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Influence of an estuarine plume and marine sewage outfall on the dynamics of coastal bacterioplankton communities

A. Cunha*, A. Almeida

Centro de Estudos do Ambiente e do Mar, Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

ABSTRACT: Marine bacterioplankton in the coastal region off Aveiro (NW Portugal) develop under the influence of the plume of a mesotrophic estuarine system (Ria de Aveiro) and, more recently, under the influence of the discharges of a marine sewage outfall (S. Jacinto). In an attempt to compare the degree of disturbance introduced by these 2 features to the abundance of sewage bacteria and heterotrophic activity of natural bacterioplankton, water samples were collected at 10 offshore and nearshore locations. Rates of ectoenzymatic activity and monomer incorporation were determined as proxies for potential heterotrophic activity of natural bacterial communities. ATP and chl *a* concentrations were used as estimates of the total abundance of phytoplankton. Faecal coliforms and total colony counts were used as indicators of sewage bacteria contamination. However, ATP, chl *a*, faecal coliforms, total colony counts, aminopeptidase activity and the maximum rate of leucine incorporation (leucine Vm) correlated negatively with linear distance to the mouth of the estuary, establishing the estuarine plume as an importance source of disturbance. Chl *a*, aminopeptidase activity and total colony counts also correlated negatively with linear distance to the sewage outfall, but no significant impact on the concentration of faecal bacteria could be detected. Compliance with the European Union Bathing Water Directive 76/160/EEC was achieved in 97% of samples. The ratio between β-glucosidase activity (polymer degradation) and glucose incorporation (monomer uptake) increased as the distance from the mouth of the estuary and from the sewage outfall increased. This indicates that, in addition to inputs of bacteria and nutrients, changes in the quality of the available organic substrates and/or sewage-related toxic effects may impose a shift in the functioning of heterotrophic bacterioplankton communities in this costal area.

KEY WORDS: Marine bacterioplankton · Marine outfall · Estuarine plume · Coastal ocean

INTRODUCTION

Numerous sources of disturbance can be related to the contamination of coastal marine ecosystems. Direct discharges of domestic and industrial effluents or contamination through rivers or estuarine waters can be associated with inputs of organic and inorganic nutrients, toxicants and microorganisms. Estuarine waters are often polluted with microorganisms discharged from recreational marinas, sewage disposal sites, sewage treatment plants, septic tanks, rainfall runoff from urban areas and other diffuse sources (McAllister et al. 1996, Lipp et al. 2001, Bernhard et al. 2003, Heath et al. 2003, Aslan-Yılmaz et al. 2004, Kelsey et al. 2004). In addition to elevated bacterial numbers, estuarine plumes often present high nutrient availability, chlorophyll concentration, biomass production and total community respiration (Malone & Ducklow 1990, Morris et al. 1995, Bianchi et al. 1999, Pakulski et al. 2000). The magnitude of the impacts of estuaries on coastal water is influenced by the rates of organic matter recycling within the estuary and by a river-driven seasonality in the balance between fresh and marine water inputs to the main body of the estuary (Cloern & Nichols 1985).

*Email: acunha@bio.ua.pt

Ocean disposal is one of the most common strategies for eliminating wastewater a few kilometres from the coastline in order to relieve the anthropogenic pressure inside estuarine systems. However, outfall discharges of untreated or partially treated wastewater into the ocean have often been associated with contamination of the water and sediments by faecal bacteria (Edwards et al. 1988, Paul et al. 1997, Obiri-Danso & Jones 1999, Delille & Gleizon 2003, Hughes 2003, Hughes & Thompson 2004) and coliphages (Paul et al. 1997). From a different perspective, organic and inorganic substrates discharged through sewage outfall are also an issue of concern and involve wider spatial ranges. Their impacts are found to cause changes in the size and structure of phytoplankton communities (Soltan et al. 2001, Parnell 2003) and of soft sediment faunal assemblages (Morris & Keough 2002).

Bacterioplankton are highly reactive towards organic matter (Taylor et al. 2003) and inorganic nutrients (Torréton et al. 2000). Extracellular degradation and nutrient uptake profiles are closely related to the quality and size of the pool of dissolved organic matter (Taylor et al. 2003). Sewage outfall provides not only considerable amounts of polymeric organic matter but also the corresponding enzymatic capabilities for its degradation, thus increasing the availability of high- and low-molecular weight organic substrates (Chappel & Goulder 1994). Discharge from sewage treatment plants was found to increase the microbial rates of leucine assimilation (Ainsworth & Goulder 2000) and affect the rates of extracellular enzymatic activity in diverse and even contradictory ways (Chappel & Goulder 1994, Montuelle & Volat 1998, Brown & Goulde 1999, Ainsworth & Goulder 2000).

