

RESEARCH ARTICLE

## Detection and dynamics of fecal indicator bacteria in two Tyrrhenian lagoons (Sabaudia and Orbetello, central Italy)

Annamaria Zoppini\*, Stefano Amalfitano

Water Research Institute, National Research Council (IRSA-CNR)  
Area della Ricerca Roma 1, Via Salaria Km 29,300, CP10, 00015 Monterotondo (Roma), Italy

\*Corresponding Author: Phone: +39 06 90672792; Fax: +39 06 90672787; E-mail: [zoppini@irsa.cnr.it](mailto:zoppini@irsa.cnr.it)

### Abstract

- 1 - Coastal lagoons are particularly vulnerable to strong anthropogenic pressure. These environments can act as sink for allochthonous material, including harmful bacteria, with the ability to reduce their impact to adjacent coastal waters.
- 2 - This study investigates fecal pollution impairment in two Italian coastal lagoons and adjacent coastal waters. We utilised methodological approaches to gain fast and more precise information on the contamination and viability of fecal indicator bacteria.
- 3 - The analyses of total coliforms (TC) and *Escherichia coli* (*E. coli*) were performed *in situ* by enzyme assays. *E. coli* live/dead cells were enumerated by immunofluorescence technique (combined with propidium iodide) for the specific detection of enteropathogenic serotypes (12 EPEC and 2 EIEC).
- 4 - Overall TC enzyme activity showed a high degree of temporal variation whereas *E. coli* enzyme activity peaked in summer in both lagoons (beta-glucuronidase, 84 and 47 nmol MUFL<sup>-1</sup>h<sup>-1</sup>, Sabaudia and Orbetello respectively). Beta-glucuronidase activity showed a high correlation with viable cells counts of *E. coli* enteropathogenic strains ( $p < 0.01$ ). Significantly lower degree of contamination was observed in the adjacent coastal waters.
- 5 - Our findings describe these lagoons as very fragile systems which hydro-morphological features allow a long time preservation of microbial contaminants, including pathogenic serotypes. These findings imply potential consequences for the human health as well as the need for protective measures of these environments.

**Keywords:** : fecal pollution, Tyrrhenian lagoons, human health risk, water quality

### Introduction

Fecal coliform pollution in the aquatic environment is a high priority problem worldwide representing a substantial human health risk (Haile et al., 1999).

Recent evidences suggest that lagoons can act as sink of harmful bacteria with the ability to reduce their impact to adjacent coastal waters (Steets and Holden 2003; Evanson and Ambrose 2006).

Coastal lagoons are quite widespread in the Mediterranean Region and in Italy 46 wetland

sites, including Sabaudia and Orbetello, have been included by UNESCO in the list of wetlands of international importance (Ramsar Convention 1971; <http://www.ramsar.org>). Sabaudia and Orbetello are threatened by pollution as located in areas extensively exploited for human activities (agriculture, cattle-breeding, fish farming and tourism) (i.e. Vollenweider et al., 1996; Lenzi et al., 2003, Innamorati and Melillo 2004). To date, published studies on Sabaudia and Orbetello report data on fecal pollution performed

by traditional methods recommended by the Italian legislation (Boccia et al. 1985; Nocciolini et al. 2000; ARPA-Lazio 2007). Concentrations of total coliforms bacteria (TC) and *Escherichia coli* (*E. coli*) have historically been used as indicators of fecal contamination (WHO 2003; APHA et al. 2005) although several limitations affect the most popular techniques based on culture and colony counting methods. The most important are: the long incubation time (up 96 h) that may result in delayed countermeasures when fecal pollution occurs; the poor detection of slow-growing or viable but not-culturable cells (VBNC) which still preserve metabolic activity and potential for virulence (Pommepuy et al. 1996) and ultimately the significant underestimation of bacterial cells enumerated by cultivation-based methods (Amann et al. 1995). Recent findings have enabled the development of reliable and fast tools to improve the knowledge on fecal indicator bacteria viability and abundance.

