as N1 and I2 in the Canal de Navegação; stations I4, I6 and I8 in Canal de Ílhavo and Rio Boco (RB, the river). Sampling along the whole profile took place at high tide (HT) and low tide (LT). Station N1 — representing the marine zone (MZ) and Station I6 — representing the brackish water zone (BZ), were sampled further during two different tidal cycles. Samples were collected from near-surface water (0.2 m depth) and from deep water (0.5 m above sediment floor), at high tide (HT) and low tide (LT) and also at intermediate intervals of 2 hours (HT+2h, HT-2h, LT-2h, LT+2h). Water samples were processed within 2-3 hours of collection.

Methods

Physical and chemical characteristics. Temperature and salinity readings were obtained with a conductivity meter (WTW — Wissenschaftlich Technische Werkstätten, Model LF 196). Dissolved oxygen concentration, expressed as percentage of saturation, was determined with an oxygen meter (WTW, Model OXI 96) equipped with a stirrer (WTW, Model BR 190). The concentration of suspended solids was determined after filtration of triplicate 0.5 l water aliquots through pre-weighted and pre-combusted Whatman GF/C filters. The filters were dried at 60ºC for 24 hours and suspended solids calculated as the increase in dry weight. The organic matter fraction in the suspended solids was determined as the loss of weight after 4 hours combustion at 525 ºC (19). Particulate organic carbon (POC) was calculated as 50% of the particulate organic matter (25).

Biological characteristics

Viruses. Viral particles were harvested from 8-18 ml water sub-samples fixed in 2% glutaraldehyde (final concentration) directly onto carbon stabilised formvar-coated 400-mesh copper grids (Labometer). Water centrifugation took place at 28 000 rpm (140000 x g) for 2 hours at 20°C in a Beckman L8-80K ultracentrifuge equipped with a swing-out rotor (SW28). Grids were then stained with 1.5% (w/v) uranyl acetate during 60 seconds (5). Viruses were enumerated on a JEOL 100CX TEM at a magnification of 100000x. View fields were shifted randomly and 100 microscope fields were counted in each preparation.

Total bacterial number (TBN). Bacterioplankton cells were counted by epifluorescence microscopy using a Leitz Laborlux K microscope equipped with a I 2/3 filter for blue light. Samples were fixed with 2% formaldehyde (final concentration), filtered through 0.2 µm black polycarbonate membranes (Poretics) and stained with 0.03% acridine orange (13). At least 200 cells or 20 microscope fields were counted in each of three replicate preparations.

Active bacteria number (ABN). Active bacterial number were determined by microautoradiography (8) after amendment of samples to 30 nM ^{3}H-leucine (Amersham, specific

activity 58-92 Ci mmol^{-1}). After 5 hours incubation at *in situ* temperature, samples were fixed with formaldehyde and triplicate subsamples (2-3 ml) of each sample (10 ml) were filtered through 0.2 μm black polycarbonate membranes. The filters were placed, face down, on slides coated with autoradiographic emulsion NTB-2 (Kodak) and exposed in total darkness, at 4°C, for 7 days. The slides were developed with Kodak Detkol (one-to-one dilution in ultrapure water) and fixed (Kodak fixer). The developed autoradiograms were stained with acridine orange solution (0.04%) and hydrated in citrate buffer (0.004 M). Microautoradiographs were examined using a combination of epifluorescence (as above) and bright-field illumination in a Leitz Laborlux microscope. Cells were counted as active if associated with three or more silver grains.

Bacterial biomass productivity (BBP). Bacterial biomass productivity was determined in 10 ml-triplicate sub-samples plus a control that was fixed by addition of formaldehyde (2% final concentration). The samples were incubated at a saturating concentration (30 nM) of ^{3}H-leucine (Amersham, specific activity 58-92 Ci mmol^{-1}) for 1 hour, at *in situ* temperature, in the dark. After incubation, sub-samples were fixed at 2% formaldehyde. Protein precipitation was performed through the addition of 1 ml 20% ice-cold TCA followed by incubation for 15 minutes on ice. Sub-samples were then filtered through 0.2 μm polycarbonate membranes (Poretics), rinsed with 2 ml 5% ice-cold TCA followed by 5 ml of 90% ice-cold ethanol (3). After standing for 3 days in scintillation cocktail UniverSol (ICN Biomedicals, USA) the radioactivity was measured in a liquid scintillation counter (Beckman LS 6000 IC). BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (29).

Chlorophyll *a* (CHLO). Chlorophyll *a* was estimated fluorimetrically (37) in a Jasco spectofluorimeter after filtration of 0,5 l triplicate sub-samples (triplicates) through Whatman GF/C filters and overnight cold extraction in 90% acetone.

Microcosm experiments

The experimental studies in microcosm conditions was devised to clarify the importance of viral infection relative to predation on the control of bacterial growth in marine or brackish water communities of the estuary. The plan implied the manipulation of viruses-to-bacterium ratio (VBR) and the virus-to-bacterium distance (VtB), through differential dilution of bacteria and viruses present in water samples after removal of predators (pre-filtration through 3 μm membranes). Differential dilution of bacteria (without modification of viral density) was done with predator-and-bacteria-free water (filtration of 3 μm filtered water through 0.2 μm membranes). Increase in the VtB distance (without VBR modification) implied dilution with predator-bacteria-and-viruses-free water (0.2 μm filtration followed by 0.02 μm filtration or microwave irradiation).

Water samples (5 l) were collected at stations N1 and at station I6 from June to September 1998. To better contrast the conditions in the two sampling stations the water was collected near HT at station N1 (in the marine zone) and near LT at station I6 (in the brackish water zone). A 0.5 l sub-sample was incubated directly (microcosm 1). The remaining volume was filtered through 3 µm cellulose acetate membranes to obtain predator-free samples. In order to avoid occlusion of filter pores the membrane was changed after filtration of each 0.5 l. Considering the high turbidity of the water the risk of large bacterial loss precluded the use of membranes with greater cut-off. Even so, filtration through 3 µm membranes decreased TBN by an average factor of 1.8. A 0,5 l portion of this water was incubated (microcosm 2). The 3 µm-filtrate leftover was filtered at low pressure through 0.2 µm polycarbonate membranes, 142 mm in diameter (A.E.B filtration unit, Model S.R.L) to generate predators-and-bacteria-free water. This filtrate was used to produce 10-fold dilutions of the 3 µm filtrates to be used in microcosm 3.

In order to obtain predators-bacteria-and-viruses-free water the previous filtrate was either filtered at low pressure through 0.02 µm polycarbonate membranes, 25 mm in diameter (membranes changed after filtration of 50 ml portions) or inactivated by exposure to microwaves (on 19 June, 16 and 23 September). In this case, 50 ml sub-samples of the 0.2 µm filtrate were exposed to 650 W emissions for 3 minutes (3 times 1 minute with intervals of 30 seconds in ice), in 100 ml screw-capped glass bottles (26). The obtained water served for a 10-fold dilution of the predator-free suspension (3 µm filtrate) causing the simultaneous dilution of bacteria and viruses and originating a 2.2-fold increase in VtB distance without changing VBR. This was the suspension present in microcosm 4.

Replicates of suspensions as used in microcosms 3 and 4 were amended with 0.05% yeast extract (final concentration) sterilised through 0.2 µm membrane filtration and autoclaving. They were used to induce high bacterial growth rates and constituted microcosm 5 and 6, respectively. Table I summarises the set of the six experiments with the indication of treatment, its effect and purpose of the experiment.

Samples of 0.5 l of the six suspensions were incubated in parallel for 24 hours at 20°C, under agitation (50 rpm in a Lab-line Orbit Shaker), in 1 l acid-washed and sterilised glass erlenmeyers. Sub-samples were taken at time 0 and after 6, 12 and 24 hours (t_0 to t_{24}) and analysed for total bacteria number and bacterial biomass productivity (in this case only on 16 and 23 September 1998).

Bacterial loss by predation after 24 hours (Lp24) was calculated as $Lp24 = 100 \times (cTBN_2 - TBN_1) / cTBN_2$, that is, as the percent difference in cell production per initial cell, after 24 hour incubation at 20ºC. In microcosm 1, predation and infection occurred at near natural conditions. In microcosm 2, the control, predators were absent. $cTBN_2$ (corrected TBN_2) corresponds to total bacterial number per ml in microcosm 2 at 24h, corrected for the initial difference in density when compared to microcosm 1. TBN_1 represents total bacterial number per ml in microcosm 1 at 24h.

The correction compensates for the inevitable initial loss of about 40% cells in microcosm 2 due to membrane filtration (3 μm nominal porosity). The higher initial bacterial density in microcosm 1 could cause an earlier depletion of nutrients during the 12-24 hour period of incubation that could be misinterpreted as predation. This was not observed, however, as bacteria in microcosm 2 always grew to higher densities than the maximum attained in microcosm 1.

Table I: Microcosm set-up for detection of bacterial predation and viral infection in conditions of different viruses-to-bacteria ratios, bacteria-to-virus distance and bacterial growth rate.

Nº	Sample treatment	Average VBR at onset	Treatment effect/purpose of experiment
1	None, whole water (W)	18	No treatment. Evolution of bacterial and viral populations in non-disturbed microcosm conditions.
2	3 μm filtration of the whole water (F_3)	18	Removal of predators. Evolution of predation (1 vs. 2).
3	Ten-fold dilution with predator-and-bacteria-free water (F_3 B_d)	180	Ten fold reduction in bacterial density in the presence of a non-modified viral suspension. Response of viral infection when viruses-to-bacteria ratio (VBR) was 10-fold increased (4 vs. 3).
4	Ten-fold dilution with predator-bacteria-and viruses-free water (F_3 B_d V_d)	18	Simultaneous ten-fold reduction in bacterial and viral density causing a 2.2-fold increase in virus-to-bacterium distance (VtB) and no alteration in VBR. Response to the experimental minimum in viral infection.
5	As F_3 B_d but added of 0.05% yeast extract at t_0 (F_3 B_d YE)	180	Substantial increase in substrate concentration. Viral control of bacterial growth in populations stimulated to high productivity when VBR was 10-fold increased (5 vs. 3).
6	As F_3 B_d V_d but added of 0.05% yeast extract at t_0 (F_3 B_d V_d YE)	18	Substantial increase in substrate concentration. Viral control of bacterial growth in populations stimulated to high productivity at the experimental minimum in viral infection (6 vs. 4).

B_d – 10-fold dilution of bacteria, V_d – 10-fold dilution of viruses; YE – yeast extract

Bacterial loss by viral infection after 12 hours (Lv12) was derived from similar calculations. It was calculated as the maximum value of infection in the conditions of the experiments, that is $Lv12 = 100 \times (cTBN_4 - TBN_3) / cTBN_4$. It expresses the percent difference in cell production per initial cell, after 12 hours incubation, taking microcosm 3 as the maximum level of viral infection and microcosm 4, the control, as the lowest level. $cTBN_4$ corresponded to total bacterial number per ml in microcosm 4 at t_{12h}, corrected for any initial difference in density when compared to microcosm 3. TBN_3 represented total bacterial number per ml in microcosm 3 at t_{12h}. The incubation beyond 12 hours was not considered because viral infection was partially masked

by the overgrowth of bacteria on nutrients released in the first round of viral lysis as already shown (15, 17).

Statistical methods

Stepwise multiple regression analysis was used to explain the variation of viruses. Temperature, salinity, depth, POC, chlorophyll and TBN were used as independent variables. SPSSWIN 7.1 was used for data analysis.

RESULTS

Field data

Physical and chemical characteristics. A clear salinity gradient was observed along the longitudinal profile. In different dates and tides the salinity values ranged from 32.7-35.8 PSU at station N1 to 2.3-17.7 PSU at RB. The temperature of the water column varied from 16.3 to 26.0 °C, increasing steadily from station N1 to station RB. The highest values were reached near LT. The water column was generally below oxygen saturation showing only occasional high oxygen levels (39-123%). Station I8 was, however, frequently oversaturated (up to 162%). Tidal currents did not develop a clear pattern of variation in the levels of dissolved oxygen. Particulate organic carbon (POC) varied between 3.0 and 12.0 mg l^{-1} and was higher near low tide.

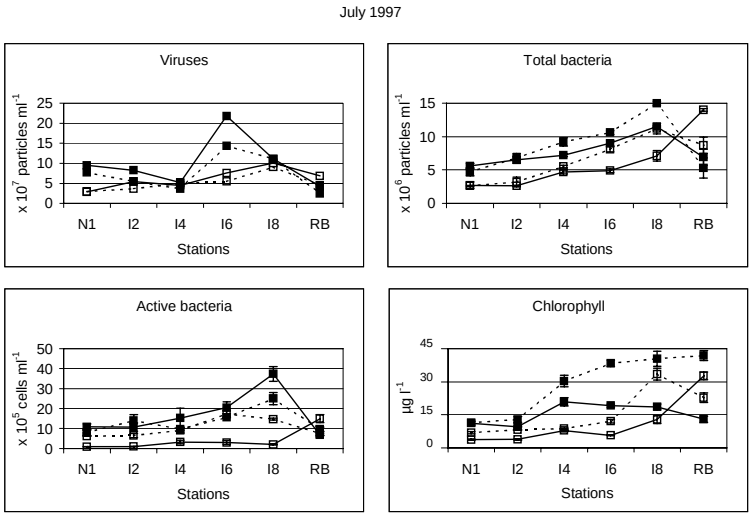


Figure 2: Profiles of variation in plankton abundance along the salinity gradient: total bacteria, active bacteria, viruses and chlorophyll in near-surface water (0.2 m below surface) and in different tidal conditions. —■— low tide (spring tide) —□— high tide (spring tide) ---■--- low tide (neap tide) ---□--- high tide (neap tide). Standard deviation is indicated by bars (sometimes is hidden under the symbols).

Biological characteristics

Viral density in the estuary varied from 2.4 to 25.0 x 10^{10} particles l^{-1}, establishing a sharp pattern of enrichment from the marine to the brackish water stations (Figure 2) where, on average, the concentration was 2 times higher. The highest values were observed, in general, near LT (figures 3 and 4). Viral density was, however, rather constant along the water column (Figure 3). The majority of the viruses showed hexagonal profile and head

diameters of 30-90 nm (average 45 nm). Tail-less viruses were numerically dominant (D and E groups in Bradley's classification). Some of them had tails (A and B groups in Bradley's classification). The range of seasonal variation in bacterial number was considerable (1.9 to 10.6 x 10^9 cells l^{-1}) but, in each sampling date, TBN levels increased only 2-3 times along the profile towards the inner section of the estuary (Figure 2). Maximum TBN values were observed near LT (Figures 3 and 4). In the marine zone TBN was frequently higher in near-surface water but in the brackish water zone the values were constant down the water column. Active bacteria number (1.0-37.3 x 10^8 cells l^{-1}) showed patterns of variation that followed TBN curves (Figures 2, 3 and 4).

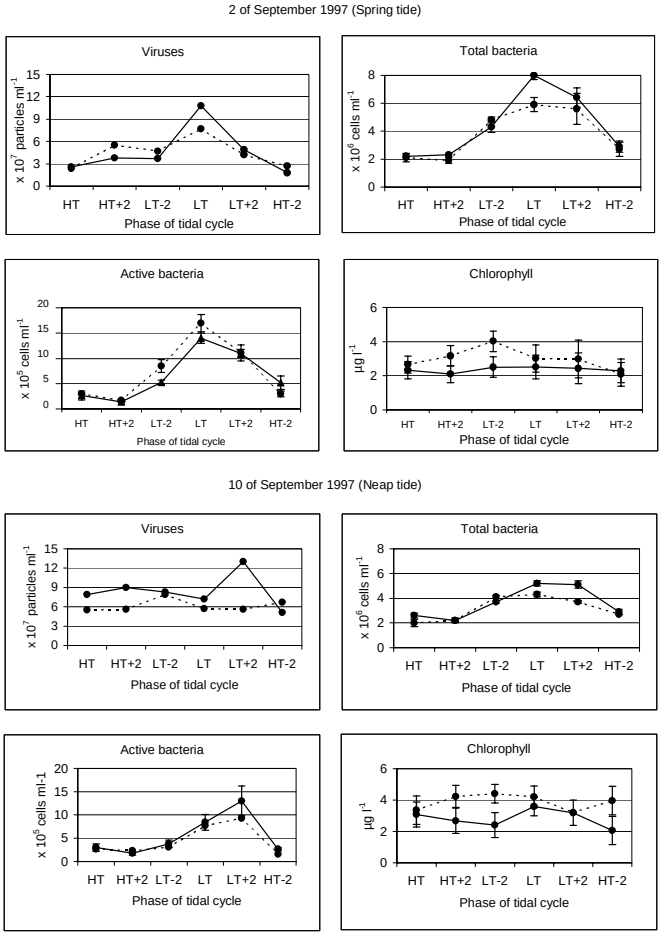


Figure 3: Tidal fluctuation in viral, bacterial and phytoplankton (chlorophyll) abundance in the marine zone (St. N1). Near-surface (0.2 m below surface) and deep water (0.5 m above sediment floor) was sampled at two hour intervals. —●— near-surface water; ---●--- deep water; HT – high tide; LT – low tide. Standard deviation is indicated by bars (sometimes is hidden under the symbols).

The ratio of viruses to total bacterial number (VBR) was similar in the two estuarine zones but was quite variable (up to 12 times) over tidal cycles with the overall range of 4.7 to 55.6 (average 18). Referred to the number of active bacteria (ABN values), VBR increased to a range of 25.8 – 323.6 (average 134.0).

Chlorophyll *a* concentration showed a clear pattern of geographical variation with a maximum of 41.9 µg l^{-1} at RB (Figure 2). CHLO levels were, in general, 2 times higher in brackish water than in marine water and the highest values were observed near LT (Figures 3 and 4). CHLO concentration was quite homogeneous down the brackish water column but in the marine zone the concentration could peak at 0,5 m from the sediment floor (Figure 3 and 4).

Statistical results

Stepwise multiple regression showed TBN as the variable that better explained the viral variation (r^2 = 0.40, $p < 0.001$, β = 0.617). Bacterial abundance and POC explained 43% of viral variation ($p = 0.046$, $\beta = -0.193$).

Microcosm experiments

During the first 6 hours of incubation, only slight variations in TBN could be detected (figures 5 and 6). No net loss was observed during the first 6 hours. Net gain in TBN was evident, in different degrees, after 12 hours incubation.

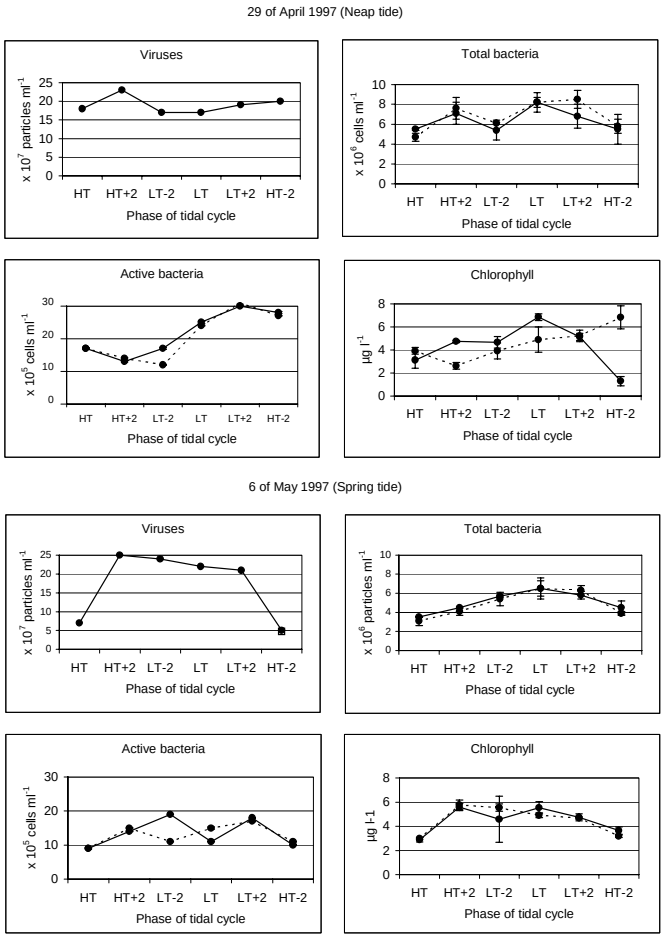


Figure 4: As in figure 3 but referred to the brackish water zone (station I6). —●— near-surface water, ----●--- deep water, HT - high tide, LT - low tide. Standard deviation is indicated by bars (sometimes is hidden under the symbols).

Loss of potential growth in cell number due to predation (microcosm 2 vs. microcosm 1) was clear in both zones (Figures 5 and 6). After 12 hours of incubation the increase of TBN in non-filtered water samples (microcosm 1) was impaired by an average factor of 69% (range 60-78%) in marine water and of 73% (range 68-78%) in brackish water (Table II) when compared to the respective microcosm 2.

Microcosm 4 versus microcosm 3 led to determination of the maximum loss effect by viral infection under the experimental conditions. After 12 hours the loss of bacterial growth corresponded, on average, to 36% (range 30-41%) in marine water and to 61% (range 49-74%) in brackish water (Figures 5 and 6 and Table II).

In microcosm 5 and 6, where yeast extract was added to samples that were otherwise similar to experiments 3 and 4, the effect of the 10-fold increase in VBR (microcosm 5) was undetectable as it was masked by the extraordinary increase in TBN (Figure 7). The increment in density reached a factor of about 5000 in marine water and of about 1500 in brackish water, regardless of the initial value of VBR.