The primary purpose of this study was to assess the effect of discharge from a marine sewage outfall on the microbial communities of the recipient water, not only in terms of inputs and dispersion of sewage bacteria but also on the rates of bacterioplankton degradation of organic matter. Since only low-molecular weight compounds and simple molecules can be directly transferred from the environment into cells, extracellular degradation is often a limiting factor in the process of organic matter in the ocean. By coupling polymer hydrolysis and monomer uptake, bacterioplankton take maximum advantage of intermittent sources of organic matter. This coupling becomes tighter in oligotrophic conditions or under high pool size ratio between polymers and monomers (Hoppe et al. 1988). Ectoenzymatic activity and monomer incorporation were used as proxies for bacterioplankton heterotrophic activity.

A second goal of this study was to compare the degree of disturbance introduced by the marine sewage outfall with the contamination associated with the plume of a mesotrophic estuarine system. For this, some indicator bacterial groups were investigated and total living plankton biomass was estimated by particulate ATP analysis.

MATERIALS AND METHODS

Study area and sampling. Sampling was conducted at 10 sites positioned intermittently along 10 km of a coastal area near the city of Aveiro (NW Portugal) under the influence of 2 important features: an estuarine plume originating at the mouth of Ria de Aveiro, a complex bar-built estuary (Pritchard 1989), and the marine sewage outfall of S. Jacinto (Fig. 1). This 3.3 km long marine outfall discharges approximately 66 000 m^3 d^{-1} of treated effluent. Of the total discharge, 90% was contributed by a pulp mill effluent treatment plant. Stns

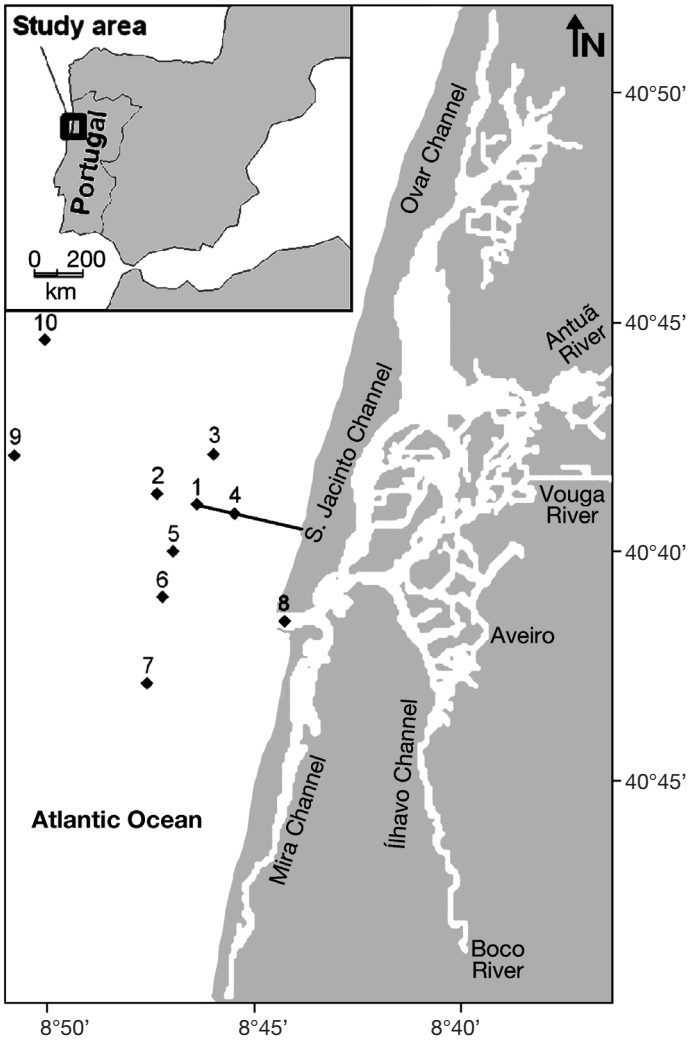

Fig. 1. Study area (NW Portugal) and sampling sites near the city of Aveiro

1 and 4 were located exactly above outfall diffusers; Stns 1 to 7 were located between the 10 and 20 m bathymetric lines; and Stns 9 and 10 were set beyond the 30 m bathymetric line.

Sampling was conducted during 8 cruises of the RV 'Andrómeda' (Portuguese Navy). The cruises were not evenly distributed during the study period because strong winds precluded sampling during autumn/winter 2001. The cruises took place in September 2000 (Cruise 1), January (Cruise 2) and June 2001 (Cruise 3), March (Cruise 4), July (Cruise 5) and December 2002 (Cruise 6), April (Cruise 7) and July 2003 (Cruise 8). Water samples were collected at the surface (0.2 m) and approximately from the middle of the water column at all sites except Site 8, where only surface samples were obtained. At Site 8, samples were collected at the beginning and end of each cruise in order to represent contrasting tidal conditions (high and low tide). Samples were kept cold during transport to the laboratory and were processes within 6 h after collection.

Physical and chemical parameters. Temperature and salinity were determined with a CTD, which was equipped with a pressure sensor that allowed the estimation of the total depth.

Microbiological parameters. Chl *a* was quantified fluorimetrically (Yentsch & Menzel 1963) in a Jasco FP-777 espectrofluorimeter. A total volume of 1 l water was filtered through Whatman GF/C filters and immediately extracted with 10 ml of 90% acetone. Determinations were performed in 3 replicates of each sample.