Fluorogenic substrates have been used to detect the presence or the activity of specific enzymes in aquatic systems (Hoppe, 1993, Zoppini et al. 2005). Specific ectoenzymes can be used for detection of TC (beta-D-galactosidase) and *E. coli* (beta-D-glucuronidase) through the utilization of culture media or kits incorporating fluorogenic or chromogenic substrates (Enzyme Substrate test, MPN; APHA, 2005, APAT-IRSA-CNR, 2003). However these specific properties of total coliforms and *E. coli* can also be exploited in rapid assays without any cultivation step to obtain an improved accuracy and a faster detection (Müller-Niklas and Herndl, 1992; Tryland et al. 1998; George et al. 2000; Garcia-Armisen et al. 2005).

The direct microscopic enumeration of *E. coli* cells by the immunological methods (Porter et al. 1996) allow more realistic counts than cultivation-based methods as well the detection of pathogenic serotypes (i.e.

Enteroinvasive, EIEC, and Enteropathogenic, EPEC) which are considered a significant and growing cause of human infection (Tozzi et al. 2003). In addition, the immunofluorescence technique combined with an indicator of the physiological state of the cell, propidium iodide (PI), allows the quantification of *E. coli* dead and live cells (Zacccone et al. 1995; Caruso et al. 2003).

The aim of this study was to evaluate the microbial pollution of the Sabaudia and Orbetello lagoons, describing the relationships occurring between environmental parameters and the viability of fecal indicator bacteria. Lagoon and coastal sites were monitored on seasonal cycle for *in situ* analysis of total coliforms and *E. coli* enzyme activities and live/dead cell abundance.

## Materials and Methods

### Study sites

The Sabaudia lagoon is located (Fig.1) on the southern coast of the Latium region (13°2'E, 41°20'N), within the Circeo National Park. It covers an area of 390 ha and a mean depth of 4 m (maximum depth 10 m). Wind action and low intensity tidal currents allow a limited water exchange mainly through Caterattino and Torre Paola channels (Perdicaro 1985). Seawater is occasionally forced into the lagoon by a pumping system located in the northern channel, whereas freshwater input is scarce, mainly consisting in drainage channels.

The Orbetello lagoon (Fig. 1) is located on the southern coast of the Tuscany region (42°30'N; 11°10'E) and it is one of the largest lagoons in the western Mediterranean (2525 ha). It consists of two communicating basins varying in depth between 1.5 to 2 m. The lagoon exchanges water with the Tyrrhenian Sea via three channels (Fibia, Nassa and Ansedonia) and it receives occasional supply of freshwater from river Albegna and drainage channels. Seawater exchange is ensured in summer by a pumping system located in the



Figure 1. Orbetello (left) and Sabaudia (right) coastal lagoons are on the Italian Western coast. The expanded views of the lagoons on the lower level show channels, sampling sites and location of main towns.

Nassa and Fibia channels forcing the water to flow through the basins.

*Sample collection*

Sampling sites (Fig.1) were selected based on results from previous studies (Boccia et al. 1985; Noccioloni et al. 2000) and comprised both lagoon and coastal waters. The sampling (monthly and bimonthly in summer) was carried out between June and December 2003, therefore included dry and wet season as well as the period of maximum and minimum tourist affluence. An additional sampling campaign was carried out between

June and December 2004, at the Sabaudia lagoon site S1 and seawater site S4, for *E. coli* enumeration by immunofluorescence technique and specific ectoenzyme activity assessment.

*Water sample analyses*

A total of 71 samples were collected in the Sabaudia and Orbetello lagoon-coastal systems. All the material entering in contact with the samples was acid washed (1N, HCl) and repeatedly rinsed with ultra pure water (Millipore, Milli-Q). All samples were placed on ice in a cooler and

analysed within few hours from collection. Temperature and salinity were determined *in situ* using probes (WTW-LF191; LS1/T-1.5). Inorganic phosphorus ( $\text{PO}_4$ ) concentration was determined by the molybdenum blue spectrophotometric method and inorganic nitrogen ( $\text{NO}_3$ ) concentration by the cadmium reduction method with a colorimetric determination of nitrite produced. Dissolved organic carbon (DOC) concentration was determined by high temperature catalytic oxidation (HTCO) using a Shimadzu TOC-5000 A analyser (Pettine et al., 2001). Chlorophyll *a* (Chl *a*) was extracted in 90% acetone and the concentration was measured by spectrophotometry according to Lorenzen (1967).