The response of bacterial productivity during the period of incubation was clear in all experiments. In general, the pattern of variation followed the variation of TBN (Figure 8). In microcosm 2, however, the increase of BBP when compared to the values in microcosm 1, occurred up to 6 hours earlier in brackish water (duplicating the initial value at t_{12}) than in marine water (1.3 increase at t_{12}). Experiment 4 (representing the lowest level of viral infection) led, in the

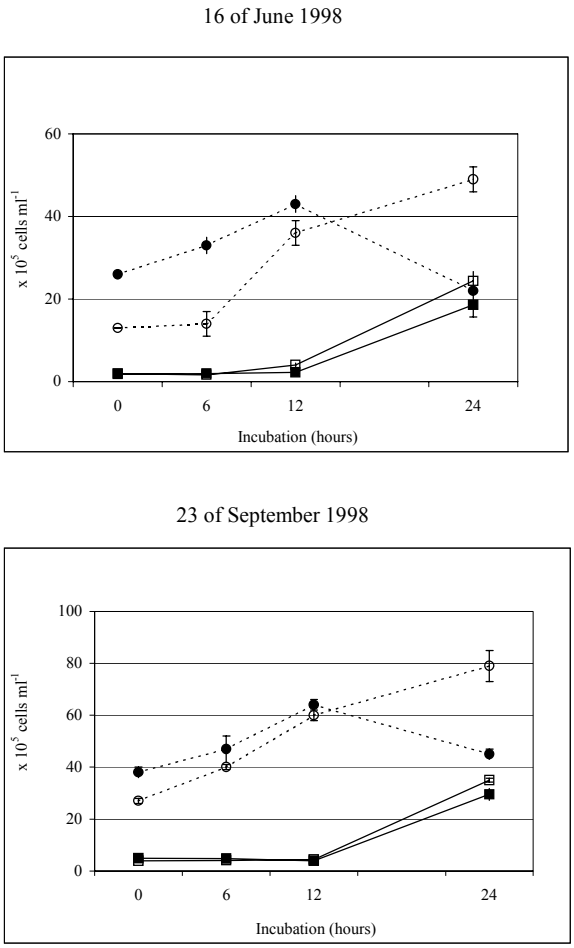


Figure 5: Evolution of bacterial abundance in marine zone according to the different microcosm experiments. ----●--- Experiment 1 (W), ----○---- Experiment 2 (F_3), —■— Experiment 3 (F_3 B_d), —□—Experiment 4 (F_3 B_d V_d). Standard deviation is indicated by bars (sometimes is hidden under the symbols).

first 12 h, to productivity curves that were similar to those obtained in experiment 3 (representing the maximum level of viral infection) both in marine and in brackish water. In the 12-24 hours period, however, growth was more intense in experiment 4, when compared to microcosms 3, in spite of the fact that initial bacterial density was similar in microcosms 3 and 4. Productivity in experiment 3 never reached the values in experiment 4.

DISCUSSION

The high density of viral community in the water column of the estuary (2.4-25.0 x 10^{10} particles l^{-1}) immediately suggests a strong overall effect on the planktonic system and, eventually, a modulation of bacterial activity. Electron microscopy showed in this work that the majority of the viruses were in the bacteriophage size-range (1). This agreed with the observation that the pattern of geographical, tidal and vertical variation of virioplankton followed bacterial abundance as confirmed by regression analysis.

The microcosm dilution experiments were constructed on serial filtration of estuarine water to remove different biological groups. It has been commented in the literature (31) that a variable fraction of viruses can be lost by 0.2 µm filtration (intended for bacterial removal) due, in part, to particle loads in environmental samples. This would result in low filtration efficiency by occlusion of filter pores and/or viral adsorption to the membrane (20). In our case, however, 0.2 µm filtration was performed through large membranes (142 mm diameter) after 3 µm filtration, in the hope of reducing these unwanted effects.

The biomass productivity in bacterial populations is usually calculated through the rate of incorporation of particular substrates. We conclude, however, that bacterial productivity may be elusive in terms of bacterial vitality, as it does not distinguish bacterial growth from viral replication. This is particularly true when VBR was increased as in our experiments. In this case,

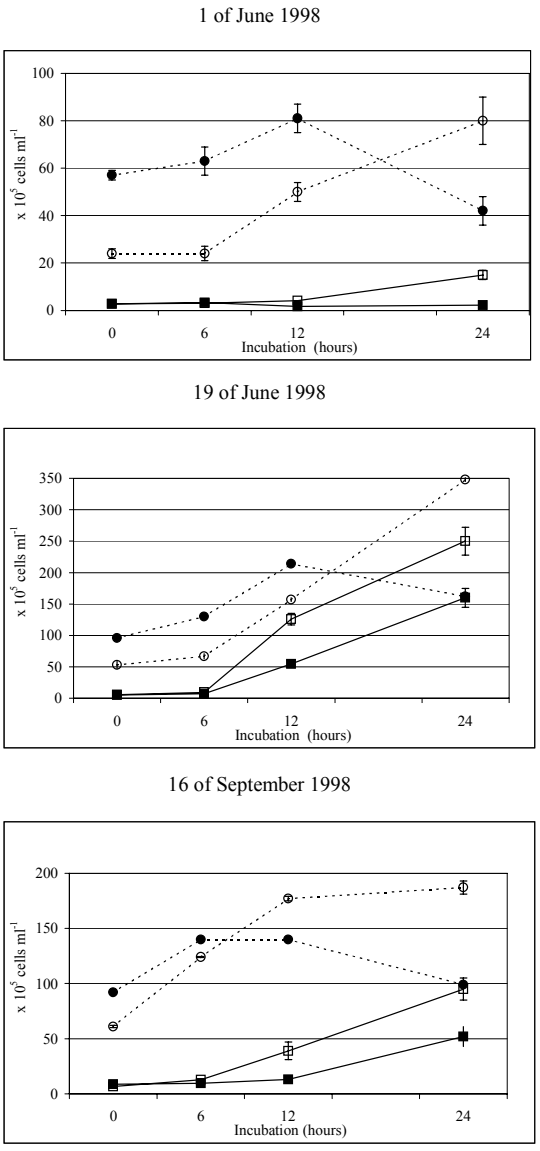


Figure 6: Evolution of bacterial abundance in brackish water according to the different microcosm experiments. ----●--- Experiment 1 (W), ----○---- Experiment 2 (F_3), —■— Experiment 3 (F_3 B_d), —□—Experiment 4 (F_3 B_d V_d). Standard deviation is indicated by bars (sometimes is hidden under the symbol).

the increment in bacterial number compared to the control was, on average, two times smaller at a time when leucine incorporation was running at similar, or even higher, rates in the experiment.

The experimental data of microcosm dilution experiments provided a first approach to the relative importance of bacterial infection and predation in the estuary as well as to the relative impact of viral infection on the growth response of bacterial communities. Average bacterial loss by predation was similar, in a percent basis, in marine (69%) and brackish water (73%). The contribution of predation to bacterial mortality fit within the ranges reported in the literature (10-80%) for coastal systems (10, 11, 30). Average bacterial loss by maximal viral infection (in the conditions of the experiment) was much higher in brackish water (61%) than in the marine zone (36%). The contribution of viral infection to bacterial mortality in the estuary (average 50%, range 30-74%) was higher or slightly higher than the published values. The frequency of infected cells (FIC) produced values in the range of 2-74% and the median at 34% (36). The enrichment of bacterial suspensions with high-molecular-weight-concentrates containing viral particles (HMWC) increased bacterial mortality by 25 to 40% (21, 23, 24, 33). The direct determination of viral production elicited the calculation of the lysed cells fraction as 1-67% of the total (10, 31). Our values are however within the range (25-100%) established on calculations of virioplankton loss rate (6, 7).

Bacterial mortality due to viral infection is controlled mainly by the encounter rate between viruses and their hosts (16). The efficiency of infection in different environments could depend on the numerical ratio between bacteriophages and bacteria. The experimental increase of VBR increased bacterial loss in marine and brackish water, an indication that the potential for viral

infection was not saturated in the estuary. We conclude that viral infection may be operative in reducing the density of estuarine bacteria below the values determined by nutrient decline and predation, a situation that drives to VBR increases.

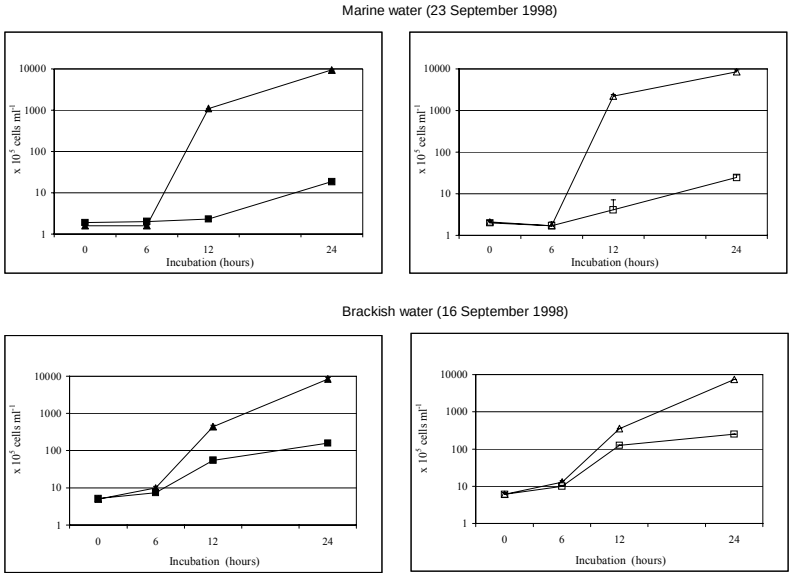


Figure 7: Impact of viral infection on abundance of estuarine bacteria at high growth rates when amended with 0.05% yeast extract at t_0. —■— Experiment 3 (F_3 B_d) —□—Experiment 4 (F_3 B_d V_d).—▲— Experiment 5 (F_3 B_d YE), —Δ— Experiment 6 (F_3 B_d V_d YE). Standard deviation is indicated by bars (sometimes is hidden under the symbols).

Any possible effect of viral lysis on the bacterial communities presenting clear enhanced growth after yeast extract addition, was masked even when the initial VBR was 10-fold greater than in the natural sample. The high density of the virioplankton did not preclude a large and rapid increase in bacterioplankton density. Growth was so great that it easily

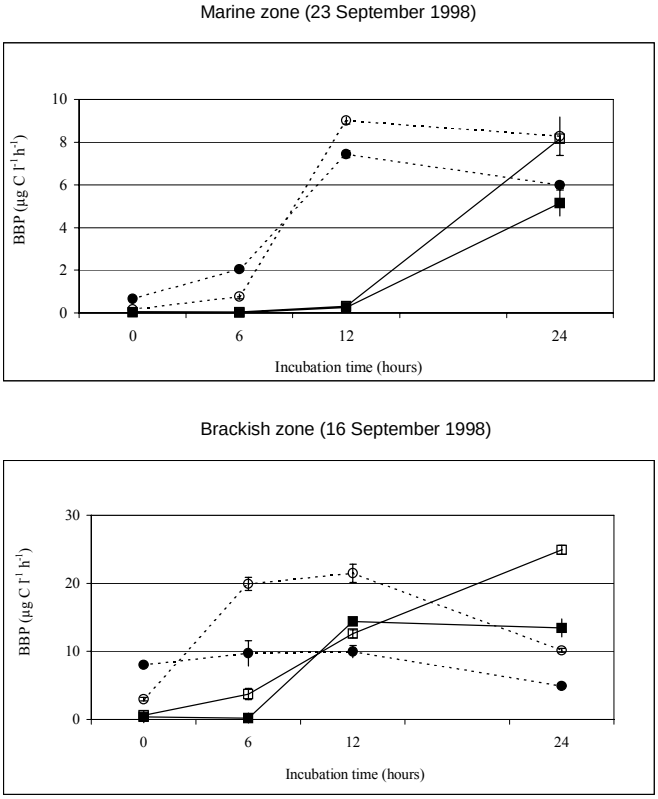


Figure 8: Bacterial biomass productivity (BBP) in marine and brackish water according to the different microcosm experiments. ----●--- Experiment 1 (W), ----○---- Experiment 2 (F_3), —■— Experiment 3 (F_3 B_d), —□—Experiment 4 (F_3 B_d V_d). Standard deviation is indicated by bars (sometimes is hidden under the symbols).

obscured any residual loss by infection. Such a quick response in bacterial density may not depend so much on the hypothetical awakening of dormant cells – a slow process – but on the immediate intensification of the growth response of already active cells. This implies that, unless other factors may come into play, and depending on the size of the food source, viral infection may lag much

behind bacterial growth until a new and higher level of dynamic equilibrium is reached. At the other end of the fluctuation process, viruses may control the low-level balance between the two communities when transiently increased VBR pushes bacterial density further down.

REFERENCES

1. Acherman HW, BuBow MS (1987) Viruses of prokaryotes: general properties of bacteriophages. CRC Press Inc., Boca Raton, Florida
2. Barrosa JA (1985) Breve caracterização da Ria de Aveiro. Jornadas da Ria de Aveiro. Volume II. Recursos da Ria de Aveiro. Edição da Câmara Municipal de Aveiro
3. Blight SP, Bentley TL, Lefevre D, Robinson C, Rodrigues R, Rowlands J, Williams P (1995) Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128: 61-75
4. Boehme J, Frisches M, Jiang S, Kellog C, Pichard S, Rose J, Steinway C, Paul J (1993) Viruses, bacteria and phytoplankton in the Southwestern Gulf of Mexico: distribution and contribution to oceanic DNA pools. Mar Ecol Prog Ser 97:1-10
5. Bratbak G, Heldal M (1993) Total counts of viruses in aquatic environments. *In*: Kemp PF, Sherr BF, Sherr EB and Cole JJ (eds.) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, New York. p 135-138
6. Bratback G, Egge JK, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms. Mar Ecol Prog Ser 93:39-48
7. Bratback G, Haslund O, Heldal M, Noess A, Roeggen T (1992) Giant marine viruses? Mar Ecol Prog Ser 85:201-202
8. Carman K (1993) Microautoradiographic detection of Microbial Activity. *In*: Kemp PF, Sherr BF, Sherr EB and Cole JJ (eds.) Handbook of Methods in Aquatic Microbial Ecology. Lewis Publishers, New York. p 397-404
9. Cochlan W, Wikner J, Stewart G, Smith D, Azam F (1993) Spatial distribution of viruses, bacteria and chlorophyll a in neritic, oceanic and estuarine environments. Mar Ecol Prog Ser 92:77-87
10. Fuhrman JA, Noble RT (1995) Viruses and protists cause similar bacterial mortality in coastal seawater. Limnol Oceanogr 40:1236-1242
11. Gonzalez JM (1999) Bacteriovory rate estimates and fraction of active bacteriovores in natural protist assemblages from aquatic systems. Appl environ Microbiol 65:1463-1469
12. Goosen NK, van Rijswijk P, Kromkamp J, Peene J (1997) Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat Microb Ecol 12:223-232.
13. Hobbie JE, Daley R, Jasper S (1977) Use of Nuclepore Filters for Counting Bacteria by Fluorescence Microscopy. Appl Environ Microbiol 33:1225-1228
14. Maranger R, Bird D, Juniper S (1994) Viral and bacterial dynamics in Artic sea ice during the spring algal bloom, near Resolute, NWT, Canada. Mar Ecol Prog Ser 111:121-127
15. Middelboe M, Jorgenson NO, Kroer N (1996) Effects of viruses on nutrient turnover and growth efficiency of noninfected marine bacterioplankton. Appl Environ Microbiol 62:1991-1997
16. Murray AG, Jackson GA (1992) Viral dynamics: a model of the effect of size, shape, motion and abundance of single-celled planktonic organisms and other particles. Mar Ecol Prog Ser 89:103-116
17. Noble RT, Middelboe M, Fuhrman JA (1999) Effects of viral enrichment on the mortality and growth of heterotrophic bacterioplankton. Aquat Microb Ecol 18:1-13
18. Page HM, Petty RL, Maede DE (1995) Influence of watershed runoff on nutrient dynamics in a Southern California salt-marsh. Estuar Coast Shelf 41:163-180
19. Parsons TR, Maita Y, Lalli CM (1989) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford
20. Paul J, Jiang S, Rose J (1991) Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl Environ Microbiol 57:2197-2204
21. Peduzzi P, Weinbauer MG (1993) The submicron size fraction of seawater containing high numbers of virus particles as bioactive agent in unicellular plankton community successions. J Plankton Res 15:1375-1386

22. Proctor L, Fuhrman J (1991) Roles of viral infection in organic particle flux. Mar Ecol Prog Ser 69:133-142
23. Proctor LM, Fuhrman JA (1992) Mortality of marine bacteria in response to enrichments of the virus size fraction from seawater. Mar Ecol Prog Ser 87: 283-293
24. Proctor LM, Fuhrman JA, Ledbetter MC (1988) Marine bacteriophages and bacterial mortality. Eos 69:1111-1112
25. Rodier J (1996) L`analyse de l`eau: eaux naturelles, eaux résiduaires, eau de mer. 8 Edition, Dunod, Paris.
26. Sanborn MR, Wan SK, Bulard R (1982) Microwave sterilization of plastic tissue culture frasks for reuse. Appl environ Microbiol 44.960-964
27. Shiah F, Ducklow HW (1994) Temperature and substrate regulation of bacterial abundance, production, and specific growth rate in Chesapeake Bay. Limnol Oceanogr 39:1243-1258
28. Silva JJ (1994) Circulação de Água na Ria de Aveiro: Contribuição para o Estudo da Qualidade da Água. Ph.D. Thesis. Universidade de Aveiro
29. Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic bacteria. Mar Ecol Prog Ser 51:201-213
30. Solic M, Krstulovic N (1994) Role of predation in controlling bacterial and heterotrophic nanoflagellate standing stocks in coastal Adriatic Sea: seasonal patterns. Mar Ecol Prog. Ser 114:219-235
31. Steward FM, Wikner J, Cohlan WP, Smith DC, Azam F (1992) Estimation of virus production in the sea. I. Method development. Mar Microb Food Webs 6:57-78
32. Steward FM, Smith DC, Azam F (1996) Abundance and production of bacteria and viruses in the Bering and Chukchi seas. Mar Ecol Prog Ser 131:287-300
33. Weinbauer M, Peduzzi P (1995a) Effect of virus-rich high molecular weight concentrates of seawater on dynamics of dissolved amino acids and carbohydrates. Mar Ecol Prog Ser.127:245-253
34. Weinbauer M, Peduzzi P (1995b) Significance of viruses versus heterotrophic nanoflagellates for controlling bacterial abundance in the northern Adriatic Sea. J Plankton Res 17:1851-1856
35. Weinbauer MG, Hoffle MG (1998) Significance of viral lysis and flagellate grazing as factors controlling bacterioplankton production in a eutrophic lake. Appl Environ Microbiol 64:431-438
36. Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 64:69-114
37. Yentsch CS, Menzel DW (1963) A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res. 10:221-231

CHAPTER 6

IS BACTERIOPLANKTON PRODUCTION IN THE RIA DE AVEIRO ESTUARY INFLUENCED BY SALT MARSHES AND BED SEDIMENT

Almeida MA, Cunha MA and Alcântara F

Departamento de Biologia - Universidade de Aveiro
Campus Universitário de Santiago, 3810-193 AVEIRO, PORTUGAL

ABSTRACT: The potential export of materials from bottom sediments and salt marshes into the water column of a shallow estuarine system of Ria de Aveiro was examined in order to evaluate their contribution to the observed high bacterial productivity in the mid section of this estuary. Vertical profiles of physical, chemical and bacterial variables were studied in the marine and brackish water zones. Transversal profiles of the same variables were studied in the brackish zone only. Although the concentrations of seston (17-241 mg l^{-1}), particulate organic carbon (3-15.5 mg l^{-1}) and chlorophyll *a* (1.2-7.0 µg l^{-1}) varied widely, the vertical and transversal profiles were rather uniform. Total bacterial number (0.2-8.5 x 10^9 cells l^{-1}) and the number of particle-attached bacteria (0.02-2.50 x 10^9 cells l^{-1}) along vertical and transversal profiles did not differ much, but bacterial productivity (0.05-14.2 µg C l^{-1} h^{-1}) and dissolved organic carbon concentration (6.0-69.2 mg l^{-1}) were frequently higher near the salt marsh margin at the brackish water transect. The increase in productivity could not be associated with runoff of particulate matter but coincided with inputs of dissolved organic carbon. The results of vertical and transversal profiles point to a minor role of particulate matter additions from the salt marsh area or from bed sediments.

Key words: bacterial productivity, shallow estuary, runoff, resuspension

Abreviations: DOC (dissolved organic carbon), HT (high tide), LT (low tide)

INTRODUCTION

The distribution of bacterial abundance and production in estuarine systems depends in part on the location, quantity and quality of the pool of organic matter used as growth substrates (Page *et al*., 1995; Goosen *et al*., 1997; Cunha *et al*., 2000). Processes involving external transport of materials to the estuary and exchange of materials between the sediment and the water column may influence the availability of substrates for bacterioplankton growth (Prahl & Coble, 1994; Goosen *et al*., 1995; Hoppe *et al*., 1996; Hopkinson *et al*., 1998; Middelboe *et al*., 1998).

The input of large quantities of allochthonous organic matter may exert a strong influence on the carbon flow in the microbial food web in aquatic systems (Coffin & Sharp, 1987). In several estuaries the external source of organic carbon was observed to be greater than primary production (Vallespinos & Mallo, 1990; Findlay *et al.* 1992). In the case of the Ria de Aveiro, a shallow temperate estuary, it has been shown (Almeida *et al.*, 2001b) that allochthonous carbon may support a significant fraction of bacterial production in the cold season and also during the warm season when phytoplankton activity is potentially sufficient to support the whole bacterial production. The high ratio of planktonic respiration to primary production in this estuary (range 2-17, average 4) also suggests the presence of important sources of allochthonous labile carbon (Almeida, MA; Cunha, MA & Alcântara, F, unpublished data). The input of allochthonous organic

matter into this estuary derives in part from rivers and from small freshwater streams but mainly from diffuse domestic and industrial discharges (Silva, personal communication). It has been reported, however, that tidal runoff from salt marshes may also carry important amounts of allochthonous organic matter into estuarine systems (Prah & Coble 1994; Hoppe et al., 1996).

The accumulation of allochthonous and autochthonous carbon on the bottom of aquatic systems, when followed by resuspension can considerably influence heterotrophic processes in the water column of shallow estuaries (Middelboe *et al*., 1998). Resuspension increases sediment-water exchanges and may result in higher concentrations of organic matter and nutrients for planktonic bacteria (Hopkinson *et al*., 1998; Middelboe *et al*., 1998). The complexity of the benthic compartment, including flow characteristics (Nowel & Jumars, 1984), sediment properties (Mehta, 1988) and biological activity (Rhoads & Boyer, 1982), influences resuspension kinetics. The minimum values of tidal current energy causing sediment resuspension have been reported for different estuarine systems (Henriksen *et al*., 1983; Blanchard *et al*., 1997). For example Lindsay *et al*. (1996) showed that, in the Ford Estuary, sediment resuspension requires a minimum current speed of 0.60 m s^{-1}. Moreover, it has been observed in estuarine systems with relatively high current speed that sediment resuspension occurred only in spring tide when the current reached speeds of 6.3-8.9 m s^{-1} (Medeiros & Kjerfve, 1993). On the other hand, Oliveira *et al*. (1993) reported resuspension events in complex shallow estuaries with much lower current speed (0.2-2.0 m s^{-1}).