Particulate ATP concentration was determined by the luciferine-luciferase reaction according to the method of Karl & Holm-Hansen (1978). Three replicates of 20 ml sea water were filtered through 0.2 µm pore polycarbonate membranes (Poretics). ATP was extracted from the suspended matter with 4 ml of boiling TRIS buffer pH 7.4 to 7.5 (Sigma-Aldrich Chemicals). Filters were removed from the solution and the extract was deep frozen (–20°C) until subsequent analysis. After the aliquots had thawed, 200 µl of the extract was transferred into cuvettes to which 200 µl of assay mix (Sigma-Aldrich Bioluminescent Assay Kit) was added. The light emission was read in a luminometer (Labsystems Luminoskan TL).

Plate counts were determined in 5 replicates via the pour-plate method, using Plate Count Agar (Difco Laboratories) amended with 1.7% (w/v) sodium chloride (Merck) as culture medium. Salinities 0, 17 and 34 were tested with this medium, and salinity 17 subsequently selected as a result of the slightly higher colony counts produced. Faecal coliforms were analysed in 3 replicates by the filter-membrane method using mFC Agar (Difco Laboratories) as culture medium.

We followed the procedure of Gocke (1977) to determine the maximum rates of leucine incorporation (leucine Vm) and glucose incorporation (glucose Vm). A final saturation concentration of 242 nM ^{14}C-glucose or 81 nM ^{14}C-leucine was added to triplicate 10 ml aliquots and a formaline-fixed blank of each sample. Incubations were conducted for 2 h at *in situ* temperature. Cells were collected on 0.2 µm polycarbonate membranes (Poretics). Radioactive-labelled glucose (SA 310 mCi mmol^{-1}) and leucine (SA 308 mCi mmol^{-1}) were obtained from Amersham-Biosciences. Substrate concentrations were chosen after kinetic analysis. Radioactivity was read in a liquid scintillation counter (Beckman LS 6000 IC) using UniverSol (ICN Radiochemicals) as scintillation cocktail.

Ectoenzymatic activity was determined fluorimetrically (Jasco FP-777 Fluorometer) as the maximum hydrolysis rate (Hm) of model substrates for β-glucosidase (4-methylumbelliferyl-β-D-glucoside; Sigma-Aldrich Chemicals) and Leu-aminopeptidase (L-leucine-7-amido-4-methyl-coumarin; Sigma-Aldrich Chemicals) according to Hoppe (1983). A saturating concentration of 500 µM was used for both substrates. Analyses were made in triplicate and incubations were conducted for 1 to 2 h at *in situ* temperature.

Statistical analysis. The significance of differences between surface and mid-water values corresponding to each of the 8 sampling cruises was assessed by ANOVA. A similar analysis was conducted with the pool of data from the 8 cruises.

In order to assess the significance of the trends of variation of microbiological descriptors in relation to effects of the outfall or estuarine plume, a correlation analysis between microbiological data and either linear distance to the mouth of the estuary or linear distance to the outfall was performed using all data and setting 0.05 as the significance threshold. Samples in which the concentration of faecal coliforms was below the quantification limit (i.e. <1 CFU 100 ml^{-1}) were excluded pair-wise from correlation analysis. Data were log-transformed only when the transformation increased the level of significance of the correlation. The normality of the transformed values was checked by the Kolmogorov-Smirnov test.

In order to determine some of the factors involved in the regulation and establishment of patterns in bacterial abundance and activity, and to explain some of the observed variability, a step-wise multiple regression analysis was performed. Temperature, salinity, depth, linear distance to the outfall and linear distance to the mouth of the estuary were used as independent variables. Biological data were log-transformed in order to ensure normal distribution. Normality was checked using the Kolmogorov-Smirnov test. All statistical analyses were performed with the SPSSWin 12.0 package.

Table 1. Total depth, linear distance to the marine sewage outfall, linear distance to mouth of the estuary and ranges of temperature and salinity. When calculated, average values are presented in parentheses

Site	Total depth (m)	Linear distance (km) to: outfall	mouth of the estuary	Temp. (°C)	Salinity
1	13	0.0	4.6	13.1–18.3 (15.37)	33.9–36.1 (35.33)
2	18	0.8	5.4	13.1–18.2 (15.35)	33.2–36.4 (35.41)
3	14	2.1	6.9	13.0–18.3 (15.24)	33.7–36.1 (35.40)
4	11	1.7	4.2	13.1–18.4 (15.21)	33.0–36.0 (35.23)
5	15	2.1	2.3	13.1–18.2 (15.44)	32.7–36.2 (35.33)
6	15	4.2	1.9	13.1–18.5 (15.42)	32.9–35.8 (35.21)
7	16	7.9	4.4	13.3–18.4 (15.79)	34.7–35.8 (35.42)
8	nd	4.6	0.0	14.1–18.7 (15.42)	30.4–36.2 (34.56)
9	37	6.7	10.4	13.0–18.1 (15.25)	32.9–36.5 (35.44)
10	34	8.0	12.0	13.0–18.4 (15.31)	33.8–36.2 (35.40)