#### *Microbial enumeration*

Total bacterial cell abundance (TBA) was determined by direct microscopic counts after 4',6-diamidino-2-phenylindole (DAPI) staining.

*E. coli* total cell enumeration was performed following the indirect immunofluorescence method combined with the fluorescent dye propidium iodide (PI) to distinguish dead cell (Zaccone et al. 1995; Caruso et al. 2003). An aliquot of fresh sample (3-8 mL of lagoon water and 60-100 mL of seawater) was stained in the dark with PI ( $0.01 \mu\text{g mL}^{-1}$  fin. conc.) and then filtered onto black polycarbonate filters (Nuclepore,  $0.2 \mu\text{m}$  pore-size, 25 mm dia.). After repeatedly rinsing by sterile phosphate buffer (pH 7.2), the cells collected on the filter were labelled with Murex (Remel Inc., USA) *E. coli* agglutinating sera (1:60 and 1:80 dilution was used for lagoon and seawater samples, respectively) with a specificity for a total of 14 *E. coli* serotypes which 12 EPEC (polyvalent 2: O26, O55, O111, O119, O126; polyvalent 3: O86, O114, O125, O127, O128; polyvalent 4: O44, O112, O124, O142) and 2 EIEC (O112 and O124). The filter was then repeatedly rinsed with PB and incubated with

goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1:120 and 1:160 dilution was used for lagoon and seawater samples respectively) for 30 min. The filter was air-dried and mounted on microscope slides with non-fluorescent oil (R.P. Cargille Lab, USA). Total *E. coli* cells (minimum 60 cells per sample on two replicated filters) were counted by epifluorescence microscopy (1000x magnification, blue light filter BP 450-490nm, FT 510 and LP 515nm). Dead *E. coli* cells (PI-positive) were identified in the same field, as red fluorescing, by switching to the green light filter (BP 515-560nm, FT 580 and LP 590).

#### *Microbial enzyme activity*

The *in situ* measurement of the potential beta-D-galactosidase (beta-gal) and beta-D-glucuronidase (beta-glu) ectoenzyme activities allowed an indirect evaluation of the TC and *E. coli* pollution respectively. In this study the protocol proposed by George et al. 2000 was modified according to experimental tests. To assay beta-gal activity 100-500 mL of water was filtered through  $0.22 \mu\text{m}$  pore-size, 47mm diameter polycarbonate filters (Nuclepore). The filters were recovered and placed in 17 mL of sterile 0.05 M phosphate buffer (PB, pH 7.2) supplemented with 0.05% of sodium laurylsulfate. Four subsamples (1.7 mL) were placed in 2 mL vials, supplemented with 0.3 mL of 4-methylumbelliferyl-beta-D-galactoside (1.18 mM final conc.) solution and incubated in a shaking water bath at  $37^\circ\text{C}$  for 3 h. To assay beta-glu activity the filter was repeatedly rinsed (see above) with 0.05 M PB (pH 6.9). Four subsamples were incubated at  $44^\circ\text{C}$  with 0.3 mL of 4-methylumbelliferyl-beta-D-glucuronide (0.48 mM final conc.). Fifty  $\mu\text{l}$  of 2 M NaOH solution was added to each 2 mL aliquot to obtain a pH > 10 before measuring the fluorescence (Jasco spectrofluorometer, Mod. FP-6200) at an excitation wavelength of 365 nm and emission at 446 nm (Hoppe,

1993). Hydrolysis rates were calculated using standards of known 4-methylumbelliferone (MUF) concentrations from 20 to 1000 nM and the ectoenzymatic activity was expressed in nmol MUF liberated L<sup>-1</sup> h<sup>-1</sup>. The autohydrolysis rate of the substrate was checked in the same assay conditions utilising boiled samples and subtracted from the MUF liberated.

The redundancy analysis (RDA) was performed with the Statistica 7.0 software package (StatSoft Inc., Tulsa, OK). The biplots were calculated by the data correlation matrix, considering all physicochemical parameters reported in Table 2 as active variables. Beta-galactosidase and beta-glucuronidase activities were used as supplementary variables. All variables were normalized using division by their standard deviations.