It is recognised that other factors including physical (e.g. temperature, water circulation) chemical (e.g. salinity, dissolved oxygen) and biological factors are involved in the modulation of the bacterioplankton metabolism (Weisse and Muller, 1998; Shiah *et al*., 1999; Schultz and Ducklow, 2000; Wommack and Colwell, 2000). Physical, chemical and biological factors as well as substrate availability interact in the regulation of bacterioplankton activity in such a way that it is often difficult to discriminate between individual effects and to assess their relative contribution. However, several studies have demonstrated that the variation in the rates of bacterial activity in estuarine and coastal systems might be regulated by temperature, with substrate supply playing a lesser role (Berman *et al*., 1994; Griffith *et al*., 1994; Shiah and Ducklow, 1995).

Different profiles of bacterial production in the estuary Ria de Aveiro have already been studied at the interfaces with the river and with the ocean (Cunha *et al*., 2000). Stimulation of bacterial productivity at the 0.5 meter water layer of the marine zone was attributed to photo-oxidation of semi-labile or refractory organic compounds and/or to increased phytoplankton exudation (Almeida *et al*., 2110a). The impact of the tide induced changes in salinity was examined experimentally and revealed the presence of two broad bacterial communities distributed longitudinally in the estuary, adapted either to high or to mid salinity ranges and influenced by tidal transport (Almeida *et al*., 2001c). In this work we intend to outline the effect of salt marshes and sediment proximity on bacterioplankton production in shallow waters and to clarify the impact of

these processes on the establishment of the relatively high profiles of bacterioplankton abundance and productivity in the mid-section of the Ria de Aveiro.

MATERIALS AND METHODS

Study site

Ria de Aveiro (Figure 1) is a mesotidal lagoon on the Northwest coast of Portugal separated from the Atlantic Ocean by a sand barrier. The tidal range at the mouth varies from 0.6 m in neap tides to 3.2 m in spring tides (Dias *et al.*, 2000). The lagoon covers an area of 66 to 83 km^2 respectively at low and high tide (Dias *et al*., 2000). It exchanges with the sea a volume of water of 89 Mm^3 in tides of 1 to 3 m range (Silva, 1994). At the mouth, current speed reaches 0.4-2.4 m s $^{-1}$ (Dias *et al*., 2000). Several rivers discharge fresh water into the lagoon with an average total input of 1.8 x 10^6 m^3 per tidal cycle (Barrosa, 1985). The Ria has a complex topography representing a multi-estuarine ecosystem associated with different inflowing rivers. The different channels are frequently flanked by low salt marshes. The main channel, Canal de Navegação, is connected with four second-order channels. In this study, we chose the Canal de Ílhavo, the smallest of the second-order channels, 15 km in length, with a water volume of 2.8 Mm^3 at low tide and 9.3 Mm^3 at high tide. This channel offers the advantage of a directly water circulation as compared with the other three small channels that have an irregular and complex geometry. Freshwater is supplied at the south end by a small river, Rio Boco. There is a considerable anthropogenic pressure along this channel, via harbour facilities in the outer section and aquaculture ponds, industrial plants, diffuse domestic sewage inputs in the mid- inner sections. The salt marshes along the channel are typically covered by *Halimione portulacoides* and, among others, by *Spartina maritima*.

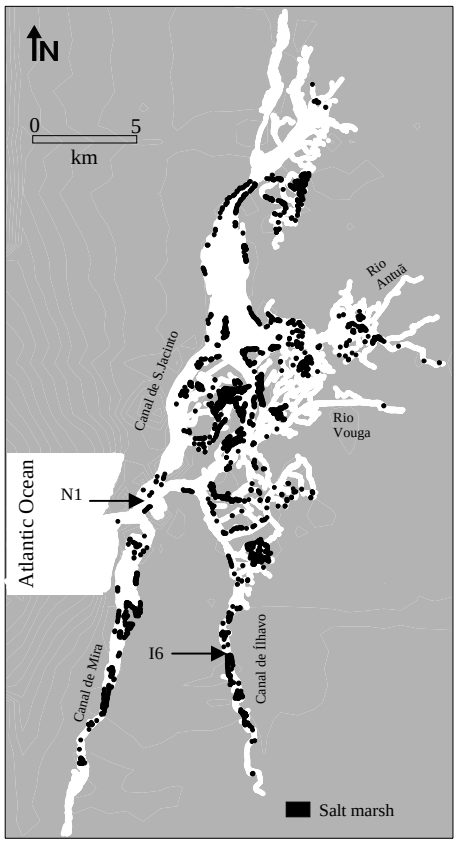


Figure 1: Ria de Aveiro with sampling stations indicated by arrows. Station N1 in Canal de Navegação represents the marine zone of the ecosystem. Station I6 in Canal de Ílhavo represents the brackish water zone. Salt marsh occurrence indicated in black.

Sampling

Evidence for sediment resuspension was studied in the marine zone and in the brackish water zone of the estuary. Salt marsh runoff was investigated only in brackish water where salt

marshes are more abundant (Figure 1). Sampling took place during the warm season and over different tidal cycles at high tide (HT) and low tide (LT) and at intervals of 2 hours (HT+2h, HT-2h, LT-2h, LT+2h). Station N1 in the centre of the main channel of the Ria de Aveiro, represented the marine zone (MZ) and was sampled over six tidal cycles (two in June 1996, two in August 1996 and two in September 1997), five of them in spring tide and one in neap tide, at four depths (0.2 m below surface; 4.5 m, 2.5 m and 0.5 m above sediment surface). Transect I6, in Canal de Ílhavo, represented the brackish water sections (BW) (Figure 1). It was sampled over four tidal cycles (April and May 1997), two in spring tide and two in neap tide, at two depths (0.2 m below surface and 0.5 m above sediment surface). The transect included 4 study sites located between the west margin and the centre of the channel – S1 close to the margin, S2 one meter from the margin, S3 half-way to the centre and S4 at the centre of the channel. In May 1997, surface and deep water samples were also collected at station N1 and at transect I6, only at low tide and high tide, for determination of dissolved organic carbon. Water samples were processed within 2-3 hours past collection.

Methods

Physical and chemical characteristics. Temperature and salinity were measured with a conductivity meter (WTW, Model LF 196). Dissolved oxygen concentration, expressed as percentage of saturation, was determined with an oxygen meter (WTW, Model OXI 96) equipped with a stirrer (WTW, Model BR 190). Depth of the water column was measured with a Sonar probe (Hondex PS-7 LCD Digital Sounder). The concentration of seston was determined after filtration of triplicate 0.5 l water sample aliquots through pre-weighted and pre-combusted Whatman GF/C filters. The filters were dried at 60ºC for 24 hours and seston content was calculated as the increase in weight due to the dried particulate matter retained. Particulate organic matter was determined through loss of weight of the dry seston after 4 hours incineration at 525 ºC (Parsons *et al.*, 1989). Particulate organic carbon (POC) was calculated as the 50% fraction of the particulate organic matter (Rodier, 1996). Samples for dissolved organic carbon (DOC) analyses were filtered through pre-combusted Whatman GF/C membranes and maintained frozen. Quantification of DOC was carried out by combustion and infrared detection in a Total Organic Carbon Analyser (Shimadzu 5000A). Chlorophyll *a* was read fluorimetrically (Yentsch & Menzel, 1963) after filtration of 0.5 l triplicate sub-samples through Whatman GF/C filters and overnight cold extraction in 90% (v/v) acetone.

Total bacterial number and particle-attached bacteria. Total and attached bacteria numbers were determined by direct counting under epifluorescence microscopy (Leitz Laborlux K microscope) with a 50 W mercury lamp, blue BP 450-490 exciter filter and LP 515 barrier filter. The samples were fixed with 2% formaldehyde (final concentration), filtered through black

polycarbonate membranes of 0.2 µm (Poretics) and stained with 0.03% acridine orange (Hobbie *et al.*, 1977). At least 200 cells or 20 microscope fields were counted in each of three replicate preparations.

Bacterial biomass productivity. Bacterial productivity was determined in 10 ml triplicate sub-samples plus a control that was immediately fixed by addition of formaldehyde (2% final concentration). The samples were incubated in the presence of a saturating concentration (30 nM) of ^{3}H-leucine (Amersham, specific activity of 58-92 Ci mmol^{-1}) for 1 hour in darkness at *in situ* temperature. After incubation, sub-samples were fixed at 2% formaldehyde. Protein precipitation was performed through the addition of 1 ml 20% ice-cold trichloroacetic acid followed by 15 minutes incubation in ice. Samples were filtered through 0.2 µm polycarbonate membranes (Poretics), rinsed twice with 2 ml of 5% ice-cold TCA and 5 ml of 90% ice-cold ethanol (Blight *et*

Table 1: Particulate matter in the marine zone – seston (mg l^{-1}), POC (mg l^{-1}) and chlorophyll *a* (Chlo) (µg l^{-1})concentrations at 0.2 m below surface (D1), 4.5 (D2), 2.5 (D3) and 0.5 m (D4) above sediment surface. ST - spring tide, NT - neap tide, HT - high tide, LT - low tide, ND - not determined.

		HT	HT+2	LT-2
		D1 D2 D3 D4	D1 D2 D3 D4	D1 D2 D3 D4
1 June 96	Seston	73-71-79-72	ND	149-131-165-157
(ST)	POC	7.5-7.4-8.2-7.7	ND	11.4-11.4-10.8-11.2
	Chlo	ND	ND	ND
3 June 96	Seston	73-69-59- 60	70-73-78-73	111-109-115-110
(ST)	POC	10.0-9.0-7.0-7.0	7.5-8.0-8.0-8.0	10-10.1-9.6-8.5
	Chlo	2.5-2.1-2.6-1.9	2.0-1.4-1.7-1.8	2.4-2.4-2.4-2.4
28 August 96	Seston	75-81-75-74	114-169-132-99	135-157-161-241
(ST)	POC	8.0-7.0-5.4-6.0	5.5-6.0-7.0-7.5	8.5-10.5-11.5-14.0
	Chlo	2.1-1.8-1.6-1.5	1.6-2.3-2.4-2.8	1.8-2.7-ND-ND
30 August 96	Seston	53- 63-48-51	93-165-102-101	222-201-189-200
(ST)	POC	4.5-6.0-6.0-5.5	8.5-12.0-9.0-8.0	13.0-11.5-11.0-12.0
	Chlo	2.8-2.0-2.2-2.2	1.7-0.2-0.6-2.2	3.2-1.0-1.0-1.2
2 September 97	Seston	17-ND-ND-21	56-ND-ND-69	36-ND-ND-51
(ST)	POC	3.0-ND-ND-3.0	7.0-ND-ND-7.0	3.0-ND-ND-5.0
	Chlo	2.3-ND-ND-2.7	2.1-ND-ND-3.2	2.5-ND-ND-4.0
10 September 97	Seston	51-ND-ND-51	58-ND-ND-62	58-ND-ND-61
(NT)	POC	10.5-ND-ND-6.0	6.0-ND-ND-7.0	7.0-ND-ND-7.5
	Chlo	3.1-ND-ND- 3.4	2.7-ND-ND-4.2	2.4-ND-ND-4.4

		LT	LT+2	HT-2
		D1 D2 D3 D4	D1 D2 D3 D4	D1 D2 D3 D4
1 June 96	Seston	63-87-83-77	69-73-121-142	67-62-72-75
(ST)	POC	5.9-7.7-7.4-6.9	7.4-7.9-10.4-10.4	6.7-7.8-7.3-6.9
	Chlo	ND	ND	ND
3 June 96	Seston	67-70- 92-89	57-94-92-96	84-78-72-64
(ST)	POC	7.5-6.0-7.6-7.5	6.5-8.0-7.5-8.0	15.5-10.0-8.5-7.5
	Chlo	2.7-2.6-1.9-2.3	3.2-2.1-2.8-1.9	1.5-1.3-1.3-2.0
28 August 96	Seston	67-59-68-85	85-79-81-79	73-89-84-82
(ST)	POC	8.0-7.0-7.0-7.0	6.0-5.0-7.0-5.0	7.0-8.5-8.0-8.0
	Chlo	ND-0.1-ND-1.2	5.5-3.9-2.4-2.3	1.3-1.7-1.8-1.7
30 August 96	Seston	68-84-86-118	69-72-80-81	60-53-55 51
(ST)	POC	7.5-9.5-7.0-8.0	5.0-4.0-4.5-4.5	4.5-5.0-5.0-5.0
	Chlo	3.6-4.3-3.5-2.4	3.1-4.4-2.2-ND	2.3-2.6-2.4-3.0
2 September 97	Seston	27-ND-ND-27	56-ND-ND-71	61-ND-ND-83
(ST)	POC	3.5-ND-ND-3.5	6.5-ND-ND-8.0	7.0-ND-ND-8.5
	Chlo	2.5-ND-ND-3.0	2.4-ND-ND-3.0	2.3-ND-ND-2.1
10 September 97	Seston	49-ND-ND-51	56-ND-ND-61	49-ND-ND-53
(NT)	POC	6.0-ND-ND-6.5	6.0-ND-ND-6.5	5.5-ND-ND-6.0
	Chlo	3.6-ND-ND-4.2	3.2-ND-ND-3.2	2.1-ND-ND-4.0

al., 1995). After standing for 3 days in the scintillation cocktail UniverSol (ICN Biomedicals, USA) the radioactivity was measured in a liquid scintillation counter (Beckman LS 6000 IC). Bacterial biomass productivity BBP was calculated from leucine incorporation rates using a ratio of cellular carbon to protein of 0.86 and a fraction of leucine in protein of 0.073 (Simon & Azam, 1989).

Statistical analysis. The significance of variation in the different characteristics between surface and bottom water layers, and between margin and centre sites of the same transect was assessed using one-way ANOVA. Three sub-samples were used for each variable at the six tidal phases in different dates. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and

Table 2: Particulate matter in the brackish water zone during the warm season – seston (mg l^{-1}), POC (mg l^{-1}) and chlorophyll *a* (Chlo) (µg l^{-1}) concentrations at 0.2 m below surface and 0.5 m above sediment in the deeper sites (in parenthesis). ST - spring tide, NT - neap tide, HT - high tide, LT - low tide, S1 - site 1, S2 - site 2, S3 - site 3, S4 - site 4.

	Date	Site	HT	HT+2	LT-2	LT	LT+2	HT-2
Seston	29 Apr 97 (NT)	S1	50	46	60	53	53	54
		S2	50	47	63	49	52	54
		S3	48 (40)	51 (54)	71 (74)	46 (46)	52 (51)	52 (55)
		S4	49 (46)	51 (57)	65 (65)	46 (44)	48 (51)	52 (57)
	6 May 97 (ST)	S1	73	64	106	64	67	71
		S2	57	68	89	78	69	75
		S3	46 (52)	78 (88)	95 (99)	71 (82)	67 (75)	63 (67)
		S4	44 (53)	77 (93)	84 (110)	59 (74)	68 (67)	63 (64)
	28 May 97 (ST)	S1	57	52	51	45	62	60
		S3	49 (52)	62 (66)	66 (60)	49 (50)	56 (63)	63 (54)
		S4	52 (54)	59 (67)	63 (66)	48 (52)	52 (58)	61 (58)
	30 May 97 (NT)	S1	53	55	55	42	42	54
		S3	51 (51)	50 (50)	57 (59)	38 (39)	47 (48)	49 (55)
		S4	49 (56)	45 (50)	55 (61)	36 (40)	44 (53)	54 (46)
POC	29 Apr 97 (NT)	S1	5.0	4.5	6.5	6.0	5.0	6.0
		S2	5.5	4.5	6.5	5.0	5.0	5.5
		S3	5.0 (4.0)	5.0 (5.0)	6.5 (7.5)	5.0 (4.5)	5.5 (5.5)	4.5 (5.0)
		S4	4.5 (4.5)	5.0 (5.0)	6.5 (6.5)	4.5 (4.0)	5.0 (5.0)	5.0 (6.0)
	6 May 97 (ST)	S1	6.5	5.0	7.0	5.5	6.5	9.5
		S2	5.0	5.5	7.0	7.0	6.5	8.0
		S3	4.5 (5.0)	5.5 (7.0)	6.5 (7.0)	6.5 (6.5)	6.5 (7.0)	9.5 (9.0)
		S4	4.5 (5.0)	6.0 (7.5)	5.5 (8.5)	5.0 (6.5)	6.0 (5.0)	9.0 (8.0)
	28 May 97 (ST)	S1	7.0	4.0	2.5	2.0	4.0	6.5
		S3	6.0 (7.5)	4.5 (4.5)	3.5 (4.0)	2.5 (3.5)	5.0 (6.0)	7.5 (6.0)
		S4	7.5 (7.5)	4.5 (4.0)	3.5 (3.5)	3.0 (4.5)	5.0 (5.5)	6.5 (5.5)
	30 May 97 (NT)	S1	7.0	8.5	5.0	4.5	5.0	7.5
		S3	7.0 (7.5)	7.5 (7.5)	6.0 (6.0)	3.5 (3.5)	5.5 (5.5)	6.0 (6.5)
		S4	7.5 (8.5)	5.5 (6.5)	5.5 (5.5)	4.0 (4.0)	4.5 (7.0)	6.5 (5.0)
Chlorophyll	29 Apr 97 (NT)	S1	2.2	3.8	5.2	6.0	5.4	2.7
		S2	3.3	3.9	4.7	7.0	5.8	1.5
		S3	3.0 (2.9)	4.6 (4.2)	4.1 (4.7)	7.0 (6.8)	5.5 (5.4)	1.3 (1.8)
		S4	3.1 (3.9)	4.8 (4.9)	4.7 (5.2)	6.8 (6.8)	5.1 (3.9)	1.3 (2.6)
	6 May 97 (ST)	S1	3.2	4.1	3.8	4.7	5.0	4.1
		S2	2.5	2.8	4.3	5.3	5.2	3.7
		S3	2.6 (3.1)	5.3 (5.9)	6.5 (4.4)	5.7 (4.7)	4.9 (5.3)	4.1 (4.0)
		S4	2.9 (3.0)	5.6 (5.7)	4.6 (5.5)	5.5 (4.9)	4.7 (4.7)	3.6 (3.3)
	22 May 97 (ST)	S1	3.2	4.1	4.9	5.3	6.1	4.4
		S3	3.0 (2.7)	3.9 (4.6)	4.8 (4.9)	5.1 (5.0)	5.9 (5.7)	3.9 (3.8)
		S4	2.8 (3.0)	4.7 (4.6)	4.8 (4.8)	5.2 (5.6)	6.0 (4.9)	4.4 (4.1)
	30 May 97 (NT)	S1	4.20	6.5	5.8	4.8	4.3	4.6
		S3	3.7 (4.0)	6.9. (7.0)	5.7 (4.5)	5.8 (5.6)	4.3 (4.5)	3.6 (3.4)
		S4	4.7 (4.2)	6.5 (6.2)	4.5 (5.4)	4.9 (4.1)	4.1 (3.7)	3.6 (3.9)

homogeneous variances (assessed by Levene test) were used. Variation in bacterial abundance and productivity was assessed by stepwise multiple regression analysis. Seston, POC, chlorophyll, temperature, salinity and oxygen concentration were used as independent variables in order to assess the environmental factors that explained bacterial abundance and production.

RESULTS

Environmental conditions. At station N1 salinity varied from 23.7 to 36.1 psu (average 34.4 psu) and from 5.4 to 34.0 psu at transect I6 (average 26.2 psu). Water temperature varied from 15.3 to 23.5°C increasing from the marine zone to the mid brackish zone. Oxygen saturation varied

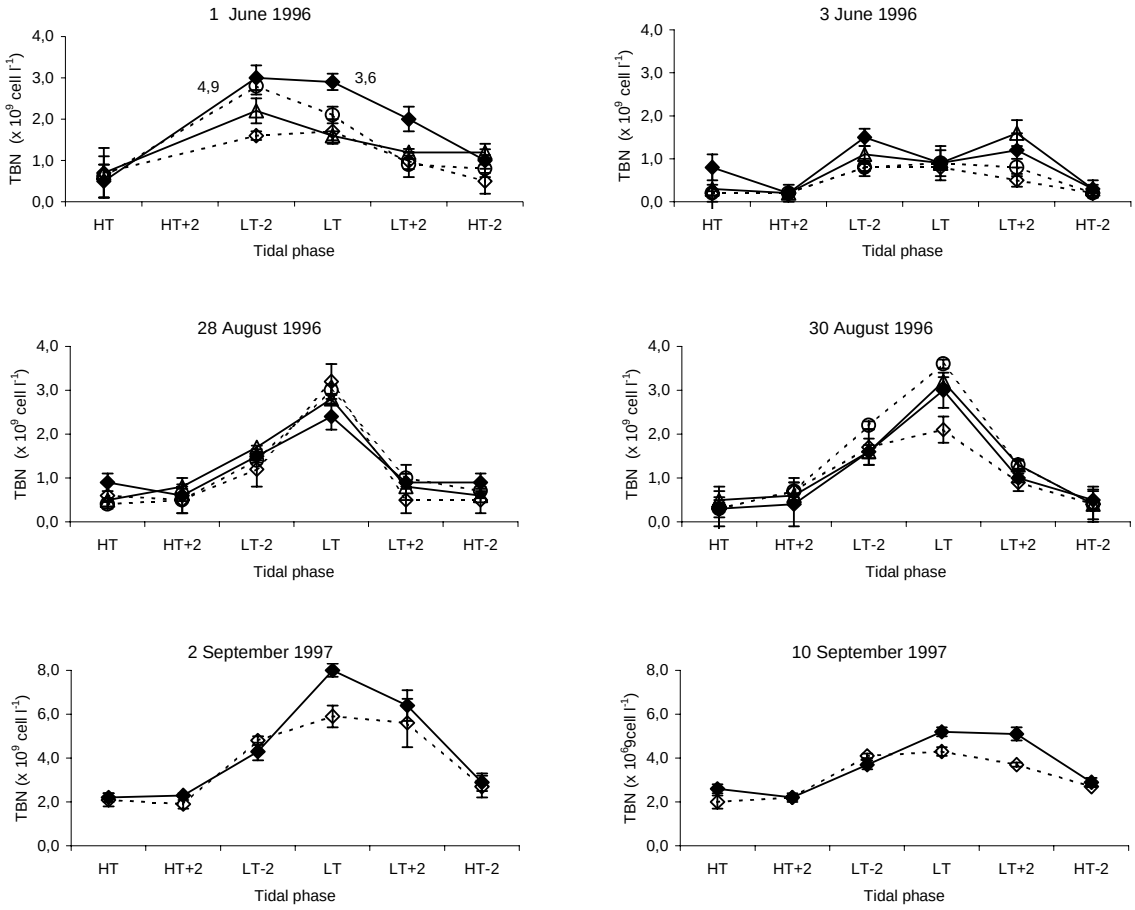


Figure 2: Tidal and vertical fluctuation of total bacterial number (TBN) at station N1. —◆— 0.2 m below surface, —▲— 4.5 m above sediment surface, ---○--- 2.5 m above sediment surface ---◇--- 0.5 m above sediment surface.

between 39 and 123%. The values were, generally, below saturation in both zones. Salinity, temperature and oxygen saturation values were similar along transects and down the water column in both zones (ANOVA $p>0.05$). Water depth varied from 4.1 to 8.9 m (average 7.4 m) at stationN1 and from 1.3 to 3.3 m (average 2.3 m) at transect I6.