RESULTS

Physical and chemical parameters

Linear distances from sampling sites to the outfall and mouth of the estuary as well as physical and chemical properties of the water column are summarized in Table 1. The total depth of the water column varied from 11 m at Stn 4 to 37 m at Stn 9. The depth at Stn 8 was not determined, owing to difficulties in anchoring. However, the total depth of the water column at this site was previously estimated to be approx. 20 m (Cunha et al. 2003). Linear distances to the outfall varied from 0 km (Stn 1) to 8 km (Stn 10), whereas distances to the mouth of the estuary varied from 0 km (Stn 8) to 12 km (Stn 10). Water temperature varied between 13.0°C at Stn 9 (early morning, July 2002) and 18.7 at Stn 8 (early afternoon, July 2001). The lowest salinity value (30.4) was registered at the mouth of the estuary (December 2002), and the highest values (36.5) were recorded at Stn 9 (December 2002).

Plankton abundance and indicator groups

Chl *a* concentrations differed between surface and mid-water samples (Fig. 2). These differences were statistically significant (ANOVA, $p < 0.05$) in January and June 2001, March and July 2002 and July 2003. However, when all data from the 8 cruises were pooled, differences between the surface and mid-water samples were no longer statistically significant ($p = 0.057$). In surface samples, values ranged from 0.12 to 12.14 µg l^{-1}, and in mid-water samples, from 0.10 to 8.10 µg l^{-1}. The lowest values were generally registered at Stns 9 and 10. At these sites, average values calculated with all the samples were 1.71 and 1.04 µg l^{-1}, respectively. The highest chl *a* concentrations were observed during in July 2002.

Concentrations of ATP also differed between surface and mid-water samples (Fig. 2). The differences were statistically significant (ANOVA, $p < 0.05$) in January 2001, July 2002, December 2002 and July 2003. Again, the differences were not significant when data from the 8 cruises were analysed together ($p = 0.091$). In surface samples, concentrations of particulate ATP varied between 0.10 and 2.17 µg l^{-1}; In mid-water samples, values were lower (0.06 to 1.62 µg l^{-1}). The highest ATP concentration was recorded in July 2003 at Stn 8. Similar to the spa-

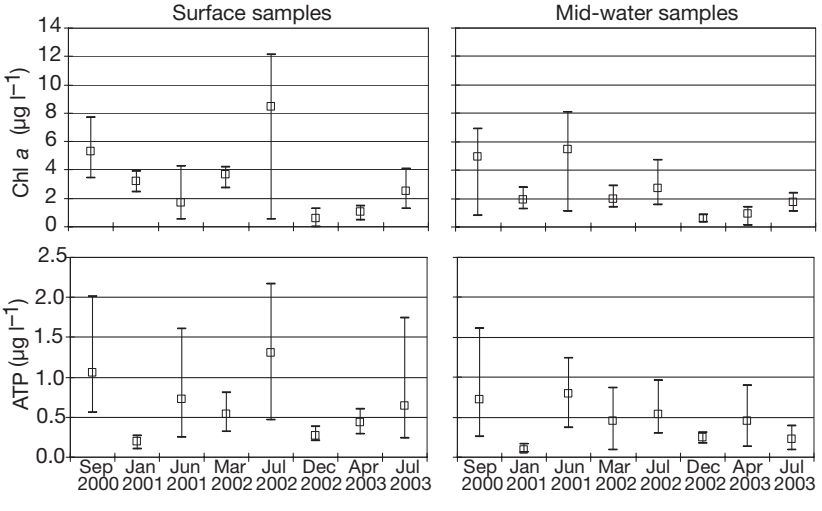

Fig. 2. Temporal variation in chl *a* and particulate ATP in surface and mid-water samples. Minimum, maximum and average (□) values from each cruise calculated from results from all sampling sites

tial pattern of chl *a*, ATP concentrations were generally lowest at Stns 9 and 10, with global average values of 0.37 and 0.34 µg l^{-1}, respectively.

Total colony counts and faecal coliforms appeared to be more abundant in mid-water samples (Fig. 3). However, the vertical variation of total colony counts was only statistically significant in December 2002 and April 2003 (ANOVA, $p < 0.05$). Total colony counts varied between 0.4 and 25 202 CFU ml^{-1} at the surface and between 64 and 25 410 CFU ml^{-1} in mid-water samples. Maximum numbers were recorded in July 2002 at Stns 2 and 7. A noticeable mid-water peak was also observed in July 2002 at Stn 2 (Fig. 3).

The concentration of faecal coliforms ranged from <1 to 2167 CFU 100 ml^{-1} at the surface and from <1 to 3167 CFU 100 ml^{-1} in mid-water samples (Fig. 3). The occurrence of peaks in abundance was in most cases only detected at 1 of the depth levels. However, with the exception of these extreme situations, differences between the surface and mid-water samples were not statistically significant (ANOVA, $p > 0.05$) when the whole data set was considered. The spatial pattern at the surface was characterized by distinct peaks at Stns 3, 8 and 9. In mid-water samples, very high values were observed at Stns 5 and 6 in December 2002 and in March 2003, respectively.

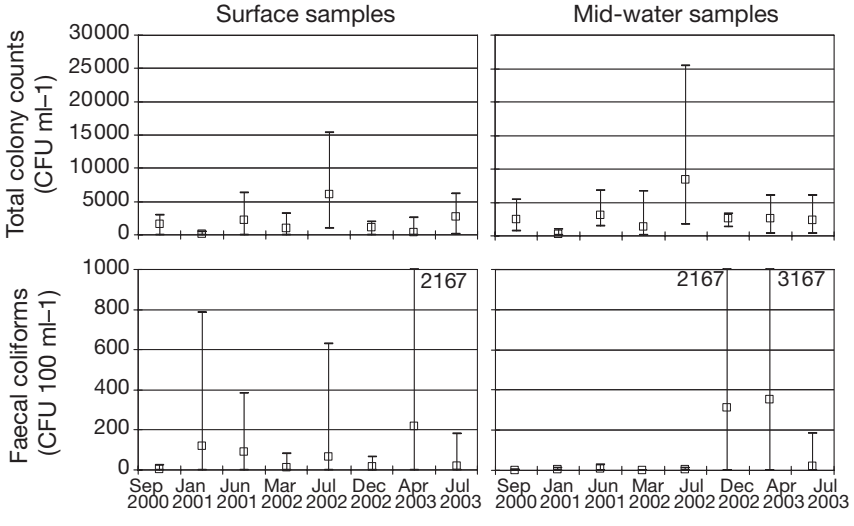

Fig. 3. Temporal variation in total colony counts and faecal coliforms in surface and mid-water samples. Minimum, maximum and average (□) values from each cruise calculated from results from all sampling sites

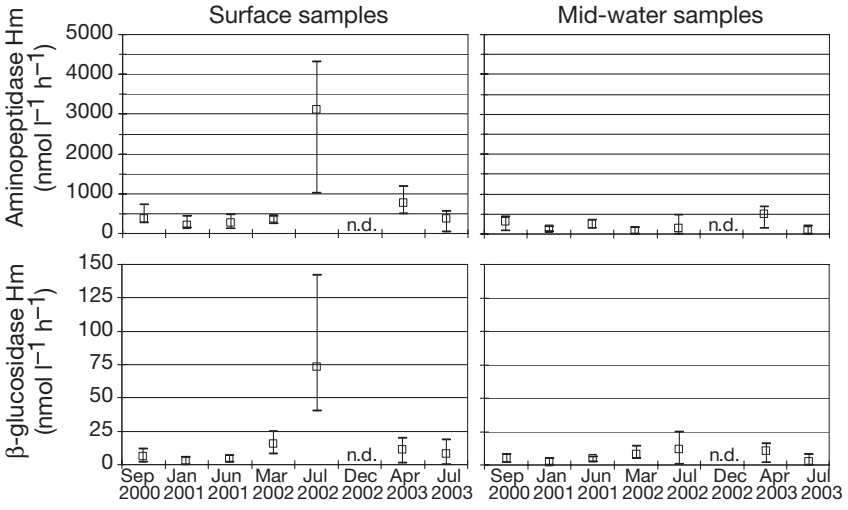

Fig. 4. Temporal variation in aminopeptidase Hm and β-glucosidase Hm in surface and mid-water samples. Minimum, maximum and average (□) values from each cruise calculated from results from all sampling sites

Bacterial heterotrophic activity

The descriptors of bacterial heterotrophic activity showed higher values at the surface (Figs. 4 & 5). Although vertical differences in aminopeptidase and β-glucosidase activities were not significant during all cruises, when data were analysed together, significant differences existed between surface and mid-water samples (ANOVA, $p < 0.05$). Aminopeptidase activity varied between 36 and 4311 nmol l^{-1} h^{-1} at the surface and between 3×10^{-2} and 672 nmol l^{-1} h^{-1} in mid-water samples (Fig. 4). β-glucosidase activity varied between 5×10^{-2} and 142 nmol l^{-1} h^{-1} at the surface and between 1×10^{-2} and 25 nmol l^{-1} h^{-1} in mid-water samples (Fig. 4). The highest activity rates were observed in July 2002. Spatially, the highest rates were recorded at Stns 1, 2 and 3 and the lowest mostly at reference Stn 9.