Seston, POC, DOC and chlorophyll. At station N1, seston concentration ranged from 17 to 241 mg l^{-1} (average 78.4 mg l^{-1}) (Table 1) and between 7 and 110 mg l^{-1} (average 58.2 mg l^{-1}) at transect I6 (Table 2). POC concentration varied between 3.0 and 15.5 mg l^{-1} (average 7.2 mg l^{-1}) at station N1 and between 2.0 and 8.5 mg l^{-1} (average 5.5 mg l^{-1}) at transect I6. The percentage of POC in the total seston was in the range of 5-20% (average 10%) at station N1 and in the range of 4.5-15.0% (average 9.7%) at transect I6. POC concentration showed patterns of variation similar to those observed for seston in both zones (Spearman`s correlation coefficient of 0.759 and 0.446 at

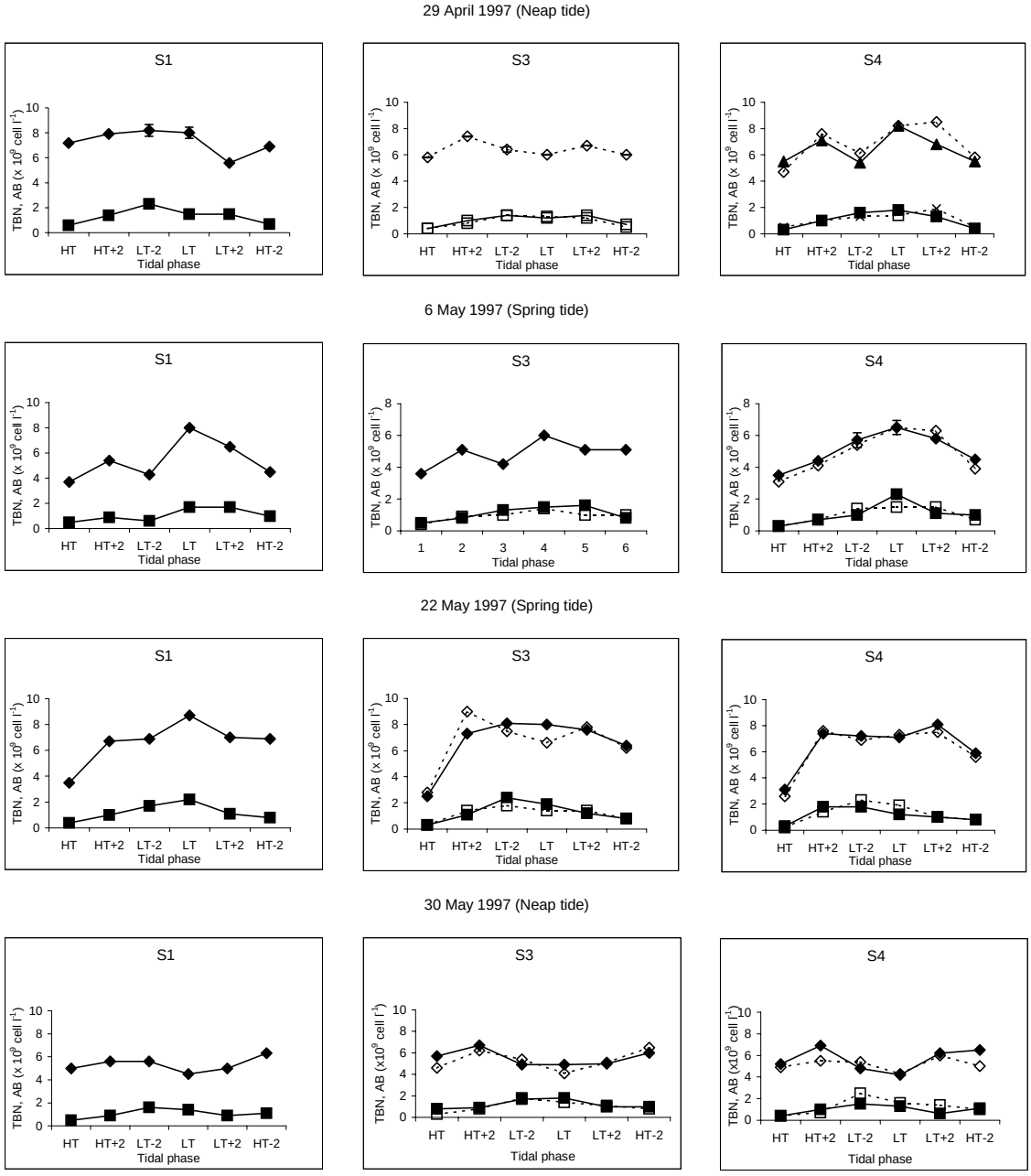


Figure 3: Tidal and vertical fluctuation of total bacterial number (TBN) and attached bacteria (AB) at sites 1, 3 and 4 of transect I6. Bacterial abundance at site 2 was similar to site 1. —◆— TBN at 0.2 m below surface, ---◇--- TBN at 0.5 m above the sediment, —■— AB at 0.2 m below surface, ---□--- AB at 0.5 m above sediment surface.

p=0.01 in the marine and brackish water zones, respectively). At station N1, seston and POC concentrations were similar between neap and spring tides (sping tide to neap tide ratio of 1.2). At transect I6, however, the values were, on average, 30 % higher in spring tide. Seston and POC concentrations were at maximum two hours before low tide and at minimum at high tide in both zones. At station N1, seston and POC concentrations were generally higher in deep water when at low tide. Surface and deep values were, however, significant (ANOVA $p<0.05$) only in 26% (seston) and 19 % (POC) of the cases. At transect I6, seston and POC values were similar (ANOVA $p>0.05$) down the water column and along the transversal profile. DOC concentration

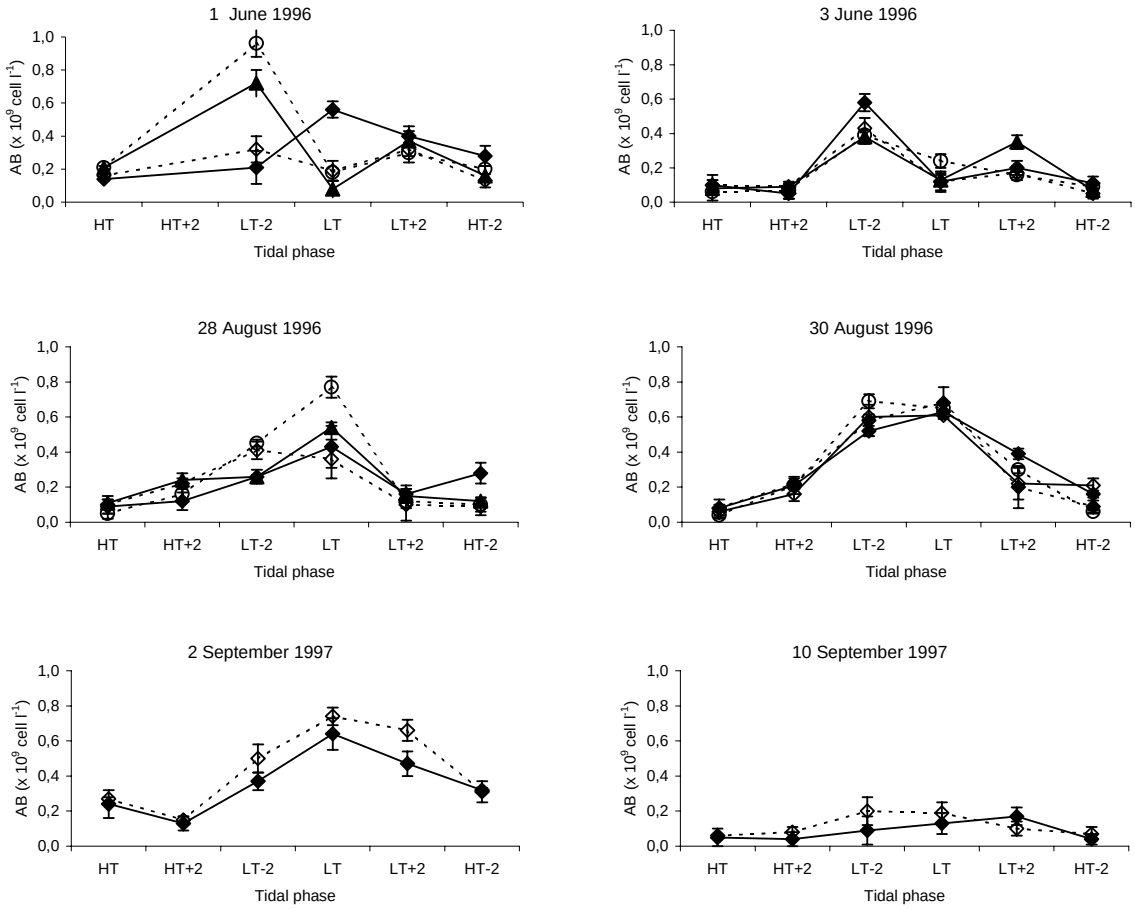


Figure 4: Tidal and vertical fluctuation of particle-attached bacteria (AB) at station N1. —◆— 0.2 m below surface, —▲— 4.5 m above sediment surface, ---○--- 2.5 m above sediment surface ---◇--- 0.5 m above sediment surface.

(6.0-69.2 mg l^{-1}) was, on average, 3 times higher at transect I6 (49.0 mg l^{-1}, on average) than at station N1 (20.4 mg l^{-1}, on average) (Table 3). Down the water column in marine and brackish water the values were rather similar. At transect I6, DOC concentration was higher at the margin site (2.3 times). Chlorophyll *a* concentration varied from 1.2 to 5.5 µg l^{-1} (average 2.6 µg l^{-1}) at station N1 (Table 1) and from 0.2 to 7.0 µg l^{-1} (average 4.6 µg l^{-1}) at transect I6 (Table 2). The

values were similar in neap and spring tides in both zones (spring tide to neap tide ratio of 1.2). The highest concentration was, in general, observed near low tide. Chlorophyll *a* concentration was not significantly different down the water column in both zones and along the I6 transect (ANOVA $p > 0.05$).

Bacterial abundance and productivity. Total bacteria number varied spatially and with tide, from 0.2 to 8.5 x 10^9 cells l^{-1} (Figures 2 and 3) in agreement with the previously observed pattern of

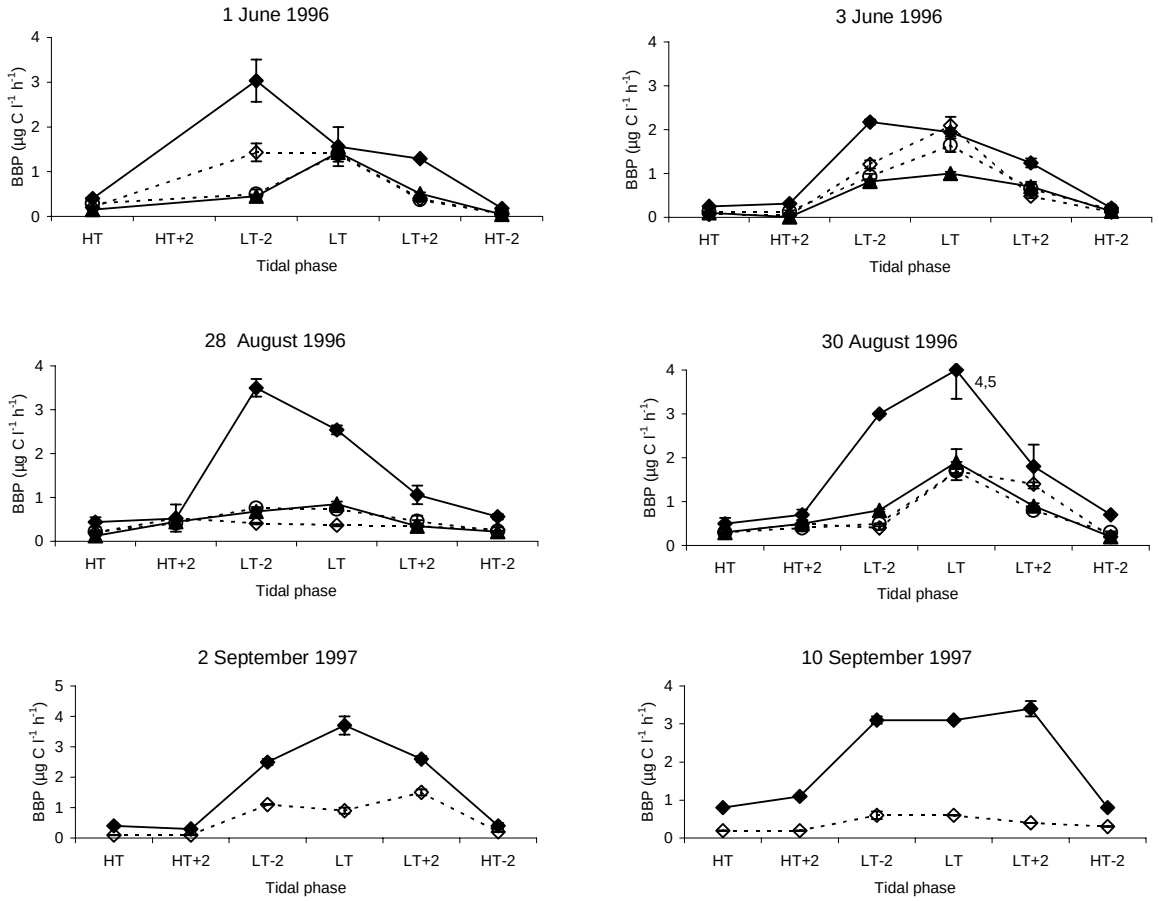


Figure 5: Tidal and vertical fluctuation of bacterial biomass productivity (BBP) at station N1. —◆— 0.2 m below surface, —▲— 4.5 m above sediment surface, ---○--- 2.5 m above sediment surface ---◇--- 0.5 m above sediment surface.

steady bacterial enrichment from the outer to the mid- and upper estuary (Cunha *et al*., 2000). Total bacterial number was, on average, 3 times higher at transect I6 when compared to station N1. At station N1, the maximum values were observed near LT (a 4 times increase in relation to the HT values) both in spring and neap tides. At transect I6, the values were on average, 1.5 times greater in neap tides than in spring tides but tidal fluctuation was not as obvious as in MZ (at transect I6, LT/ HT values averaged 1.7). The small fluctuations in total bacterial number determined at different layers of the water column in MZ (Figure 2) were found to be non significant (ANOVA

p>0.05). In the case of the transect I6 (Figures 3), bacterial abundance was also found to be similar at the different sites (ANOVA p>0.05). Total number of particle-attached bacteria varied from 0.02 to 2.5 x 10^9 cells l^{-1} (Figures 3 and 4). Particle-attached bacteria followed, in general, the variation of total bacterial number in both estuarine zones. The fraction of particle-attached bacteria in total abundance was similar in both zones (average 19%, range 2-53%).

Bacterial productivity varied spatially and with tide within the broad range of 0.05 to 14.2 µg C l^{-1} h^{-1} (Figures 5 and 6). The values were, on average, 3.5 times higher at transect I6 than at station N1 and reached maximum values near LT in both zones (60 and 30% average increases in

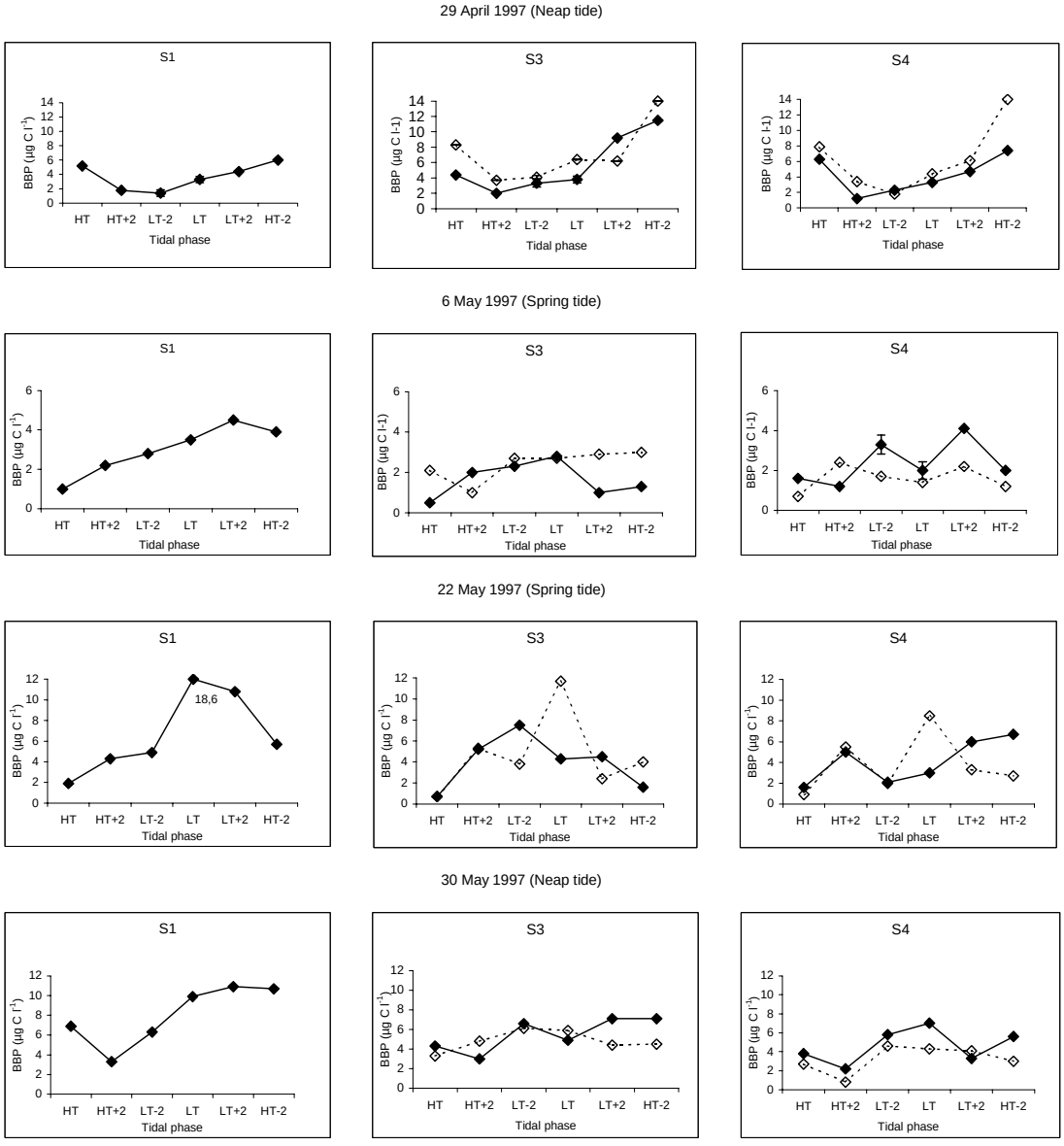


Figure 6: Tidal and vertical fluctuation of bacterial biomass production (BBP) at sites 1, 3 and 4 of transect I6. Bacterial biomass productivity at site 2 was similar to site 1. —◆— 0.2 m below surface, ---◇--- 0.5 m above sediment surface.

Table 3: Dissolved organic carbon (mg l^{-1}) in the marine and brackish water zones at 0.2 m below surface. In the marine zone, site S1 close to the margin south, site S2 at the centre of the channel and site S3 close to the margin north. In the brackish water zone, site S1 close to the margin west, site S4 at the centre of the channel and site S4 close to the margin east. HT - high tide, LT - low tide, ND – not determined.

	Site	HT	LT
Marine zone	S1 surface	ND	29.6
(Station N1)	S1 bottom	ND	34.8
	S2 surface	30.5	6.0
	S2 bottom	24.8	7.7
	S3 surface	27.0	11.0
	S3 bottom	19.3	13.3
Brackish water zone	S1 surface	69.2	74.1
(Station I6)	S4 surface	50.4	52.0
	S4 bottom	44.6	38.8
	S5 surface	29.9	31.2

MZ and BZ, respectively). In station N1, bacterial productivity was significantly (ANOVA $p<0.05$) higher at near-surface water (3.3 times on average). This reaction to surface conditions was not observed in transect I6, where bacterial productivity was similar (ANOVA $p>0.05$) down the water column (Figures 5 and 6). With respect to transversal variation, the values were, on average, 1.5 times greater at the margin site (ANOVA $p<0.05$ in 54% of the 24 studied cases).

Multiple stepwise regression analysis showed that temperature was most powerful in explaining the variation of bacterial abundance (55 %) and of bacterial productivity (38 %). Temperature, chlorophyll and POC together explained 58 % of the bacterial abundance variation (Table 4). Temperature and salinity together explained 41 % of the bacterial productivity variation. The variation of the number of particle-attached bacteria was explained best by salinity (44 %). Salinity and POC together explained 50 % of the particle-attached bacteria variation. Total bacterial number and particle-attached bacteria were negatively correlated with POC. Particle-

Table 4: Multiple stepwise regression analysis between total bacterial number (TBN), number of particle-attached bacteria (AB), bacterial biomass productivity (BBP) and physico-chemical variables. β: standardized coefficient of regression; p: level of significance of the slopes in the regression equations; POC: particulate organic carbon; CHLO: chlorophyll *a*, SAL: salinity; TEMP: temperature.

	Independent variables	Regression equation	Adjusted r^2
Log TBN	LogTEMP (β=0.619; p=0.000)	LogTBN=9.544+0.293TEMP+0.311	0.583
	LogCHLO (β=0.161; p=0.038)	LogCHLO-0.034LogPOC	
	LogPOC (β=-0.148; p=0.043)		
Log AB	LogPOC (β=-0.256; p=0.008)	LogAB=8.592-0.462LogPOC-	0.501
	SAL (β=-0.640; p=0.000)	0.537SAL	
Log BBP	TEMP (β=0.419; p=0.001)	LogBBP=-2.222+0.230TEMP-	0.410
	SAL (β=-0.273; p=0.028)	0.05SAL	

-attached bacteria were also negatively correlated with salinity. Total bacterial number was positively correlated with temperature and chlorophyll. Bacterial productivity was negatively and positively correlated with salinity and temperature, respectively.

DISCUSSION

The origin and degradability of organic matter in the Ria de Aveiro ecosystem are issues of great ecological and management interest. It has been reported that estuarine systems with enriched sediments and productive flooded margins may develop patterns of tidally induced transfer of organic matter and nutrients between their different compartments (Prah & Coble, 1994; Goosen *et al*., 1995; Hoppe *et al*., 1996; Hopkinson *et al*., 1998; Middelboe *et al*., 1998). In the case of the Ria de Aveiro, the extension of the bordering salt marshes and the considerable turbidity of the mid- and inner-sections of the estuary raised the need for considering the eventual relevance of sediment resuspension and runoff in the support of a brackish water bacterial community 3 times denser and 3.5 times more productive than the community at the outer estuary.

When looking for resuspension events, the comparison of deep and surface water at station N1 and transect I6 failed to show (in a total of 10 tidal cycles) any evidence for the expected effects. In fact, seston, POC and chlorophyll *a* concentrations were, in general, similar at the different levels of the water column. Bacterial density (total and particle-attached bacteria) and productivity were also rather constant along the vertical profiles in both zones (excluding the 0.5 m surface layer of the marine zone, a phenomenon which is explained by a different logic (Almeida *et al*., 2001a). This was observed even at spring tide when current speed and the area of the flooded margins increased. At the marine water, where tidal current speed can reach values of 2.4 m s^{-1} (Dias *et al*., 2000), higher than those required for resuspension in other estuarine systems (Oliveira *et al*., 1993; Lindsay *et al*., 1996), the nature of the sediments – coarse and medium sand – resisted resuspension. Given the sequence of tide induced vertical profiles of seston, the observed increase in seston and POC concentrations in deep water at low tide is interpreted as the result of particle deposition in slack water. At the brackish water transect I6, the 0.4 m s^{-1} current speed (Dias *et al.*, 2000) was insufficient to resuspend even the fine sand sediment or the organic matter that it contained – 2% (w/w) (Cunha *et al*., 1999).

When the transport of particulate matter through runoff was investigated at ebb tide in the neighbourhood of a salt marsh, no evidence could be found along the transect for inputs of seston or enrichment in POC or chlorophyll *a*. Although bacterial density followed this same pattern, bacterial productivity increased within the 1 m range from the interface with the salt marsh, denoting the presence of stimulation factors. Newell & Krambeck (1995) found also an increase in bacterioplankton productivity (2 fold) after tidal inundation of a cordgrass saltmarsh. The authors

interpreted these results as due to the increased availability on nutrient from cordgrass shoots and/or the marsh sediment.

Statistical analysis showed that POC was not a suitable variable for explaining bacterial growth variability. Temperature and chlorophyll were the variables that better explained bacterial abundance variation (55 % of the variation). Although POC was a variable that also explained bacterial density, the relation was negative and only increased the variance accounted by 2 %. Moreover, the variation in bacterial productivity was better explained by other variables than POC. For instance, salinity and temperature were responsible for 41% of the total variation. On the other hand, we had noticed (Cunha *et al.*, 2000) that the increase in POC was associated with the increase in ß-glucosidase activity but not with the rate of glucose incorporation. The complex, high molecular weight pool of POC fails to stimulate bacterial growth in a short range of time. The positive relationship between bacterial productivity and temperature suggests that bacterial activity might primarily be regulated by temperature, with substrate supply playing a lesser role as observed in other eutrophic and mesotrophic ecosystems (Berman et al., 1994; Griffith et al., Shiah and Ducklow, 1995).

Even though DOC distribution in the water column was not fully characterized, it appears to be the major form of organic carbon supporting bacterioplankton production in this shallow estuary as already observed in other aquatic systems (Hopkinson *et al*., 1998; Jorgensen *et al.*, 1999; Sanüdo-Wilhelmy & Taylor, 1999; Wikner *et al*., 1999). In fact, DOC concentration was 3 times higher than POC along the estuary. The data available at transect I6 indicates that DOC may be the only contribution of salt marsh runoff (2.3 times increase of DOC at the margin) to the high bacterial activity at the mid-estuary although it may have only fringe spatial effects. In fact, the rough estimate of the volume of runoff may not support a widespread impact of the ebb tide DOC input in the receiving stream. The brackish water sections of the lagoon sum up, depending on tidal phase, a volume of water of 51-126 x 10^6 m^3, 2 to 4 times greater than the volume calculated for the deeper outer estuary. The flooded margins cover a maximum area of 17 x 10^6 m^2 (Dias *et al.*, 2000). Considering that the flooding water is no more than 0.3 m deep, the maximum total volume of runoff is about 5 x 10^6 m^3 in each tidal cycle, amounting to a maximum of 10 % of the total brackish water volume at low tide. The doubling in DOC concentration near the margin of the transect does not give enough support to the average 3 and 3.5 times increase, respectively, in bacterial abundance and productivity in brackish water; but the DOC may have a positive effect on bacterial productivity out of proportion to its relative increase in concentration.

In view of these results and building on previous studies that revealed planktonic respiration to be 4 times greater than primary production (Almeida, MA & Cunha, MA, unpublished data), we conclude that most of the organic matter supporting bacterial activity in the Ria de Aveiro estuary must be allochthonous. Contributions from the sediment compartment or from flooded margins proved to be unnoticeable or localized, without any influence.

REFERENCES

Almeida MA, Cunha MA, Alcântara F (2001a) Factors influencing bacterial production in a shallow estuarine system. Microb Ecol (in press)

Almeida MA, Cunha MA, Alcântara F (2001b) Seasonal change in the proportion of bacterial and phytoplankton production along a salinity gradient in a shallow estuary. Hydrobiologia (in press)

Almeida MA, Cunha MA, Alcântara F (2001c) Physiological responses of marine and brackish water bacterial assemblages in a tidal estuary (Ria de Aveiro, Portugal). Aquat Microb Ecol 25:113-125

Barrosa JA (1985) Breve caracterização da Ria de Aveiro. Jornadas da Ria de Aveiro. Volume II. Recursos da Ria de Aveiro. Edição da Câmara Municipal de Aveiro

Berman T, Hoppe H, Gocke K (1994) Responses of aquatic bacterial production to substrate enrichment. Mar Ecol Prog Ser 104:173-184

Blanchard GF, Sauriau PG, Gall VC, Gouleau D, Garet MJ, Olivier F (1997) Kinetics of tidal resuspension of microbiota: testing the effects of sediment cohesiveness and bioturbation using flume experiments. Mar Ecol Prog Ser 151:17-25

Blight SP, Bentley TL, Lefevre D, Robinson C, Rodrigues R, Rowlands J, Williams P (1995) Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128: 61-75

Coffin RB, Sharp JH (1987) Microbial trophodynamics in the Delaware. Mar Ecol Prog Ser 41:253-266

Cunha MA, Almeida MA, Alcântara F (1999) Compartments of oxygen consumption in a tidal mesotrophic estuary (Ria de Aveiro, Portugal). Acta Oecologica. 20 (4):227-235

Cunha MA, Almeida MA, Alcântara F (2000) Patterns of variation of ectoenzimatic and heterotrophic bacterial activities along a salinity gradient in a shallow tidal estuarine. Mar Ecol Prog Ser 204:1-12

Cunha MA, Almeida MA, Alcântara F (2001) Short-time responses of the natural planktonic bacterial community of the changing water properties in an estuarine environment: ectoenzimatic activity, glucose incorporation and biomass production. Microb Ecol (in press)

Dias JM, Lopes JF, Dekeyser I (2000) Tidal propagation in the Ria de Aveiro lagoon, Portugal. Phy Chem Earth 25:369-374

Findlay S, Pace ML, Lints D, Howe K (1992) Bacterial metabolism of organic carbon in the tidal freshwater Hudson Estuary. Mar Ecol Prog Ser 89:147-153

Goosen NK, Rijswijk P, Brockmann U (1995) Comparison of heterotrophic bacterial production rates in early spring in the turbid estuaries of the Scheldt and the Elbe. Hydrobiologia 311:31-42

Goosen NK, van Rijswijk P, Kromkamp J, Peene J (1997) Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat Microb Ecol 12:223-232

Griffith P, Shiah F, Gloersen K, Ducklow H, Fletcher M (1994) Activity and distribution of attached bacteria in Chesapeake Bay. Mar Ecol Prog Ser 108:1-10

Henriksen K, Rasmussen M, Jensen A (1983) Effects of bioturbation on microbial nitrogen transformation in the sediment and fluxes of amonium and nitrate to the overlyng water. Ecol Bull 35:193-205

Hobbie JE, Daley R, Jasper S (1977) Use of Nuclepore Filters for Counting Bacteria by Fluorescence Microscopy. Appl Environ Microbiol 33:1225-1228

Hopkinson C, Giblin AE, Garritt RH, Tucker J, Hullar MA (1998) Influence of benthos on growth of planktonic estuarine bacteria. Aquat Microb Ecol 16:109-118

Hoppe HG, Gocke K, Alcântara F (1996) Shifts between autotrophic and heterotrophic processes in a tidal lagoon (Ria de Aveiro, Portugal). Arch Hydrobiol Spec Issues Advanc Limnol 48:39-52

Jorgensen NO, Kroer N, Coffin RB, Hoch MP (1999) Relations between bacterial nitrogen metabolism and growth efficiency in an estuarine and in an open-water ecosystem. Aquat Microb Ecol 18:247-261

Lindsay P, Ball PW, West JR (1996) Influence of tidal range and river discharge on suspended particulate matter fluxes in the Forth Estuary (Scotland). Est Coast Shelf Sci 42:63-82

Medeiros C, Kjerfve B (1993) Hydrology of a tropical estuarine system: Itamaracá, Brazil. Est Coast Shelf Sci 36:495-515

Mehta AJ (1988) Laboratory studies on cohesive sediment deposition and erosion. *In*: Dronkers J, van Leussen V (eds.) Physical processes in estuaries. Spring-Verlag, Berlin, p 427-445

Middelboe M, Kroer N, Jorsensen NO, Pakulski D (1998) Influence of sediment on pelagic carbon and nitrogen turnover in a shallow Danish estuary. Aquat Microb Ecol 14:81-90

Newell SY, Krambeck C (1995) Responses of bacterioplankton to tidal inundations of a saltmarsh in a flume and adjacent mussel enclosures. J Exp Mar Biol Ecol 190:79-95

Nowell AR, Jumars PA (1984) Flow environments of aquatic benthos. Ann Rev Ecol Syst 15:303-328

Oliveira AM, Kjerve B (1993) Environmental responses of a tropical coastal lagoon system to hydrological variability: Mundaú-Manguaba, Brazil. Estuar Coast Shelf Sci 37:575-591

Page HM, Petty RL, Maede DE (1995) Influence of watershed runoff on nutrient dynamics in a Southern California salt marsh. Est Coast Shelf Sci 41:163-180

Parsons TR, Maita Y, Lalli CM (1989) A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, Oxford

Prah FG, Coble, PG (1994) Input and behaviour of dissolved organic carbon in the Columbia River Estuary. *In*: Dyer K, Orth R (eds.) Changing particle fluxes in estuaries: implications from science to management. ECSA22/ERF Symposium. Olsen & Olsen Press, Fredensborg, p 451-457

Rhoads DC, Boyer LF (1982) The effect of marine benthos on physical properties of sediments: a successional perspective. *In*: McCall PL, Tevesz MJS (eds.) Animal-sediment relations: the biogenic alteration of sediments. Plenum Press, New York, p 3-52

Rodier J (1996) L`analyse de l`eau: eaux naturelles, eaux résiduaires, eau de mer. 8 Edition, Dunod, Paris.

Sanudo-Wihhelmy AS, Taylor, GT (1999) Bacterioplankton dynamics and organic carbon partitioning in the lower Hudson River estuary. Mar Ecol Prog Ser 182:17-27

Shiah FK, Ducklow HW (1995) Multiscale variability in bacterioplankton abundance, production, and specific growth rate in a temperate salt-marsh tidal creek. Limnol Oceanogr 40(1):55-56

Silva JJ (1994) Circulação de Água na Ria de Aveiro: Contribuição para o Estudo da Qualidade da Água. Ph.D. Thesis. Universidade de Aveiro

Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic bacteria. Mar Ecol Prog Ser 51:201-213

Vallespinos F, Mallo S (1990) Bacteria as transformers of the arena of play of other organisms. Oecologia 10:215-222

Wikner J, Cuadros R, Jansson M (1999) Differences in consumption of allochthonous DOC under limnic and estuarine conditions. Aquat Microb Ecol 17:289-299

Yentsch CS, Menzel DW (1963) A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res 10:221-231

CHAPTER 7

DISCUSSION

Bacterial abundance (2.6-15.3 x 10^9 cells l^{-1}) and productivity values (0.05-18.6 µg C $l^{-1}h^{-1}$) in the estuary Ria de Aveiro are within the range obtained in other Portuguese estuaries (1.2 to 50.0 x 10^9 cells l^{-1} and 1.19 to 4.83 6 µg C l^{-1} h^{-1}, respectively) (Barbosa, 1991; Bordalo *et al.*, 1993; Caeiro, 1996; Gocke, personal communication) and agree with those determined in other coastal waters (Fucks *et al.*, 1994; Gossen *et al.*, 1997; Bouvy *et al.*, 1998; Hoppe *et al.*, 1998). The number of active bacteria (0.1-3.7 x 10^9 cells l^{-1}) falls also in the same order of magnitude registered for other coastal waters (Lovejoy *et al.*, 1996; Tuomi, 1997; Kisand and Noges, 1998; Kornas *et al.*, 1998; Smith, 1998; Vosjan and van Noort, 1998). The authors have no knowledge of any other study quantifying the total active bacterial number in Portuguese estuaries and its proportion within the total community.

In the Ria de Aveiro, as already observed in other systems (del Giorgio and Scarborough, 1995; Ullrich *et al.*, 1996; Choi *et al.*, 1999; Sherr *et al.*, 1999), a large fraction of the total cell count consists of metabolically inactive bacteria that do not participate in growth. Most of the bacteria (about 70 %) were apparently inactive. Although it has been hypothesized that the fraction of active cells seems to increase in parallel with the productivity of the system (del Giorgio *et al.*, 1995), in the Ria de Aveiro, where bacterial production increases by a factor of 3.5 from the marine to the brackish water zones, the proportion of metabolically active bacteria in the two zones was similar. It has been shown, however, that temperature, salinity, predation and viral infection also play a role in determining the relative size of the active and inactive fractions of bacterioplankton in aquatic systems (Proctor and Fuhrman, 1990; Shiah and Ducklow, 1994; Rigsbee *et al.*, 1996). Moreover, the fraction of inactive bacteria did not respond to nutrient amendment within the period of time of 3 hours. When different substrates were added to the bacterial communities of the marine and of the brackish water zones, the proportion of active bacteria was not different from the controls. The bacterial responses were similar when simple inorganic (e.g. phosphate) or organic nutrients (e.g. glucose, amino acids, vitamins) were added, or when a complex yeast extract base or even phytoplankton exudates (from diatoms of Ria de Aveiro or from a commercial formula) were used. The fraction of active bacteria was similar after 10 minutes, 1, 2 or 3 hours of stimulation attempts. It was concluded that the large fraction of inactive bacteria may correspond essentially to the presence of dead cells. However, these results, confronted with the positive reactions obtained with natural water in diffusion chambers, may indicated that a combination of factors may be required to induce bacterial activation. On the other hand, the negative results of the stimulation attempts could, however, be related with the high toxicity of the CTC (Ullrich *et al.*, 1996; Karner and Fuhrman, 1997), which would impair the detection of the awakening of cell activity. We may also think that the activation response of bacterial cells may be a slow process that can only be

revealed by CTC-reduction after longer exposures. In fact, Choi *et al*. (1999) demonstrated that substantial fractions (30 to 85%) of inactive marine bacterial cells, in what concerns the electron transport system, could become active within 1 to 2 days after addition of organic matter and/or increase in water temperature.

In Ria de Aveiro, in the Douro Estuary (Bordalo *et al.*, 1998) and in some non-portuguese estuaries (Wright and Coffin, 1983; Kornas *et al.*, 1998) the peaks of bacterial abundance and productivity were observed in the mid-section of the estuary. In the Ria de Aveiro, these peaks were reported to the absolute size of the active bacteria population and to the intensity of their activity rather than to the relative size of the active fraction. Total and active bacterial numbers increased between the outer and the mid-section by the same factor (3 times) and, consequently, the fraction of active bacteria remained similar in both sections (26.6 %). The factor of increase of bacterial productivity was higher (3.5 times, on average). Specific productivity per cell was similar throughout the estuary but specific productivity per active cell was 19 % higher in the mid-section.

In the brackish water zone, bacterial density and productivity were constant down the water column, as expected for shallow estuarine systems (Ducklow and Shiah, 1993; Fuks *et al.*, 1994; Jellet *et al.*, 1996; Talbot *et al.*, 1997). In the marine zone, with the average water depth of 7 m, bacterial productivity was significantly higher at surface. Moreover, the stratification of bacterial productivity in the marine zone occurred in the absence of significant physical or chemical variation down the water column. Although a first approach to the explanation of this fact suggested that the increased values at surface water could be associated with phyto- and bacterioplankton transport in ebb water, other biological parameters (i.e. total and active bacterial numbers and chlorophyll concentration) were relatively constant along the water column. Moreover, turnover rate of glucose and ectoenzimatic activity, determined in the same samples of the marine water, were also constant down the water column (Cunha MA, unpublished data). Even though, phytoplankton distribution could not directly explain the higher bacterial productivity in the half-meter layer of surface water, it was found that irradiation may enhance phytoplankton exudation (Zlotnik and Dubinsky, 1989; Feuillade *et al.*, 1990) and consequently bacterial productivity in this water layer. In addition, the higher bacterial productivity at surface water could be stimulated by photochemical transformation of recalcitrant dissolved organic matter into labile compounds that may stimulate locally and transiently the growth of active bacteria. Lindell *et al.* (1995) suggest that solar irradiance (UV-B radiation) may cleave recalcitrant high molecular weight dissolved organic matter into smaller fractions, and thereby facilitate bacterial utilisation with the result of an increased bacterial biomass production. Fuks *et al.* (1994), in the estuary of the Krka River, also found enhanced productivity at the surface layer of the water column, but only at one sampling station, a fact that was interpreted as the result of organic matter transformation. It is

well known, however, that the exposure to solar irradiance can influence negatively the production and metabolism of heterotrophic bacteria by photoinhibition (Pakulski *et al.*, 1998; Pauz and Hernd, 1999). The results of Herndl *et al.* (1993) suggest that bacteria do not adapt to UV-radiation as reported for phytoplankton (Helbling *et al.*, 1992). These authors found a reduced activity (as much as 40 % reduction) in bacteria after exposure to UV-B, and suggested that this was attributed to photolysis of extracelular enzymes. The photochemical transformation of recalcitrant organic matter in aquatic systems may then compensate for the loss of enzymatic cleavage of dissolved organic matter. In fact, in the Ria de Aveiro the vertical variation of bacterial productivity in the marine zone (possibly due to photochemical transformation and/or enhanced phytoplankton exudation) was of the same magnitude as the longitudinal variation between the brackish and the marine water.

In the brackish water zone the much higher turbidity that reduced light penetration and, consequently photodegradation and/or phytoplankton exudation, may preclude the increase of productivity in surface bacterioplankton. On the other hand, diffusion from the close sediment may increase bacterial productivity in deeper water and consequently, obscure the increase induced in the surface water layer by photodegradation of recalcitrant organic matter and/or phytoplankton exudation.

Short-term variation of bacterial production associated with tidal currents was observed in both zones of the estuary but in brackish water the fluctuation was not, as evident as in the marine zone. The fluctuation in bacterial productivity during the tidal cycle resulted not only from the fluctuation in total bacterial number (and active bacterial number), but also from the rapid response of active cell productivity to the changing water conditions. The variation associated with the tidal cycle was not, then, the result of simple mixing and dilution during tidal transport. The exposure of marine and brackish water communities to extremely contrasting conditions (assays in diffusion chambers) showed that the marine and the brackish water communities responded quickly and differently to tidal fluctuation of environmental factors. A decrease was observed in the fraction size of active bacteria and in bacterial productivity of the brackish water community incubated in marine water. Contrastingly, an increase in the fraction of active bacteria and in bacterial productivity of the marine community was observed when it was incubated in brackish water. The positive response of marine bacteria to the brackish water conditions was even higher when the salinity was artificially brought to 34 psu. Brackish water bacteria, however, responded negatively to the increase in salinity. Moreover, in the reversed experiment the marine community response showed to be reversible (bacteria rapidly re-acquired their previous characteristics) but the response of the brackish water community was irreversible (bacteria did not recover from the negative effect of exposure to marine water). These results suggested the existence of distinct bacterial communities adapted to the environmental conditions that prevail at the two zones of the estuary.

The short-term reactivity of the two communities to changes in salinity and in nutrient content of the water column is compatible with the hypothesis of activation and inactivation of bacteria during tidal transport, and has implications in the interpretation of estuarine profiles of bacterial activity.

The seasonal effects of bacterial abundance and production in the estuary were quite clear, with higher values in the warm season, and agree with other observations in other temperate systems (Heinanen and Kuparinen, 1991; Di Siervi *et al.*, 1995; Yanada *et al.*, 2000). The different climatic conditions were also reflected in the bacterioplankton cell-specific growth rate and in the ratio between bacterio- and phytoplankton production. The specific growth rate increased during the warm season, as well as the specific growth rate of the phytoplankton, but the ratio of bacterio- to phytoplankton production decreased significantly. The seasonal change in this ratio indicated that bacterial production might be essentially supported by phytoplankton carbon during the warm season (21 % of primary production could be potentially sufficient to support the whole bacterial production) but, in the cold season, the total primary production would be either required or even insufficient to support bacterial production. In this season the estuary turns into a mostly heterotrophic system.