The rates of monomer incorporation (Fig. 5) were also significantly higher at the surface (ANOVA, $p < 0.05$) in September 2000 and in March and July 2002, but in pooled data, only the rate of glucose incorporation showed significant vertical variation (ANOVA, $p < 0.05$). The ranges of values registered in surface samples were from 0.04 to 5.59 nmol l^{-1} h^{-1} for leucine incorporation and from 0.02 to 5.15 nmol l^{-1} h^{-1} for the incorporation of glucose. The corresponding ranges in mid-water samples were from 1.4×10^{-4} to

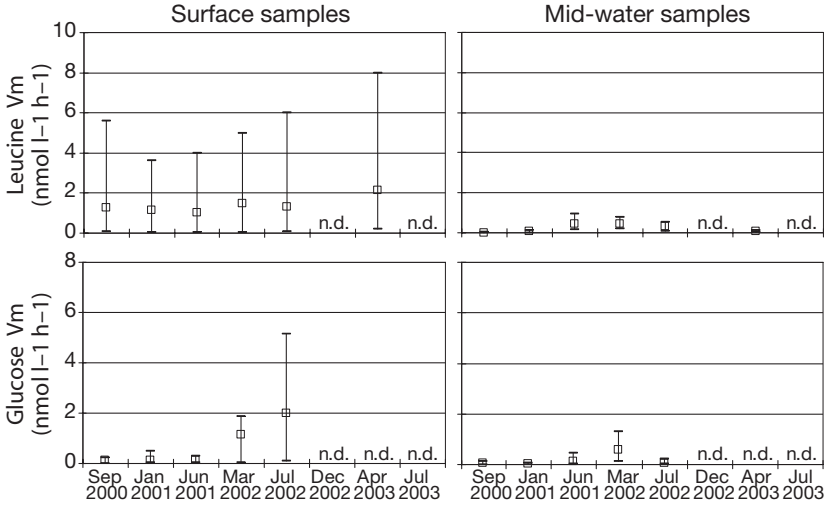

Fig. 5. Spatial and temporal variation in leucine Vm and glucose Vm in surface and mid-water samples. Minimum, maximum and average (□) values from each cruise calculated from results from all sampling sites

0.93 nmol l^{-1} h^{-1} and from 7.5×10^{-3} to 1.30 nmol l^{-1} h^{-1}, respectively. The maximum rates of monomer incorporation were observed at Stn 2 in July 2002, and minimal rates at Stn 9 in September 2000.

Correlation analysis

Significant correlations between microbiological parameters and linear distance to the mouth of the estuary or the outfall are presented in Table 2.

Chl *a* (log-transformed), aminopeptidase activity and total colony counts were significantly and negatively correlated with linear distances to the mouth of the estuary and to the outfall. On the contrary, the ratio between β-glucosidase activity and glucose incorporation was positively correlated with linear distances to both features. Leucine Vm (log-transformed) and faecal coliform abundance were negatively correlated with linear distance to the mouth of the estuary, but no significant correlation was observed with linear distance to the outfall.

Table 2. Results of correlation analysis (Pearson's coefficient) between microbiological variables and linear distance to mouth of the estuary or to the marine sewage outfall. Values of chlorophyll concentration and leucine Vm were log-transformed to increase the significance of the correlation. $^{*}p < 0.05$; $^{**}p < 0.01$; ns: not significant

	Linear distance (km) to: mouth of the estuary	outfall
ATP	−0.212* (n = 148)	ns
Log chl *a*	−0.297** (n = 146)	−0.182* (n = 146)
Aminopeptidase Hm	−0.212* (n = 130)	−0.146* (n = 130)
β-glucosidase Hm	ns	ns
Log leucine Vm	−0.242** (n = 121)	ns
Glucose Vm	ns	ns
β-glucosidase/glucose ratio	0.331** (n = 91)	0.273** (n = 91)
Aminopeptidase/leucine ratio	ns	ns
Colony counts	−0.331** (n =145)	−0.273** (n = 145)
Faecal coliforms	−0.198* (n = 101)	ns

Linear regression analysis

The results of the step-wise multiple regression analysis are presented in Table 3. No model could be found to describe the variability of faecal coliform abundance using the available set of independent variables. The variation of depth — alone or in combination with other variables — appeared to be a significant source of variability of the rates of bacterial heterotrophic activity and total colony counts. However, theses independent variables only explained a fraction (5 to 32%) of the total variability of microbiological parameters observed in this study.

DISCUSSION

Spatial patterns of bacterioplankton abundance and activity

The main goals of this study were to collate field evidence of the influence of a marine sewage outfall and plume of mesotrophic estuarine water on the abundance and activity of marine communities of northern Portuguese coastal waters, as well as on the sanitary quality of these waters (assessed by levels of indicator bacteria).

Microbial parameters fluctuated largely with site, depth and time, making it difficult to recognize spatial patterns. Linear distance to the outfall was significantly correlated with colony counts, aminopeptidase activity and the ratio between β-glucosidase activity and the rate of glucose incorporation. Surprisingly, the correlation with the concentration of faecal coliforms was not significant. The increase in the abundance of enteric bacteria in recipient waters is one of the most frequently reported effects of sewage discharge through marine outfalls. The treated effluent that is

Table 3. Regression equations for the explanation of variation in microbiological parameters obtained from step-wise multiple regression analysis. Dependent variables are Leu-AMPase (Hm of leu-aminopeptidase), β-GLCase (Hm of β-glucosidase), glucose Vm, leucine Vm, Leu-AMPase/leucine Vm ratio (ratio between rates of aminopeptidase activity and leucine incorporation), β-GLCase/glucose Vm ratio (ratio between rates of β-glucosidase activity and glucose incorporation), TCC17 (total colony counts in medium of salinity 17), FC (faecal coliforms). Independent variables are temperature (T), salinity (S), depth (D), and linear distance to mouth of the estuary (LDE). LDO: linear distance to outfall