Among the set of variables studied (temperature, salinity, dissolved oxygen, seston, POC, Chlorophyll *a*), temperature was the factor that most influenced bacterial abundance in the estuary. Bacterial productivity was mostly dependent on temperature and salinity. In the marine zone, depth was also an important factor of bacterial productivity variation as already mentioned. The positive relation of bacterial abundance and productivity with temperature and the negative relation with salinity and depth help to explain the higher values found at low tide and during the warm season. In the brackish water the fraction of the total variation in bacterial density and productivity explained by this set of variables was, however, much smaller than the determined value for marine water. These results suggest the relevance of other variables, namely the size of the supply of authchonous and allochthonous organic matter and the presence of biological control.

In the Ria de Aveiro the availability of the substrates was one of the main factors of bacterioplankton variability. The strength of the bottom-up control by substrate supply varied within the system, as found in other systems (Shiah and Ducklow, 1995; Shiah *et al.*, 1999). In the brackish water zone, primary production and the estimated allochthonous inputs of organic matter were greater than in the marine zone. Nevertheless, when yeast extract was added to brackish water, the bacterial community responded intensively and quickly. This suggests that the lability of the available organic matter was low and, consequently, that the incorporation of this organic matter by bacteria was dependent of lengthy enzymatic cleavage. In the marine zone the addition of yeast extract also increased bacterial growth after 12 hours of treatment. Bacterial density reached values similar to those obtained in brackish water when added of yeast extract. The marine

bacterial community also showed a positive response *in situ*, increasing significantly the productivity at the surface possibly due to enhanced availability of labile organic matter after stimulation of phytoplankton exudation and/or photochemical transformation of recalcitrant organic matter. On the other hand, the experimental analysis in diffusion chambers showed that the marine bacterial community when exposed to brackish water reacted with enhanced growth. This positive response was shown to be due to improved throphic conditions and not to the decrease in salinity. It was noticed that the marine bacteria response was stronger when the salinity of the envolving brackish water was increased from 18 psu to 34 psu. In estuarine systems salinity gradients are superimposed on throphic gradients making difficult to distinguish the different sets of effects on the communities.

In Ria de Aveiro, allochthonous organic matter seems to amount to an important source of organic carbon. Its contribution to bacterial production was greater in the brackish water zone of the estuary. This confirms the results of others authors who found that in the mid- and inner-estuary the coupling between bacterial production and primary production was lower than in the outer estuary (Shiah and Ducklow, 1995). As mentioned above, during the cold season primary production could not support bacterial production. As a matter of fact, the bacterial production/primary production ratio varied from 1.0 to 3.6, indicating that other carbon sources were very representative in the estuary. In the warm season, although an average of 21 % of primary production could be potentially sufficient to support the whole bacterial production, in the brackish water zone the estimated allochthonous carbon supply could be responsible for a significant fraction (41 %) of bacterial biomass growth. The geographical and seasonal variation in the production values showed that, on an annual basis and on a per water volume unit, the balance of secondary to primary carbon production was different in the two zones of the estuary. The marine zone was a predominantly autotrophic water mass and the brackish water zone was almost at balance in the auto- and heterotrophic growth. Other studies revealed planktonic respiration to be four times greater than primary production in these estuary (Almeida MA and Cunha MA, unpublished data), a finding that supports the conclusion that most of the organic matter supporting bacterial activity in this estuary is allochthonous.

Particulate organic matter is frequently referred as an important source of organic matter in estuarine systems (Murrel *et al.*, 1999). In Ria de Aveiro, however, when the transport of particulate allochthonous matter through runoff was investigated in the neighborhood of a salt marsh in the brackish water zone, no evidence could be found along the transect for inputs of seston or enrichment in POC or chlorophyll. The concentration of DOC was, however, 2.3 times higher at the margin. These results suggest that DOC may be the only contribution of salt marsh runoff for bacterial growth in the water mass. In fact, bacterial productivity increased within the 1 m range from the interface with the salt marsh denoting the presence of stimulation factors. However, the rough estimate of the volume of runoff provided by the total flooded area in relation

to the total volume of the brackish water may not withstand a widespread impact of the ebb tide DOC inputs in the receiving stream. The maximum total volume of runoff amounts to only 10 % of the total brackish water volume at low tide. Consequently, the input of organic matter by runoff could not possibly support the average 3 and 3.5 increase, respectively, in bacterial density and productivity observed in the brackish water zone. The input of allochthonous organic matter to the estuary derives mainly from diffuse domestic and industrial discharges (Silva, personal communication). In fact, along the Canal de Ílhavo there is a considerable anthropogenic impact associated with harbour activities in the outer- and mid-sections, aquaculture pounds, industries, domestic sewage discharges and small agriculture explorations in the mid- and inner sections of the estuary.

The contribution of organic matter from the sediment compartment through resuspension proved to be unnoticeable in the marine and in the brackish water zones of the estuary. At the marine zone, where tidal current speed can reach values of 2.4 m s^{-1} (Dias *et al.*, 2000), higher than those required for resuspension in other estuarine systems (Oliveira *et al.*, 1993; Lindsay *et al.*, 1996), the nature of the sediments (coarse and medium sand) resisted resuspension. At the brackish water zone, the 0.4 m s^{-1} current speed (Dias *et al.*, 2000) was insufficient to resuspend even the fine sediments.

Although the export of particulate materials from bottom sediments was not detected in the estuary, in the brackish water zone diffusion from sediments may increase the pool of organic matter and nutrients in the water column. Not even in the brackish water zone, where organic matter content of the sediments was higher (2.9 % of the dry weigh) than in the marine zone (0.5 % of the dry weigh) and the water column depth was low (1.3 m, on average), could be detected stratification of bacterial productivity as evidence of enrichment at the bottom. If, however, bacterial productivity is strongly stimulated at the surface (as in the marine zone) then the lack of stratification had to be interpreted as evidence for significant sediment contribution to bacterial production. Experimental approaches to this subjected will be required.

Even though DOC distribution in the water column of the estuary was not fully characterised, it appears to be the major organic carbon form regulating bacterial production in this shallow estuary, as already observed in other aquatic systems (Hopkinson *et al.*, 1998; Jorgensen *et al.*, 1999; Sanudo-Wilhehmy and Taylor, 1999; Wikner *et al.*, 1999). In fact, DOC was 3 times as abundant than POC along the estuary and its concentration was 3 times greater in brackish water than in the marine zone. It is also thought that it was the only major carbon contribution of salt marsh runoff into the estuary. The negative association of POC with bacterial abundance and productivity suggests its considerable recalcitrance in this estuary. The complex high molecular weight pool of POC fails to stimulate bacterial growth in a short period of time. The low fraction (19 %) of particle-attached bacteria in the Ria de Aveiro confirms the small relevance of particulate matter as a source of bacterioplankton carbon. Previous determinations (Almeida and Alcântara,

1992) showed even a lower fraction (9 %) of bacteria attached to particles in this estuary and that, curiously, the smaller particles showed to be more densely colonized.

In Ria de Aveiro viral infection may greatly control bacterioplankton density and productivity. The high density of the viral community (2.4-25.0 x 10^{10} particles l^{-1}) in the water column of the estuary (18 times higher than baterioplankton, on average) suggests a strong overall effect on the planktonic system and, eventually, on bacterial density and activity. It was found that the majority of the viruses were in the bacteriophage size-range and the patterns of geographical, tidal and vertical variation of virioplankton followed bacterial abundance. The experimental data of microcosm dilution experiments showed that the average bacterial loss by maximal viral infection (in the conditions of the experiment) was much higher in the brackish water zone (61 %) than in the marine zone (36 %). The contribution of maximal viral infection to bacterial mortality in the estuary (average 50 %, overall range 30-74 %) was higher or slightly higher than published values (Steward *et al.*, 1992; Bratback *et al.*, 1993; Peduzzi and Weinbauer, 1993; Weinbauer and Peduzzi, 1995a; Fuhrman and Noble, 1995). The effect of viral lysis on bacterial communities with enhanced growth (after yeast extract addition) was masked even when the initial ratio of viruses to bacteria was ten fold greater than in the natural samples. The higher density of the virioplankton did not repress the large and rapid increase in bacterial density. This unexpected result can be attributed to the very rapid growth of non-infected bacteria. In this case, marine viruses would be, essentially, selective agents acting on short term temporal fluctuations in the composition of bacterial communities but not so much in intensely growing bacterial community.

The contribution of predation to bacterial mortality fits within the range reported in the literature (10-80 %) for coastal systems (Fuhrman and Noble, 1995; Gonzalez, 1999; Posch *et al.*, 1999) and, contrary to viral infection, was similar in marine (69 %) and brackish (73 %) waters. In brackish water, the effects of viral lysis and predation to bacterial mortality were similar but in the marine zone predation almost doubled the loss by viral infection. The high level of viral lysis in brackish water, where the throphic conditions are richer and where it could be expected to find an increased percentual value of active bacteria, may keep under control the size of this fraction, which, in fact, is similar all over the estuary.

Physical, chemical, nutritional and biological pressures are exerted on the bacterioplankton and it shows to be in different balance in the marine and brackish water zones of the Ria de Aveiro.

COMPLEMENTARY WORK IN THE IMMEDIATE FUTURE

Summing up all the results of this thesis one can see that important aspects of the regulation of bacterial activity in this estuary are still not yet fully understood and some remarks can be made towards further work.

Dissolved organic matter seems to play, as expected, a major role in the regulation of bacterioplankton activity in the Ria. It would be interesting to follow the spatial and seasonal profiles of dissolved organic matter in order to evaluate the relative size of the different sources along the estuary. The major composition of particulate and dissolved carbon could be analysed against the expression of ectoenzimatic activity as well as against the strength of the exchanges at the interfaces. The increased bacterial productivity found at the surface of the marine zone, mainly at ebb tide, suggests a degradation process that may gain further relevance if continued to the nearshore. On the other hand, some doubts still persist about the occurrence of a similar process in the shallow brackish water zone of the estuary. This would turn insulation a major factor of bacterial productivity.

Laboratory experiments demonstrated that when the viruses-to-bacteria ratio was artificially increased, the virioplankton could play a major role in the regulation of bacterial density. In order to draw a realistic picture of the contribution of viral infection to bacterial mortality in the estuary it will be necessary to find a way to circumvent the difficulty of separating loss due to viral infection from obscuring bacterial growth in experimental cosmos.

The identification of the bacteriophage fraction in the total virioplankton would bring preliminary and useful information on the control of infection. Up to now, it has not been reported any attempt to distinguish bacteriophages in the whole of the marine virioplankton.

The marine and brackish water bacterial communities in this estuary responded differently and within short-time intervals to shifts in environmental factors. The distinct response of the two communities can be interpreted as resulting from intrinsic differences in the composition of the bacterial assemblages and/or as deriving mostly from similar bacterial assemblages responding to changing environmental factors. Cross-hybridization of genomic DNA of the two bacterial communities may provide relevant information to the clarification of this issue. Since viruses are host specific, cross-hybridization of DNA of the virioplankton present in the two communities would further help to distinguish the alternative hypotheses.

For a better assessment of the trophic conditions prevailing in Ria de Aveiro and in order to integrate the processes occurring in the estuary, the construction of a numerical model of the system would be decisively important. The distinct estuarine interfaces, namely with the ocean, the freshwater and the bottom sediments, are certainly areas of enhanced instability and, consequently, of different bacterial activity, deserving further analysis. In this analysis are included, among others, the bacterioplankton dynamics, namely in terms of bacterial productivity, size of the active bacterial fraction and viral activity.

In 2000, an interdisciplinary study was launched in order to construct the first model of the Ria de Aveiro. The project "Water quality modeling of the Ria de Aveiro lagoon (MODELRIA)", coordinated by the University of Aveiro, counts with the participation of the Instituto Superior Técnico and Hidromod. The validation of the model will take also into account results of previous work developed by the University of Aveiro, including the contribution of the microbial compartment, an important component of the aquatic food web.

REFERENCES

Abreu S, Duarte A (1997) Biogest First Scientific Report - Environment & Climate Programme. Contract Nº ENV4-CT96-0213 (partner 6 participation)

Acherman HW, BuBow MS (1987) Viruses of prokaryotes: general properties of bacteriophages. CRC Press Inc., Boca Raton, Florida

Acinas SG, Antón J, Rodríguez-Valera F (1999) Growth rate paradox of *Salmonella typhimurium* within host macrophages. J Bacteriol 175:3744-3748

Alcântara F, Almeida A, Cunha A (1994) Qualidade Microbiológica da Água da Ria de Aveiro (1993). Relatório do Projecto Polaveiro, Universidade de Aveiro

Alcântara F, Cunha A, Ferreira C (1996) Matéria orgânica e actividade enzimática extracelular em sedimentos estuarinos. *In:* Proceedings of the 5th National Conference on the quality of the environment, 10-12 April, Aveiro, Portugal, Vol 2, p 1465-1474

Almeida MA, Alcântara F (1992) Bacterial colonization of seston particles in brackish waters (Ria de Aveiro, Portugal). Mar Ecol Prog Ser 89:165-173

Almeida MA, Cunha MA, Alcântara F (2001a) Factors influencing bacterial production in a shallow estuarine system. Microb Ecol (in press)

Almeida MA, Cunha MA, Alcântara F (2001b) Seasonal change in the proportion of bacterial and phytoplankton production along a salinity gradient in a shallow estuary. Hydrobiologia (in press)

Almeida MA, Cunha MA, Alcântara F (2001c) Physiological responses of marine and brackish water bacterial assemblages in a tidal estuary (Ria de Aveiro, Portugal). Aquat Microb Ecol 25:113-125

Amon RM, Brenner R (1998) Seasonal pattern of bacterial abundance and production in the Mississippi River plume and their importance for the fate of enhanced primary production. Microb Ecol 35:289-300

Anderson A, Larsson U, Hagstrom A (1986) Size-selective grazing by a microflagellate on pelagic bacteria. Mar Ecol Prog Ser 33:51-57

Anderson TR, Williams PJ (1998) Modelling the seasonal cycle of dissolved organic carbon at Station E1 in the English Channel. Coastal Shelf Sci 46:93-109

Azam F, Hodson RE (1977) Size distribution and activity of marine microheterotrophs. Limnol Oceanogr 22:492-501

Azam F (1998) Microbial control of oceanic carbon flux: the plot thickens. Science 280:694-696

Bano N, Moran MA, Hodson RE (1997) Bacterial utilization of dissolved humic substances from a freshwater swamp. Aquat Microb Ecol 12:233-238

Bano N, Moran MA, Hodson RE (1998) Photochemical formation of labile organic matter from two components of dissolved organic carbon in a freshwater wetland. Aquat Microb Ecol 16:95-102

Barbosa A (1991) Spatial and temporal variation of bacterioplankton abundance and biomass in a coastal lagoon (Ria Formosa, Southeastern Portugal). Kieler Meeresforsch 8:66-73

Barrosa JA (1985) Breve caracterização da Ria de Aveiro. Jornadas da Ria de Aveiro, Volume II, Recursos da Ria de Aveiro, Edição da Câmara Municipal de Aveiro

Bell RT, Kuparinen J (1984) Assessing phytoplankton and bacterioplankton production during early spring in Lake Erken, Sweden. Appl Environ Microbiol 48:1221-1230

Berger B, Hoch B Kavka G, Herndl GJ (1995) Bacterial metabolism in the River Danube: parameters influencing bacterial production. Freshwater Biol 34:601-616

Berman T, Holm-Hansen, O (1974) Release of photoassimilated carbon as dissolved organic matter by marine phytoplankton. Marine Biology 28:305-310

Berman T, Hoppe H, Gocke K (1994) Responses of aquatic bacterial production to substrate enrichment. Mar Ecol Prog Ser 104:173-184

Berman T, Kaplan B, Chava S, Viner Y, Sherr BF, Sherr EB (2001) Metabolically active bacteria in Lake Kinneret. Aquat Microb Ecol 23:213-224

Bianchi A, Giuliano L (1996) Enumeration of viable bacteria in the marine pelagic environment. Appl Environ Microbiol 62:174-177

Bidle KD, Fletcher M (1995) Comparison of free-living and particle associated bacterial communities in the Chesapeake Bay by stable low-molecular-weight RNA analysis. Appl Environ Microbiol 61:944-952

Bie MJ, Speksnijder AG, Kowalchuk GA, Schuurman T, Zwart G, Stephen JR, Diekmann OE, Laanbroek H (2001) Shifts in the dominant populations of ammonia-oxidizing ß-subclass Proteobacteria along the eutrophic Schelde estuary. Aquat Microb Ecol 23:225-236

Bird DF, Kalff J (1993) Protozoan grazing and size-activity structure in limnetic bacterial communities. Can J Fish Aquat 50:370-380

Bjornsen PK (1986) Automatic determination of bacterioplankton biomass by image analysis. Appl Environ Microbiol 51:1199-1204.

Blackburn N, Ulla LZ, Hagstrom A (1996) Cycling of marine dissolved organic matter. II. A model analysis. Aquat Microb Ecol 11:79-90

Blackburn N, Fenchel T, Mitchel J (1998) Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science 282:2254-2256

Blanchard GF, Sauriau PG, Gall VC, Gouleau D, Garet MJ, Olivier F (1997) Kinetics of tidal resuspension of microbiota: testing the effects of sediment cohesiveness and bioturbation using flume experiments. Mar Ecol Prog Ser 151:17-25

Blight SP, Bentley TL, Lefevre D, Robinson C, Rodrigues R, Rowlands J, Williams P (1995) Phasing autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128: 61-75

Bloem J, Ellenbroek FM, Bar-Gilissen M, Cappenberg T (1989) Protozoan grazing and bacterial production in stratified lake Vechten estimated with fluorescently labelled bacteria and by thymidine incorporation. Appl Environ Microbiol 55:1787-1795

Bode A, Barquero S, Varela M, Braun J, Armas D (2001) Pelagic bacteria and phytoplankton in oceanic waters near the Canary Island in summer. Mar Ecol Prog Ser 209:1-17

Boehme J, Frisches M, Jiang S, Kellog C, Pichard S, Rose J, Steinway C, Paul J (1993) Viruses, bacteria and phytoplankton in the Southwestern Gulf of Mexico: distribution and contribution to oceanic DNA pools. Mar Ecol Prog Ser 97:1-10

Bordalo AA, Caeiro CC (1993) Plancton microbiano do estuário do Rio Douro: Produção primária e biomassa. Bol UCA, vol 1, p 296-313

Bordalo AA, Pinto MM, Carvalho LM (1998) Dinâmica sazonal e espacial de variáveis abióticas e bióticas no estuário do Rio Douro. Actas do 1º Simpósio Interdisciplinar sobre processos estuarinos, Universidade do Algarve, Faro, p 47-48

Borsheim KY (2000) Bacterial production rates and concentrations of organic carbon at the end of the growing season in the Greeland Sea. Aquat Microb Ecol 21:115-123

Bouvy M, Arfi R, Cecchi P, Corbin D, Pagano M, Saint-Jean L, Thomas S (1998) Trophic coupling between bacterial and phytoplanktonic compartments in shallow tropical reservoirs (Ivory Coast, West Africa). Aquat Microb Ecol 15:25-37

Bratback G, Haslund O, Heldal M, Noess A, Roeggen T (1992) Giant marine viruses? Mar Ecol Prog Ser 85:201-202

Bratbak G, Heldal M (1993) Total counts of viruses in aquatic environments. *In*: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds.) Handbook of Methods in Aquatic Microbial Ecology, Lewis Publishers, New York, p 135-138

Bratback G, Egge JK, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and termination of algal blooms. Mar Ecol Prog Ser 93:39-48

Bratbak G, Jacobsen A, Heldal M (1998) Viral lysis of *Phaeocystis pouchetii* and bacterial secondary production. Aquat Microb Ecol 16:11-16

Bushaw-Newton Kl, Moran MA (1999) Photochemical formation of biologically available nitrogen from dissolved humic substances in coastal marine systems. Aquat Microb Ecol 18:285-292

Bussmann I (1999) Bacterial utilization of humic substances from the Arctic Ocean. Aquat Microb Ecol 19:37-45

Button DK, Robertson BR (1993) Use of high-resolution flow cytometry to determine the activity and distribution of aquatic bacteria. *In*: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds.), Handbook of Methods in Aquatic Microbial Ecology, Lewis Publishers, New York, p 163-173

Button DK, Robertson BR, Juttner F (1996) Microflora of a subalpine lake: bacterial populations, size and DNA distribution, and their dependence on phosphate. FEMS Microb Ecol 21:87-101

Caeiro C (1996) Actividade depuradora bacteriana na interface água/sedimento em ambientes sedimentares estuarinos com diferentes cargas orgânicas. M. Sc. Thesis, Universidade de Aveiro

Campbell R (1983) Microbial ecology. Blackwell Scientific Publications, Oxford

Carlson P, Granéli E, Tester P, Boni L (1995) Influences of riverine humic substances on bacteria, protozoa, phytoplankton, and copepods in a coastal plankton community. Mar Ecol Prog Ser 127:213-221

Carlson CA, Ducklow HW (1996) Growth of bacterioplankton and consumption of dissolved organic carbon in the Sargasso Sea. Aquat Microb Ecol 10:69-85.

Carman K (1993) Microautoradiographic detection of Microbial Activity. *In*: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds.), Handbook of Methods in Aquatic Microbial Ecology, Lewis Publishers, New York, p 397-404

Chin-Leo G, Benner R (1992) Enhanced bacterioplankton production and respiration at intermediate salinities in the Mississippi River plume. Mar Ecol Prog Ser 87:87-103

Cho BC, Azam F (1988) Major role of bacteria in biogeochemical fluxes in the ocean`s interior. Nature 332:441-443

Cho BC, Azam F (1990) Biogeochemical significance of bacterial biomass in the ocean`s euphotic zone. Mar Ecol Prog Ser 63:253-259

Choi JW, Sherr BF, Sherr EB (1999) Dead or alive? A large fraction of ETS-inactive marine bacterioplankton cells, as assessed by reduction of CTC, can become ETS-active with incubation and substrate addition. Aquatic Microb Ecol 18:105-115

Cochlan W, Wikner J, Stewart G, Smith D, Azam F (1993) Spatial distribution of viruses, bacteria and chlorophyll a in neritic, oceanic and estuarine environments. Mar Ecol Prog Ser 92:77-87

Coffin RB, Sharp JH (1987) Microbial trophodynamics in the Delaware. Mar Ecol Prog Ser 41:253-266

Cole JJ, Findlay S, Pace ML (1988) Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Mar Ecol Prog Ser 43:1-10

Cole JJ, Caraco NF (1993) The pelagic food web of oligotrophic lakes. *In*: Ford TE (ed.), Aquatic microbiology, An ecological approach. Blackwell Scientific Publication, Boston, p 101-111

Cole JJ, Pace Ml (1995) Why measure bacterial production? A reply to the comment by Jahnke & Craven. Limnol Oceanogr 40:441-444

Conan P, Turley C, Stuff E, Pujo-Pay M, Wambeke F (1999) Relationship between phytoplankton efficiency and the proportion of bacterial production to primary production in the Mediterranean Sea. Aquat Microb Ecol 17:131-144

Coveney MF, Wetzel RG (1995) Biomass, production, and specific growth rate of bacterioplankton and coupling to primary production in an oligotrophic lake. Limnol Oceanogr 40:1187-1200

Crump BC, Armbrust EV, Barross JA (1999) Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary and the adjacent coastal ocean. Appl Environ Microbiol 65:3192-3204

Cunha MA, Almeida MA, Alcântara F (1999) Compartments of oxygen consumption in a tidal mesotrophic estuary (Ria de Aveiro, Portugal). Acta Oecologica. 20 (4):227-235

Cunha MA, Almeida MA, Alcântara F (2000) Patterns of variation of ectoenzimatic and heterotrophic bacterial activities along a salinity gradient in a shallow tidal estuarine. Mar Ecol Prog Ser 204:1-12.

Cunha MA, Almeida MA, Alcântara F (2001) Short-time responses of the natural planktonic bacterial community of the changing water properties in an estuarine environment: ectoenzimatic activity, glucose incorporation and biomass production. Microb Ecol (in press)

Currie D, Kalff J (1984) The relative importance of bacterioplankton and phytoplankton in phosphorus uptake in freshwater. Limnol Oceanogr 29:311-321

Cushing DH (1989) A difference in structure between ecosystems in strongly stratified waters and in those are only weakly stratified. J Plankton Res 11:1-13

Davey HM, Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analysis. Microbiol Rev 60:641-696

De Long EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. Limnol Oceanogr 38:924-934

del Giorgio PA, Scarborough G (1995) Increase in the proportion of metabolically active bacteria along gradients of enrichment in freshwater and marine plankton: implications for estimates of bacterial growth and production rates. J Plankton Res 17: 1905-1924

del Giorgio PA, Gasol JM, Vaque D, Mura, P, Agusti S (1996) Bacterioplankton community structure: protists control net production and the proportion of active bacteria in a coastal marine community. Limnol Oceanogr 41: 1169-1179

del Giorgio P, Prairi YT, Bird DF (1997) Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. Microb Ecol 34:144-154

del Giorgio P, Cole J (1998) Bacterial growth efficiency in natural aquatic systems. Annual Rev Ecol Syst 29:503-541.

Di Siervi MA, Mariazzi AA, Donadelli JL (1995) Bacterioplankton and phytoplankton production in a large patagonian reservoir (Republic Argentina). Hydrobiologia 297:123-129

Dias JM, Lopes JF, Dekeyser I (2000) Tidal propagation in the Ria de Aveiro lagoon, Portugal. Phys Chem Earth 25:369-374

Ducklow HW (1982) Chesapeake Bay nutrient and plankton dynamics. I. Bacterial biomass and production during spring tidal destratification in the York River, Virginia, estuary. Linmol Oceanogr 27:651-659

Ducklow HW, Kirchman DL (1983) Bacterial dynamics and distribution during a spring bloom in the Hudson River plume, USA. J Plankton Res 5:333-355

Ducklow HW (1992) Factors regulating bottom-up control of bacterial biomass in open ocean plankton communities. Archiv fur Hydrobiologie 37:207-217

Ducklow HW, Carlson CA (1992) Oceanic bacterial production. Adv Microb Ecol 12:113-181

Ducklow HW, Shiah FK (1993) Bacterial production in estuaries. *In:* Ford TE (ed.), Aquatic Microbiology, Blackwell Scientific Publications, Boston, p 261-287

Ducklow H, Carlson C, Smith W (1999) Bacterial growth in experimental plankton assemblages and seawater cultures from the *Phaeocystis antarctica* bloom in the Ross Sea, Antarctica. Aquat Microb Ecol 19:215-227

Dufour PH, Torréton JP (1996) Bottom-up and top down control of bacterioplankton from eutrophic to oligotrophic sites in the tropical Northeastern Atlantic Ocean. Deep-Sea Res 43:1305-1320

Dufour P, Berland B (1999) Nutrient control of phytoplankton biomass in atoll lagoons and Pacific Ocean waters: studies with factorial enrichment bioassays. J Exp Mar Biol Ecol 234:147-166

Eppley RW, Harrison WG, Chisholm SW, Stewart E (1977) Particulate organic matter in surface waters off Southern California and its relationships to phytoplankton. J Mar Res 35:671-696

Es Van FB, Meyer-Reil LA (1982) Biomass and metabolic of heterotrophic marine bacteria. *In:* Marshall KC (ed.), Advanced Microbiol Ecology, Vol 6, Plenum Press, New York, p 111-170

Esteves VI (1995) Extracção e caracterização de substâncias húmicas de diferentes ambientes aquáticos. Ph.D. Thesis, Universidade de Aveiro

Fandino LB, Riemann L, Steward GF, Long RA, Azam F (2001) Variations in bacterial community structure during a dinoflagellate bloom analysed by DGGE and 16S rDNA sequencing. Aquat Microb Ecol 23:119-130

Fernandez MM, Binai M, An Wambeke F (1994) Bacterial biomass, heterotrophic production and utilisation of dissolved organic matter photosynthetically-produced in the Almeria-Oran front. J Mar Sys 5:313-325

Ferrier-Pagès C, Furla P (2001) Pico- and nanoplankton biomass and production in the two largest atoll lagoons of French Polynesia. Mar Ecol Prog Ser 211:63-76

Feuillade M, Feuillade J, Fiala V (1990) The effect of light on the release of organic compounds by the cyanobacterium *Oscillatoria rubescens.* Aquat Sci 52:348-359

Findlay S, Pace ML, Lints D, Cole J, Caraco NF, Peierls B (1991) Weak coupling of bacterial and algal production in a heterotrophic ecosystem: the Hudson River estuary. Limnol Oceanogr 36:268-278

Findlay S, Pace ML, Lints D, Howe K (1992) Bacterial metabolism of organic carbon in the tidal freshwater Hudson Estuary. Mar Ecol Prog Ser 89:147-153

Fuhrman JA, Azam F (1982) Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar Biol 66:109-120

Fuhrman J (1992) Bacterioplankton roles in cycling of organic matter: the microbial food web. *In:* Falkowski PG, Wood-head AD (eds.), Primary productivity and biogeochemical cycles in the sea, Plenum press, New York, pp 361-383

Fuhrman JA, Suttle CA (1993) Viruses in marine planktonic systems. Oceanography 6:51-63

Fuhrman JA, Noble RT (1995) Viruses and protists cause similar bacterial mortality in coastal seawater. Limnol Oceanogr 40:1236-1242

Fukami K, Simidu U, Taga N (1985) Microbial decomposition of phyto- and zooplankton in seawater. II. Changes in the bacterial community. Mar Ecol Prog Ser 21:7-13

Fuks D, Devescovi M, Precali R, Krstulovic N, Solic M (1991) Bacterial in the highly stratified estuary of the Krka River. Mar Chem 32:333-346

Fuks D, Precali R, Devercovi M (1994) Bacterial production in the stratified Karstic estuary of the Krka River. Acta adriat 34 (1/2):21-28

Gasol JM, del Giorgio PA, Massana R, Duarte CM (1995) Active versus inactive bacteria: size-dependence in a coastal marine plankton community. Mar Ecol Prog Ser 128:91-97

Gasol JM, Doval MD, Pinhassi J, Caldéron-Paz JI, Guixa-Boixareu N, Vaqué D, Pedrós-Alió C (1998) Diel variations in bacterial heterotrophic activity and growth in the northwestern Mediterranean Sea. Mar Ecol Prof Ser 164:107-124

González JM, Sherr EB, Sherr BF (1990) Size selective grazing on bacteria by natural assemblage of estuarine flagellates and ciliates. Appl Environ Microbiol 40:156-160

González JM, Sherr EB, Sherr BF (1993) Differential feeding by marine flagellates on growing versus starving, and on motile versus non-motile, bacterial prey. Mar Ecol Prog Ser 102:257-267

González JM (1999) Bacteriovory rate estimates and fraction of active bacteriovores in natural protist assemblages from aquatic systems. Appl Environ Microbiol 65:1463-1469

Goosen NK, van Rijswijk P, Brockmann U (1995) Comparison of heterotrophic bacterial production rates in early spring in the turbid estuaries of the Scheldt and the Elbe. Hydrobiologia 311:31-42
Goosen NK, van Rijswijk P, Kromkamp J, Peene J (1997) Regulation of annual variation in heterotrophic bacterial production in the Schelde estuary (SW Netherlands). Aquat Microb Ecol 12:223-232
Griffith P, Shiah F, Gloersen K, Ducklow H, Fletcher M (1994) Activity and distribution of attached bacteria in Chesapeake Bay. Mar Ecol Prog Ser 108:1-10
Haas LW (1977) The effect of the spring-neap tidal cycle on the vertical salinity structure of the James, York and Rappahannock Rivers, Virginia, USA. Est Coast Mar Sci 5:483-496
Hagstrom A, Larson U, Horstedt P, Normark S (1979) Frequency of dividing cells, a new approach to the determination of bacterial growth rates in aquatic environments. Appl Environ Microbiol 37:805-812
Hagstrom A, Pinhassi J, Zweifel UL (2000) Biogeographical diversity among marine bacterioplankton. Aquat Microb Ecol 21:231-244
Hahn MW, Hofle MG (1998) Grazing pressure by a bacterivorous flagellate reverses the relative abundance of *Comamonas acidovorans* PX54 and *Vibrio* strain CB5 in chemostat cocultures. Appl Environ Microbiol 64:1910-1918
Hahn MW, Moore ER, Hofle MG (1999) Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Appl Environ Microbiol 65:25-35
Hall JA, Vincent WF (1990) Vertical and horizontal structure in the picoplankton communities of a coastal upwelling system. Marine Biology 106:465-471
Hasegawa T, Koike I, Mukai H (2000) Estimation of dissolved organic nitrogen release by micrograzers in natural planktonic assemblages. Plankton Biol Ecol 47:23-30
Heinanen A (1991) Bacterial numbers, biomass and productivity in the Baltic Sea: a cruise study. Mar Ecol Prog Ser 70:283-290
Heinanen A, Kuparinen J (1991) Horizontal variation of bacterioplankton in the Baltic Sea. Appl Environ Microbiol 57:3150-3155
Helbling EW, Villafame V, Ferrario M, Holm-Hansen O (1992) Impact of natural ultraviolet radiation on rates of photosynthesis and on specific marine phytoplankton species. Mar Ecol Prog Ser 80:89-100
Helbling EW, Eilertsen HC, Villafane VE, Holm-Hansen O (1996a) Effects of UV-radiation on post-bloom phytoplankton populations in Kvalsund, North Norway. Photochem Photobiol 33:255-259
Helbling EW, Chalker BE, Dunlap WC, Holm-Hansen O (1996b) Photoacclimation of Antarctic marine diatoms to solar ultraviolet radiation. J Exp Mar Biol Ecol 204:85-101
Heldal M, Bratback G (1991) Production and decay of viruses in aquatic environments. Mar Ecol Prog Ser 72:205-212
Hennes KP, Suttle TA, Chan AM (1995) Fluorescentlylabeled virus probes show that natural viruses populations can control the structure of marine microbial communities. Appl Environ Microbiol 61:3623-3627
Henriksen K, Rasmussen M, Jensen A (1983) Effects of bioturbation on microbial nitrogen transformation in the sediment and fluxes of ammonium and nitrate to the overlying water. Ecol Bull 35:193-205
Herndl GJ, Muller-Nicklas G, Frick J (1993) Major role of ultraviolet-B in controlling bacterioplankton growth in the surface layer of the ocean. Nature 361:717-719
Hobbie JE, Daley R, Jasper S (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225-1228
Hobbie JE (1988) A comparison of the ecology of planktonic in fresh and salt water. Limnol Oceanogr 33:750-764
Hoch MP, Kirchman DL (1993) Seasonal and inter-annual variability in bacterial production and biomass in a temperate estuary. Mar Ecol Prog Ser 98:283-295
Hofle M, Bretar I (1995) Taxonomic diversity and metabolic activity of microbial communities in the water column of the central Baltic Sea. Limnol Oceanogr 40:868-874
Hofle M, Bretar I (1996) Genotyping of heterotrophic bacteria from the central Baltic Dea by use of low-molecular-weight RNA profiles. Appl Environ Microbiol 62:1383-1390
Hollibaugh JT (1994) Relationship between thymidine metabolism, bacterioplankton community metabolic capabilities and sources of organic matter used for growth. Microb Ecol 28:117-131
Holligan PM, Williams PJ, Purdie D, Harris RP (1984) Photosynthesis, respiration and nitrogen supply of plankton populations in stratified, frontal and tidally mixed shelf waters. Mar Ecol Prog Ser 17:201-213
Hopkinson C, Giblin AE, Garritt RH, Tucker J, Hullar MA (1998) Influence of benthos on growth of planktonic estuarine bacteria. Aquat Microb Ecol 16:109-118

Hoppe HG (1978) Relations between active bacteria and heterotrophic potential in the sea. Neth J Sea Res 12:78-98

Hoppe HG, Gocke K, Alcântara F (1996) Shiffs between autotrophic and heterotrophic processes in a tidal lagoon (Ria de Aveiro, Portugal). Arch Hydrobiol Spec Issus Advanc Limnol 48:39-52

Hoppe H, Giesenhagen H, Gocke K (1998) Changing patterns of bacterial substrate decomposition in a eutrophication gradient. Aquat Microb Ecol 15:1-13

Jellet JF, Li WK, Dickie PM, Boraie A, Keplay PE (1996) Metabolic activity of bacterioplankton communities assessed by flow cytometry and single carbon substrate utilization. Mar Ecol Prog Ser 136:213-225

Jiang SC, Paul JH (1994) Seasonal and diel abundance of viruses and occurrence of lysogeny/bacteriocinogeny in the marine environment. Mar Ecol Prog Ser 104:163-172

Jokiel Pl, York RH (1984) Importance of ultraviolet radiation in photoinhibition of microalgal growth. Limnol Oceanogr 29:192-199

Jorgensen NO, Tranvik L, Edling H, Granéli W, Lindell M (1998) Effects of sunlight on occurrence and bacterial turnover of specific carbon and nitrogen compounds in lake water. FEMS Microbiol Ecol 25:217-227

Jorgensen NO, Kroer N, Coffin RB, Hoch MP (1999) Relations between bacterial nitrogen metabolism and growth efficiency in an estuarine and in an open-water ecosystem. Aquat Microb Ecol 18:247-261

Joux F, LeBaron P (1997) Ecological implications of an improved direct viable count method for aquatic bacteria. Appl Environ Microbiol 63:3643-3647

Jurgens K, Pernthaler J, Schalla S, Amann R (1999) Morphological and compositional changes in a planktonic bacterial community in response to enhanced protozoan grazing. Appl Environ Microbiol 65:1241-1250

Karentz D, Bothwell L, Coffin R, Hanson A, Herndl G, KIlham S, Lesser M, Lindel M, Moeller R, Morris D, Neale P, Sanders R, Weiler C, Wetzel R (1994) Impact of UV-B radiation on pelagic freshwater ecosystems: Report of working group on bacteria and phytoplankton. Arch Hydrobiol Beih 43:31-69

Karner M, Herndl GJ (1992) Extracellular enzymatic activity and secondary production in free-living and marine-snow-associated bacteria. Mar Biol 113:341-347

Karner M, Fuhrman JA (1997) Determination of active marine bacterioplankton: a comparison of universal 16Sr RNA probes, autoradiography, and nucleoid staining. Appl Environ Microbiol 63:1208-1213

Kirchman DL, Rich JH (1997) Regulation of bacterial growth rates by dissolved organic carbon and temperature in the equatorial Pacific Ocean. Microb Ecol 33:11-20

Kisand V, Noges T (1998) Seasonal dynamics of bacterio- and phytoplankton in large and shallow eutrophic lake Vortsjarv, Estonia. Int Ver Hydrobiol 83:205-216

Kornas K, Kapiris K, Thessalou-Legaki M, Nicolaidou A (1998) Quantitative relationships between phytoplankton, bacteria and protists in an Aegean semi-enclosed embayment (Maliakos Gulf, Greece). Appl Environ Microbiol 15:255-264

Kroer N (1993) Bacterial growth efficiency on natural dissolved organic matter. Limnol Oceanogr 38:1282-1290

Kuosa H, Kivi K (1989) Bacteria and heterotrophic flagellates in the pelagic carbon cycle in the northern Baltic Sea. Mar Ecol Prog Ser 53:93-100

Lee CH, Kudo I, Yanada M, Maita Y (2001) Bacterial abundance and production and heterotrophic nanoflagellate abundance in subartic coastal waters (Western North Pacific Ocean). Aquat Microb Ecol 23:263-271

Lignell R (1990) Excretion of organic carbon by phytoplankton: its relation to algal biomass, primary productivity and bacterial secondary productivity in the Baltic Sea. Mar Ecol Prog Ser 68:85-99

Lindell MJ, Granéli W, Tranvik LJ (1995) Enhanced bacterial growth in response to photochemical transformation of dissolved organic matter. Linmol Oceanogr 40:195-199

Lindell MJ, Edling H (1996) Influence of light on bacterioplankton in a tropical lake. Hydrobiologia 323:67-73

Lindsay P, Ball PW, West JR (1996) Influence of tidal range and river discharge on suspended particulate matter fluxes in the Forth Estuary (Scotland). Est Coast Shelf Sci 42:63-82

Lovejoy C, Lejandre L, Klein B, Tremblay J, Ingran R, Therriault J (1996) Bacterial activity during early winter mixing (Gulf of St. Lawrence, Canada). Aquat Microb Ecol 10:1-13

Malinsky N Legrand C (1996) Excretion of dissolved organic carbon by phytoplankton of different sizes and subsequent bacterial uptake. Mar Ecol Prog Ser 132:249-255

Mann CJ, Wetzel RG (1996) Dissolved organic carbon and its utilisation in a riverine Wetland ecosystem. Biogeochemistry 31:99-120

Maranger R, Bird D, Juniper S (1994) Viral and bacterial dynamics in Artic sea ice during the spring algal bloom, near Resolute, NWT, Canada. Mar Ecol Prog Ser 111:121-127

Maramger R, Bird D (1995) Viral abundance in aquatic systems: a comparison between marine and fresh waters. Mar Ecol Prog Ser 121:217-226

Martinez J, Smith D, Steward D, Azam F (1996) Variability in ectoydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. Aquatic Microb Ecol 10:223-230

Medeiros C, Kjerfve B (1993) Hydrology of a tropical estuarine system: Itamaracá, Brazil. Est Coast Shelf Sci 36:495-515

Mehta AJ (1988) Laboratory studies on cohesive sediment deposition and erosion. *In:* Dronkers J, van Leussen V (eds.), Physical processes in estuaries, Spring-Verlag, Berlin, p 427-445

Methé BA, Hiorns WD, Zehr JP (1998) Contrast between marine and freshwater bacterial composition: analyses of communities in Lake George and six other Adirondack lakes. Limnol Oceanogr 43:368-374

Meyer-Reil LA (1978) Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl Environ Microbiol 36:506-512

Middelboe M, Jorgenson NO, Kroer N (1996) Effects of viruses on nutrient turnover and growth efficiency of noninfected marine bacterioplankton. Appl Environ Microbiol 62:1991-1997

Middelboe M, Kroer N, Jorsensen NO, Pakulski D (1998) Influence of sediment on pelagic carbon and nitrogen turnover in a shallow Danish estuary. Aquat Microb Ecol 14:81-90

Miller RV, Sayler GS (1992) Bacteriophage-host interactions in aquatic systems. *In*: Wellington EM, Van Elsas JD (eds.), Genetic interactions among microorganisms in the natural environment. Pergamon Press, New York, p 176-193

Miller Wl, Moran MA (1997) Interaction of photochemical and microbial processes in the degradation of refractory dissolved organic matter from a coastal marine environment. Limnol Oceanogr 42:1317-1324

Moreira MH, Queiroga H, Machado MM, Cunha MR (1993) Environmental gradients in a southern estuarine system: Ria de Aveiro, Portugal, Implications for soft bottom macrofauna colonization. Neth J Aquat Ecol 27:465-482

Morita RY (1997) Bacteria in oligotrophic environments. Chapman and Hall, New York.