Microbiological descriptor	Independent variables	Regression equation	Adjusted R^2
Leu-AMPase	D (β = –0.571; p = 0.000)	log Leu-AMPase = 2.714 – 0.029D	0.320
β-GLCase	D (β = –0.274; p = 0.002)	log β-GLCase = 0.930 – 0.016D	0.067
Leucine Vm	D (β = –0.578; p = 0.000) T (β = –0.442; p = 0.000) LDE (β = – 0.179; p = 0.040)	log leucine Vm = 2.876 – 0.037D – 0.214T – 0.044LDO	0.266
Glucose Vm	D (β = –0.728; p = 0.000) T (β = –0.444; p = 0.000) S (β = 0.394; p = 0.000)	log glucose Vm = –9.735 – 0.048D – 0.168T + 0.338S	0.309
Leu-AMPase/leucine Vm ratio	T (β = 0.377; p = 0.000) S (β = 0.294; p = 0.002)	log(Leu-AMPase/leucine Vm) = 0.410 + 0.166T + (1.26×10^{-5})S	0.203 0.111
β-GLCase/glucose Vm ratio	D (β = 0.350; p = 0.001)	log(β-GLCase/glucose Vm) = 1.466 + 0.016D	
TCC17	D (β = 0.234; p = 0.006)	log TCC17 = 2.852 + 0.018D	0.048
FC		No model	

discharged through the S. Jacinto outfall is mostly composed of industrial effluent from a paper mill, containing only a minor fraction (<20%) of domestic sewage. The concentrations of faecal coliforms in the treated sewage prior to discharge ranged between 2×10^3 and 145×10^3 CFU 100 ml^{-1} during the study period (data not shown), which is well below the typical range of 10^6 to 10^8 CFU 100 ml^{-1} (Hirn 1980, George et al. 2002). Sewage is discharged at a depth of approximately 15 m, and water circulation in the outfall area is mainly driven by tide and wind. Numerical models simulating the conservative dispersion indicate that, in spring tides, the plume tends to disperse very little and independently from wind direction. In neap tide conditions, the plume shows wider dispersion, especially with west or north-westerly wind. The dispersion of the coliform plume shows similar trends but, as a consequence of bacterial mortality, tends to be much more restricted to the vicinity of the outfall (Ramos et al. 2003). The lack of a significant correlation between the abundance of this group of indicator bacteria and distance to the outfall may result from their low abundance and reduced viability in what is essentially industrial sewage, combined with the rapid mortality in marine water (Sherwin et al. 2000, Noble et al. 2004); however, to a large extent it probably reflects a complex pattern of horizontal and vertical dispersion.

The abundance of culturable heterotrophic bacteria increased in the vicinity of the outfall. This can result from the combined effects of the direct discharge of heterotrophic bacteria and stimulation of the autochthonous community by the release of sewage-derived organic substrates. Aminopeptidase activity is usually closely related to bacterial abundance (Cunha et al. 2000, Zaccone et al. 2002). This activity has been reported to be largely unresponsive to some external effects, namely the influence of a fish farm effluent (Brown & Goulder 1999), but it can be inhibited by ammonia, nitrate or toxicants present in treated sewage (Montuelle & Vollat 1998). In this study, the effect of the increased availability of organic substrates seems to have overcome the influence of inhibitors eventually discharged through the outfall.

An important aspect of the influence of the sewage outfall on patterns of organic matter utilization by heterotrophic bacterioplankton is the increase of the ratio between polysaccharide hydrolysis (β-glucosidase) and monomer incorporation (glucose incorporation) with increasing distance from the outfall. Considering that the paper mill effluent is the major component of the treated sewage discharged through the S. Jacinto outfall, the tighter coupling between extracellular degradation of polysaccharides and glucose incorporation can be related to inputs of residual cellulose or cellulose-related products containing a relatively low proportion of monomers. The fingerprinting of the organic matter in the effluent revealed a high diversity of compounds, some of which are characteristic of the paper mill industry (A. Duarte pers. comm.). In this scenario, extracellular activity would assume a major role in the supply of simple substrates to a dense heterotrophic community, and would also limit the rates of degradation of sewage-derived organic compounds.

The effects of estuarine exports on the characteristics of plankton communities at the adjacent coastal area were more significant than the influence of the S. Jacinto marine sewage outfall. Linear distance to the mouth of the estuary correlated negatively with ATP and chl *a*, indicating that the estuarine plume is characterized by higher biomass of plankton. Similar to the effect of the sewage outfall, colony counts also increased with decreasing distance to the mouth of the estuary; however, in this case, the increase was accompanied by a significant increase in the concentration of faecal coliforms. A net flux of plankton and potential bacterial heterotrophic activity from the estuary to the ocean was previously demonstrated for this system (Cunha et al. 2003). The results obtained in the present study show that exports include faecal bacteria and that estuarine waters negatively influence the microbiological quality of coastal waters. However, only 14 samples from a total of 138 analysed failed to comply with the guide level of 500 CFU 100 ml^{-1} established by the European Union Bathing Water Quality Directive 76/160/EEC, and only 3 of these reached the mandatory value of 2000 CFU 100 ml^{-1}.