Mousseau L, Gosselin M, Levasseur M, Demers S, Fauchot J, Roy S, Villegas P, Mostajir B (2000) Effects of ultraviolet-B radiation on simultaneous carbon and nitrogen transport rates by estuarine phytoplankton during a week-long mesocosm study. Mar Ecol Prog Ser 199:69-81

Murray AE, Jackson GA (1992) Viral dynamics: a model of the effect of size, shape, motion and abundance of single-celled planktonic organisms and other particles. Mar Ecol Prog Ser 89:103-116

Murray AE, Hollibaugh JT, Orrego C (1996) Phylogenetic composition of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl Environ Microbiol 62:2676-2680

Murray AE, Preston CM, Massana R, Taylor RT, Blakis A, Wu K, DeLong EF (1998) Seasonal and spatial variability of bacterial and archael assemblages in the coastal waters near Anvers Island, Antarctica. Appl Environ Microbiol 64:2585-2595

Murrel MC, Hollibaugh JT, Solver MW, Wong PS (1999) Bacterioplankton dynamics in northern San Francisco Bay: role of particle association and seasonal freshwater flow. Linmol Oceanogr 44:295-308

Newell SY, Krambeck C (1995) Responses of bacterioplankton to tidal inundations of a saltmarsh in a flume and adjacent mussel enclosures. J Exp Mar Biol Ecol 190:79-95

Noble RT, Fuhrman JA (1997) Virus decay and its cause in coastal waters. Appl Environ Microbiol 63:77-83

Noble RT, Fuhrman JA (1999) Breakdown and microbial uptake of marine and other lysis products. Aquat Microb Ecol 20:1-11

Noble RT, Middelboe M, Fuhrman JA (1999) Effects of viral enrichment on the mortality and growth of heterotrophic bacterioplankton. Aquat Microb Ecol 18:1-13

Novitsky JA (1983) Heterotrophic activity throughout a vertical profile of seawater and sediment in Halifax Harbor, Canada. Appl Environ Microbiol 45:1753-1760

Nowell AR, Jumars PA (1984) Flow environments of aquatic benthos. Ann Rev Ecol Syst 15:303-328

Oliveira AM, Kjerve B (1993) Environmental responses of a tropical coastal lagoon system to hydrological variability: Mundaú-Manguaba, Brazil. Est Coast Shelf Sci 37:575-591

Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Appl Environ Microbiol 63:3367-3373

Ovreas L, Jensen S, Daae FL, Torsvik V (1998) Microbial community changes in a perturbed agricultural soil investigated by molecular and physiological approaches. Appl Environ Microbiol 64:2739-2742

Pace ML, Cole JJ (1994) Comparative and experimental approaches to top-down and bottom-up regulation of bacteria. Microb Ecol 28:181-193
Pace ML, Cole JJ (1996) Regulation of bacteria by resources and predation tested in whole-lake experiments. Limnol Oceanogr 41:1448-1460
Page HM, Petty RL, Maede DE (1995) Influence of watershed runoff on nutrient dynamics in a Southern California salt marsh. Est Coast Shelf Sci 41:163-180
Painchaud J, Lefaivre D, Therriault JC (1987) Box model analysis of bacterial fluxes in the St Lawrence estuary. Mar Ecol Prog Ser 41:241-252
Pakulsky JD, Aas P, Jeffrey W, Lyons M, Waasbergen V, Mitchell D, Coffin R (1998) Influence of light on bacterioplankton production and respiration in a sub-tropical coral reef. Aquat Microb Ecol 14:137-148
Palumbo AV, Fergunson RL (1978) Distribution of suspended bacteria in the Newport River estuary, North Carolina. Est Coast Mar Sci 7:521-529
Panzenbock M, Mobes-Hansen B, Albert R, Herndl G (2000) Dynamics of phyto- and bacterioplankton in a high Artic lake on Franz Joseph Land archipelago. Aquat Microb Ecol 21:265-273
Parsons TR, Takahashi M, Hargrave B (1984) Biological oceanic processes, 3rd ed, Pergamon Press, Oxford.
Parsons TR, Maita Y, Lalli CM (1989) A Manual of Chemical and Biological Methods for Seawater Analysis, Pergamon Press, Oxford
Paul J, Jiang S, Rose J (1991) Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. Appl Environ Microbiol 57:2197-2204
Paul JH (1999) Microbial gene transfer: an ecological perspective. J Microbiol Biothecnol 1:45-50
Pausz C, Herndl GJ (1999) Role of ultraviolet radiation on phytoplankton extracellular release and its subsequent utilization by marine bacterioplankton. Aquat Microb Ecol 18:85-93
Peduzzi P, Herndl GJ (1992) Zooplankton activity fueling the microbial loop: differential growth response of bacteria from oligo- and eutrophic waters. Limnol Oceanogr 37:1087-1092
Peduzzi P, Weinbauer MG (1993) The submicron size fraction of seawater containing high numbers of virus particles as bioactive agent in unicellular plankton community successions. J Plankton Res 15:1375-1386
Pereira G, Alcântara F (1993) Culturability of *Escherichia coli* and *Stretrococcus faecalis* in batch culture and *in situ* in estuarine water (Portugal). Wat Res 27:1351-1360
Pernthalen J, Sattler B, Simek K, Schwarzenbacher A (1996) Top-down effects on the size-biomass distribution of a freshwater bacterioplankton community. Aquat Microb Ecol 10:255-263
Pinhassi J, Azam F, Hemphala J, Long R, Martinez J, Zweifel U, Hagstrom A (2000) Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. Aquat Microb Ecol 17:13-26
Pomeroy LR (1991) Status and future needs in protozoan ecology. *In:* Reid PC, Turley CM, Burkhill PH (eds.), Protozoan and their role in marine processes, NATO ASI Series G: Ecological sciences, Vol 25, Springer-Verlag, Heidelberg, p 475-492
Pomeroy LR, Sheldon JE, Sheldon WE (1994) Changes in bacterial numbers and leucine assimilation during estimations of microbial respiratory rates in seawater by the precision Winkler method. Appl Environ Microbiol 60:328-332
Pomeroy LR, Weibe WJ (1998) Energetics of microbial food webs. Hydrobiologia 159:7-18
Pomeroy LR, Wiebe WJ (2001) Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat Microb Ecol 23:187-204
Porter J, Diaper J, Edwards C, Pickup R (1995) Direct measurements of natural planktonic bacterial community viability by flow citometry. Appl Environ Microbiol 61:2783-2786
Posch T, Simek K, Vrba J, Pernthaler J, Nedoma J, Sattler B, Sonntag B, Psenner R (1999) Predator-induced changes of bacterial size-structure and productivity studied on an experimental microbial community. Aquat Microb Ecol 18:235-246
Prah FG, Coble, PG (1994) Input and behaviour of dissolved organic carbon in the Columbia River Estuary. *In:* Dyer K, Orth R (eds.), Changing particle fluxes in estuaries: implications from science to management, ECSA22/ERF Symposium, Olsen & Olsen Press, Fredensborg, p 451-457
Prichard DW (1967) What is an estuary: physical viewpoint. *In:* Lauff GE (ed.), Estuaries, American Association for the Advancement of Sciences, Publication 83, Washington, p 3-5
Proctor LM, Fuhrman JA, Ledbetter MC (1988) Marine bacteriophages and bacterial mortality. Eos 69:1111-1112
Proctor LM, Fuhrman JA (1990) Viral mortality of marine cyanobacteria and bacteria. Nature 343: 60-62
Proctor LM, Fuhrman J (1991) Roles of viral infection in organic particle flux. Mar Ecol Prog Ser 69:133-142

Proctor LM, Fuhrman JA (1992) Mortality of marine bacteria in response to enrichments of the virus size fraction from seawater. Mar Ecol Prog Ser 87: 283-293

Proctor LM, Okubo A, Fuhrman JA (1993) Calibrating estimates of phage-induced mortality in marine bacteria: ultrastructural studies of marine bacteriophage development from one-step growth experiments. Microb Ecol 25:161-182

Quinn JP (1984) The modification and evaluation of some cytochemical techniques for the enumeration of metabolically active heterotrophic bacteria in the aquatic environment. J Appl Bacteriol 57: 51-57

Rehnstam AS, Backman S, Smith DC, Azam M, Hagstrom A (1993) Blooms of sequence-specific culturable bacteria in the sea. FEMS Microbiol Ecol 102:161-166

Rheinheimer G (1981) Investigations on the role of bacteria in the food web of the Western Baltic. Kieler Meeresforsch., Sonderh 5:284-290

Rheinheimer G (1985) Aquatic microbiology. John Wiley & Sons, New York

Reitner B, Herzig A, Herndl G (1999) Dynamics in bacterioplankton production in a shallow, temperate lake (Lake Neusiedl, Austria): evidence for dependence on macrophyte production rather than on phytoplankton. Aquat Microb Ecol 19:245-254.

Rhoads DC, Boyer LF (1982) The effect of marine benthos on physical properties of sediments: a successional perspective. *In:* McCall PL, Tevesz MJS (eds.), Animal-sediment relations: the biogenic alteration of sediments, Plenum Press, New York, p 3-52

Riegsbee W, Simpson L, Oliver J (1996) Detection of the viable but nonculturable state in *Escherichia coli* 0157:h7. J Food Saf 16:255-262

Riemann B, Sondergaard M (1986) Regulation of bacterial secondary production in two eutrophic lakes and in experimental enclosures. J Plankton Res 8:519-536

Riemann B, Christoffersen K (1993) Microbial trophodynamics in temperate lakes. Mar Microb Food Webs 7:69-100

Rivkin R, Anderson M, Lajzerowicz C (1996) Microbial processes in cold oceans. I. Relationship between temperature and bacterial growth rate. Aquat Microb Ecol 10:243-254

Rodier J (1996) L`analyse de l`eau: eaux naturelles, eaux résiduaires, eau de mer. 8 Edition, Dunod, Paris.

Rodriguez GG, Phipps D, Ishiguro K, Ridgway HF (1992) Use a fluorescent redox probe for direct visualisation of actively respiring bacteria. Appl Environ Microbiol 58:1801-1810

Sanborn MR, Wan SK, Bulard R (1982) Microwave sterilization of plastic tissue culture frasks for reuse. Appl Environ Microbiol 44:960-964

Sanders RW, Porter KG, Bennett SJ, DeBaise AE (1989) Seasonal patterns of bacterivory by flagellates, ciliates rotifers and cladocerans in a freshwater planktonic community. Limnol Oceanogr 34:673-687

Santos EB, Duarte AC (1998) The influence of pulp and paper mill effluents on the composition of the humic fraction of aquatic organic matter. Wat Res 32:597-608

Sanudo-Wihhelmy AS, Taylor, GT (1999) Bacterioplankton dynamics and organic carbon partitioning in the lower Hudson River estuary. Mar Ecol Prog Ser 182:17-27

Scavia D, Laird GA (1987) Bacterioplankton in Lake Michigan: dynamics, controls, and significance to carbon flux. Limnol Oceanogr 32: 1017-1033.

Schultz G, Ducklow H (2000) Changes in bacterioplankton metabolic capabilities along a salinity gradient in the York River estuary, Virginia, USA. Aquat Microb Ecol 22:163-174

Schut F, Prins R, Gottschal J (1997) Oligotrophy and pelagic marine bacteria: facts and fiction. Aquatic Microb Ecol 12:177-202

Sherr BF, Sherr EB, Pedrós-Alió C (1989) Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine water. Mar Ecol Prog Ser 54:209-219

Sherr BF, Sherr EB, McDaniel J (1992) Effects of protistan grazing on the frequency of dividing cells in bacterioplankton. Appl Environ Microbiol 58: 2381-2385

Sherr BF, del Giorgio P, Sherr EB (1999) Estimating abundance and single-cell characteristics of respiring bacteria via the redox dye CTC. Aquat Microb Ecol 18:117-131

Shiah FK, Ducklow HW (1994) Temperature and substrate regulation of bacterial abundance, production, and specific growth rate in Chesapeake Bay. Limnol Oceanogr 39:1243-1258

Shiah FK, Ducklow HW (1995) Multiscale variability in bacterioplankton abundance, production, and specific growth rate in a temperate salt-marsh tidal creek. Limnol Oceanogr 40(1):55-56

Shiah FK, Ducklow HW (1997) Bacterioplankton growth responses to temperature and chlorophyll variations in estuaries measured by thymidine:leucine incorporation rate. Aquat Microb Ecol 13:151-159

Shiah FK, Liu K, Gong G (1999) Temperature versus substrate limitation of heterotrophic bacterioplankton production across trophic and temperature gradients in the east China Sea. Aquat Microb Ecol 17:247-254

Shiah FK, Chen TY, Gong GC, Chen CC, Chiang KP, Hung JJ (2001) Differential coupling of bacterial and primary production in mesotrophic and oligotrophic systems of the East China Sea. Aquat Microb Ecol 23:273-282

Silva J (1994) Circulação de água na Ria de Aveiro: contribuição para o estudo da qualidade da água. Ph.D. Thesis, Universidade de Aveiro

Simek K (1986) Bacterial activity in a reservoir determined by autoradiography and its relationships to phyto- and zooplankton. Int Revne Ges Hydrobiol 71:593-612

Simek K J, Pernthaler J, Posh T, Hartman P, Nedoma J, Psenner R (1997) Morphological and compositional shifts in na experimental bacterial community influenced by protists with contrasting feeding modes. Appl Environ Microbiol 63:587-595

Simon M, Azam F (1989) Protein content and protein synthesis rates of planktonic bacteria. Mar Ecol Prog Ser 51:201-213

Simon M, Wunsch C (1998) Temperature control of bacterioplankton growth in a temperate large lake. Aquat Microb Ecol 16:119-130

Smith DC, Simon M, Alldredge Al, Azam F (1992) Intense hydrolytic enzyme activity on marine aggregates and implications for rapid particle dissolution. Nature 359:139-142

Smith JJ, McFeters GA (1997) Mechanisms of INT (2-(-idophenyl-3-(4-nitrophenyl)-5phenyl tetrazolium chloride) reduction in *Escherichia coli* K-12. J Microbiol Methods 29:161-175

Smith EM (1998) Coherence of microbial respiration rate and cell-specific bacterial activity in a coastal planktonic community. Aquat Microb Ecol 16:27-35

Solic M, Krstulovic N (1994) Role of predation in controlling bacterial and heterotrophic nanoflagellate standing stocks in coastal Adriatic Sea: seasonal patterns. Mar Ecol Prog. Ser 114:219-235

Sommaruga R, Conde D (1997) Seasonal variability of metabolic active bacterioplankton in the euphotic zone of the hypertrophic lake. Microb Ecol 13:241-248

Stahl DA, Key R, Flesher B, Smith J (1992) The phylogeny of marine and freshwater caulobacters reflects their habitat. J Bacteriol 174:2193-2198

Steemann-Nielsen E (1952) The use of radioactive carbon (^{14}C) for measuring organic production in the sea. J Cons Int Expl Mer 18:117-140

Steward FM, Wikner J, Cohlan WP, Smith DC, Azam F (1992) Estimation of virus production in the sea. I. Method development. Mar Microb Food Webs 6:57-78

Steward FM, Smith DC, Azam F (1996) Abundance and production of bacteria and viruses in the Bering and Chukchi seas. Mar Ecol Prog Ser 131:287-300

Stoderegger K, Herndl GJ (1998) Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol Oceanogr 43:877-884

Strom SL, Benner R, Ziegler S, Dagg MJ (1997) Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol Oceanogr 42:1364-1374

Suttle C, Chan A, Cottrell M (1990) Infection of phytoplankton by viruses and reduction of primary productivity. Nature 347:467-469

Suttle CA (1994) The significance of virus to mortality in aquatic microbial communities. Microb Ecol 28:237-243

Suzuki MT (1999) Effect of protistan bacterivory on coastal bacterioplankton diversity. Aquat Microb Ecol 20:261-272

Tabor PS, Neihof RA (1982) Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl Environ Microbiol 44 (4): 945-953

Tabor PS, Neihof RA (1984) Direct determination of activities for microorganisms of Chesapeake Bay populations. Appl Environ Microbiol 48: 1012-1019

Talbot V, Giuliano L, Bruni V, Bianchi M (1997) Bacterial abundance, production and ectoproteolytic activity in the Strait of Magellan. Mar Ecol Prog Ser 154:293-302

Thingstad TF, Skjoldal EF, Bohne RF (1993) Phosphorus cycling and algal-bacterial competition in Sandsfjord, western Norway. Mar Ecol Prog Ser 99:239-259

Thingstad TF, Lignell R (1997) Theoretical models for the control of bacterial growth rate, abundance, diversity and carbon demand. Aquat Microb Ecol 13:9-27

Thingstad TF, Havskum H, Kaas H, Nielsen T, Riemann B, Lefevre D, Williams B (1999) Bacteria-protist interactions and organic matter degradation under P-limited conditions: analysis of an enclosure experiment using a simple model. Limnol Oceanogr 44:62-79

Thorsen BK, Enger O, Norland S, Hoff K (1992) Lon-term starvation survival of *Yersinia rukeri* at different salinities studied by microscopical and flow cytometric methods. Appl Environ Microbiol 58:1624-1628

Toolan T, Wehr JD, Findlay S (1991) Inorganic phosphorus stimulation of bacterioplankton production in a mesoeutrophic lake. Appl Environ Microbiol 57:2074-2078
Torréton JP, Talbot V, Garcia N (2000) Nutrient stimulation of bacterioplankton growth in Tuamotu atoll lagoons. Aquat Microb Ecol 21:125-137
Tranvick LJ (1992) Allochthonous dissolved organic matter as an energy source for pelagic bacteria and the concept of the microbial loop. Hydrobiologia 229:107-114
Tuomi D (1997) Bacterial carbon production in the northern Baltic: a comparison of thymidine incorporation and FDC based methods. Mar Ecol Prog Ser 153:59-66
Ullrich S, Karrasch B, Hoppe H, Jeskulke K, Mehrens M (1996) Toxic effects on bacterial metabolism of the redox dye 5-cyano-2, 3- ditolyl tetrazolium chloride. Appl Environ Microbiol 62:4587-4593
Ullrich S, Karrash B, Hoppe HG (1999) Is the CTC dye technique an adequate approach for estimating active bacterial cells. Aquat Microb Ecol 17:207-209
Vadstein O, Jensen A, Olsen Y, Reinertson H (1988) Growth and phosphorus status of limnetic phytoplankton and bacteria. Limnol Oceanogr 33:489-503
Vadstein O, Olsen Y, Reinestsem H (1993) The role of planktonic bacteria in phosphorus cycling in lakes - sink and link. Limnol Oceanogr 38:1539-1544
Valdés M, Albright LJ (1981) Survival of heterotrophic activities of Fraser River and Strait of Georgia bacterioplankton within the Fraser River plume. Mar Biol 64:231-241
Vallespinos F, Mallo S (1990) Bacteria as transformers of the arena of play of other organisms. Oecologia 10:215-222
Vaqué D, Gasol J, Marrasé C (1994) Grazing rates on bacteria: the significance of methodology and ecological factors. Mar Ecol Prog Ser 109:263-274
Vosjan J, van Noort G (1998) Enumerating nucleoid-visible marine bacterioplankton: bacterial abundance determined after storage of formalin fixed samples agrees with isopropanol rinsing method. Aquat Microb Ecol 14:149-154
Weinbauer M, Peduzzi P (1995a) Effect of virus-rich high molecular weight concentrates of seawater on dynamics of dissolved amino acids and carbohydrates. Mar Ecol Prog Ser 127:245-253
Weinbauer M, Peduzzi P (1995b) Significance of viruses versus heterotrophic nanoflagellates for controlling bacterial abundance in the northern Adriatic Sea. J Plankton Res 17:1851-1856
Weinbauer MG, Hoffle MG (1998) Significance of viral lysis and flagellate grazing as factors controlling bacterioplankton production in a eutrophic lake. Appl Environ Microbiol 64:431-438
Weisse T (1997) Growth and production of heterotrophic nanoflagellates in a meso-eutrophic lake. J Plankton Res 19:703-722
Weisse T, Muller H (1998) Planktonic protozoa and the microbial food web in Lake Constance. Arch Hydrobiol Spec Issues Adv Limnol 53:223-254
Wetzel RG (1984) Detrital dissolved and particulate organic carbon functions in aquatic ecosystems. Bull Mar Sci 35:503-509
Wetzel RG (1992) Gradient-dominated ecosystems: sources and regulatory functions of dissolved organic matter in freshwater ecosystems. Hydrobiologia 229:181-198
Wetzel RG, Hatcher PG, Bianchi TS (1995) Natural photolysis by ultraviolet irradiance of recalcitrant dissolved organic matter to simple substrates for rapid bacterial metabolism. Limnol Oceanogr 40:1369-1390
White PA, Kalff J, Rasmussen B, Gasol JM (1991) The effect of temperature and algal biomass on bacterial production and specific growth rate in freshwater and marine habitats. Microb Ecol 21:99-118
Wiebe WM, Pomeroy LR (1992) Bacterial growth in the cold: Evidence for an enhanced substrate requirement. Appl Environ Microbiol 58:359-364
Wiggins BA, Alexander M (1985) Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl Environ Microbiol 49:19-23
Wikner J, Rassoulzadegan F, Hagstrom A (1990) Periodic bacterivore activity balances bacterial growth in the marine environment. Limnol Oceanogr 35:313-324
Wikner J, Cuadros R, Jansson M (1999) Differences in consumption of allochthonous DOC under limnic and estuarine conditions. Aquat Microb Ecol 17:289-299
Wilcox RM, Fuhrman JA (1994) Bacterial viruses in coastal sea water: lytic rather than lysogenic production. Mar Ecol Prog Ser 114:35-45
Williams PJ (1981) Incorporation of microheterotrophic processes into the classical paradigm of the planktonic food web. Kieler Meeresforsch 5:1-28
Williams PJ (1990) The importance of losses during microbial growth: commentary on the physiology, measurement and ecology of the release of dissolved organic material. Mar Microb Food Webs 4:175-206

William SC, Hong Y, Danavall DC, Howard-Jones MH (1998) Distinguishing between living and non-living bacteria: evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. J Microbiol Methods 32:225-236

Witek Z, Ochocki S, Maciejowska M, Pastuszak M, Nakonieczny J, Podgórska B, Kownacka JM, Mackiewicz T, Wrzesinska-Kwiecien M (1997) Phytoplankton primary production and its utilization by the pelagic community in the coastal zone of the Gulf of Gdansk (southern Baltic). Mar Ecol Prog Ser 148:169-186

Wommack KE, Hill RT, Muller TA, Colwell RR (1996) Effects of sunlight on bacteriophage viability and structure. Appl Environ Microbiol 62:1336-1341

Wommack KE, Ravel J, Hill RT, Chun J, Colwell RR (1999) Populations dynamics of Chesapeake Bay virioplankton: total-community analysis by pulse-field gel electrophoresis. Appl Environ Microbiol 65:231-240

Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. Microbiol Mol Biol Rev 64:69-114

Wright RT, Coffin RB (1983) Planktonic bacteria in estuaries and coastal waters of northern Massachusetts: spatial and temporal distribution. Mar Ecol Prog Ser 11:205-216

Wright RT, Coffin RC (1984) Factors affecting bacterioplankton density and productivity in salt marsh estuaries. *In:* Klug MJ, Reddy CA (eds.), Current perspectives in microbial ecology, American Society for Microbiology, Washington DC, p. 485-494

Yanada M, Yokokawa T, Lee, CW, Tanaka H, Kudo I, Maita Y (2000) Seasonal variation of two different heterotrophic bacterial assemblages in subartic coastal seawater. Mar Ecol Prog Ser 204:289-292

Yentsch CS, Menzel DW (1963) A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. Deep-Sea Res 10:221-231

Zimmermann R, Iturriaga R, Becker-Birck J (1978) Simultaneous determination of the total number of bacteria and the number therefore involved in respiration. Appl Environ Microbiol 36:926-935

Zlotnik I, Dubinsky (1989) The effect of light and temperature on DOC excretion by phytoplankton. Limnol Oceanogr 34:831-839

Zweifel U, Hagstrom A (1995) Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). Appl Environ Microbiol 61: 2180-2185