Estuarine waters also influenced the patterns of organic matter utilization. Aminopeptidase activity was more intense closer to the estuary, consistent with the spatial pattern of variation in colony counts. The parallel effect was not observed in β-glucosidase activity, an attribute less common to marine bacteria than aminopeptidase (Zaccone et al. 2002). However, and similar our observations from the vicinity of the sewage outfall, there was a significant decrease in the coupling between β-glucosidase activity and glucose incorporation with increasing distance from the mouth of the estuary. This effect might again be related to an unfavourable proportion of monomers in the aged and impoverished pool of organic matter that generally reaches the lower sections of estuaries (Raymond et al. 2000, Yamashita & Tanoue 2003).

Regulation of bacterial abundance and activity

One objective of this study was to estimate the relative importance of a marine outfall and an estuarine plume as sources of variability of the abundance and rates of heterotrophic activity of bacterial communities in a temperate coastal area. Two main results from the results of the multiple step-wise regression can be noted: (1) a fraction of 5 to 32% of the total variability could be explained by the set of independent variables available; (2) depth or depth-related factors such as light and primary productivity, rather than proximity to the estuarine plume or sewage outfall, are relevant sources of variability in this coastal ecosystem.

Variability in the rates of ectoenzymatic activity are mostly related to depth, which alone explains 32% of the variability of aminopeptidase activity and 7% of the variability of β-glucosidase activity. However, most of the variability remained unexplained, and is most probably related to the size and nature of the pool of organic matter and in the way in which it links to primary production.

Depth, temperature and salinity appear to be relevant factors in the regulation of rates of monomer incorporation, confirming that this activity is subject to marked seasonal fluctuation. Peaks in particulate ATP, colony counts, ectoenzymatic activity and monomer incorporation occurred during summer and were particularly marked in July 2002, matching the maximum chl *a* concentration. This can be interpreted as an indication that primary production modulates temporal profiles of variation in abundance and activity of heterotrophic bacterioplankton in this coastal area. The coupling between primary production and secondary bacterial activity most probably underlies the depth-related factors that emerged from the regression analysis as important sources of variability. The temporal fluctuation of faecal coliforms did not form a regular pattern, and some very high concentrations occurred in mid-water samples in December 2002 and April 2003 without any apparent relationship to other descriptors of bacterial abundance and activity. These fluctuations may be related to particular hydrodynamic conditions that may have altered the pattern of vertical and horizontal dispersion of the outfall emission and estuarine plume. The wind and tidal currents are major forcing mechanisms in this region. During north and north-westerly winds, the polluted plume is to a great extent limited to the source, whereas the plume may reach coastal beaches under the influence of westerly winds (Ramos et al. 2003). The complex temporal variation in the concentration of faecal coliforms may be related to sudden changes in wind direction and in the orientation of the contaminated plume.

Linear distance to the mouth of the estuary influences the rate of leucine incorporation, but not that of glucose incorporation. The size and composition of the DOM pool are dominant factors in the regulation of the relative abundance of the major heterotrophic bacterial groups, and in the way in which these groups use the available substrates, and most certainly underlie seasonal and spatial patterns (Kirchman et al. 2004). However, DOM quantification and characterization was not attempted in the present study.

Depth explained only 5% of the variability in total colony counts and, with the available set of independent variables, it was not possible to construct a model for variability in abundance of faecal coliforms. In the

first sampling campaign, dissolved organic carbon (DOC) was analysed in all samples (data not shown). If these data were incorporated into a regression model, DOC together with salinity would explain 46% of the variability in abundance of faecal coliforms. This can be interpreted as an indication of a concomitant dispersion of organic matter and bacteria, or that organic loading is affecting the survival of enteric bacteria in the environment such as observed by Chandran & Hatha (2005). Salinity is often cited to be a significant source of mortality of faecal bacteria in the environment (Monfort & Baleux 1994, Yang et al. 2000). However, in this study, salinity only varied between 30.4 and 36.5 and was not a relevant source of variability. This indicates that other factors, such as light intensity, particulate organic matter availability, toxicants and physical forcing by currents or wind, may be of major importance in the distribution and mortality of enteric bacteria.

CONCLUSIONS

The estuarine plume of Ria de Aveiro and sewage discharge from the S. Jacinto outfall were found to cause an increase in the rates of aminopeptidase activity and in the number of culturable bacteria, probably as a response to the availability of organic matter, in particular proteinaceous substrates. Rates of β-glucosidase activity or monomer incorporation did not reflect distance from the outfall or estuary, but our results indicate a tighter coupling between β-glucosidase activity and glucose incorporation in the vicinity of the sewage outfall and also of the estuarine plume, denoting an indirect affect of the changing quality of the available organic substrates. The estuarine plume of Ria de Aveiro was identified to be a major source of living plankton and enteric bacteria.

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