## Results

### *Physical, chemical and biological features*

Generally, the morphological and hydrological conditions of these systems determined the variability in physical, chemical and biological parameters (Table 1). The shallow water column and a reduced exchange with the sea determine in both lagoons a seasonal cycle with maxima values of the temperature (maximum 31°C at Sabaudia) and salinity (maximum 46.9 at Orbetello) in summer. Drastic hypoxia conditions affected Sabaudia in early fall (0.8 mg DO L<sup>-1</sup>, S2 site) and Orbetello in the summer period (2.7 mg DO L<sup>-1</sup>, O2 site). A drastic drop of the pH value was observed in early fall sampling at Sabaudia (mean S1, S2; S3 sites 7.61±0.5). Overall, both lagoon waters were rich in nutrients (NO<sub>3</sub> and PO<sub>4</sub>), largely exceeding coastal water concentrations. In the Sabaudia lagoon sites PO<sub>4</sub> concentration increased linearly in summer (Pearson correlation, r=0.75; p<0.01; n=15) up to rise to the maximum value in September (0.49 μM PO<sub>4</sub> at S1 site), whereas NO<sub>3</sub>

concentration linear increase (Pearson correlation, r=0.91; p<0.01; n=12) occurred between October and December (max conc. 26.7 μM NO<sub>3</sub>, S2 site). The Orbetello seawater sites showed sporadic high NO<sub>3</sub> concentrations (max 15.4 and 10.6 μM, O1 and O4 sites respectively) with overall a mean value higher than that in the lagoon. Large DOC concentration characterised the water of both lagoons with different trends. Orbetello showed the most elevated DOC concentrations (t-test, p<0.001) characterised by a scattered trend (max. 884 μM DOC, O3 site, in October) whereas at Sabaudia it followed an increasing trend (max. 503 μM DOC, S1 site, in July) in the period of time comprised between June and July (Pearson correlation, r=0.77, p<0.05, n=12). The range of DOC concentrations in the Sabaudia seawater site was typical of oligotrophic waters (<137 μM DOC) whereas in the Orbetello seawater sites it showed high variability with concentrations up to 399 μM DOC (O4 site). The range of Chl *a* concentrations measured in this study describes the elevated trophic level of these basins and the oligotrophic state of the nearby seawater sites as usually reported for the Tyrrhenian coastal waters (Pettine et al. 2007).

Mean Chl *a* concentration (15.5±12.2 μg L<sup>-1</sup>) obtained at the Sabaudia lagoon sites significantly overcame (t-test, p<0.001) the values obtained at Orbetello (5.4±7.1 μg Chl *a* L<sup>-1</sup>). Peaks were observed in both lagoons in July, with maxima values at Sabaudia (43 μg Chl *a* L<sup>-1</sup>, S2 site). Orbetello seawater site O4 makes exception to this trend as sporadic Chl *a* (12 μg Chl *a* L<sup>-1</sup>) and DOC peak concentrations (399 μM DOC) were observed in late September and in December respectively.

The analysis of the TBA contributes to describe the higher trophic level of the Sabaudia lagoon with respect to Orbetello (Table 1). In the former TBA followed a linear increasing trend (Pearson correlation r=0.82,

Table 1 - Physical, chemical and biological characteristics of the Sabaudia and Orbetello coastal lagoons and adjacent seawater sites. Numbers are arithmetic means of values measured during the 2003 survey, with standard deviations (in brackets) and range of values. Stars denote values found to be significantly different between lagoons at  $p < 0.001$  using Students' unpaired t-tests. ( $\text{NO}_3$  = nitrate;  $\text{PO}_4$  = phosphate; DOC = dissolved organic carbon; Chl *a* = chlorophyll *a*; TBA = total bacterial cell abundance; beta-gal = beta-galactosidase and beta-glu = beta-glucuronidase, ectoenzyme activities indicative of TC and *E. coli* contamination respectively).

	<b>Sabaudia</b>		<b>Orbetello</b>	
	<i>lagoon</i> S1,S2,S3	<i>seawater</i> S4	<i>lagoon</i> O2,O3	<i>seawater</i> O1,O4
Temperature (°C)	24.4 (6.1) 12.6-31.0	22.3 (3.6) 16.1-26.5	22.2 (7.2) 6.7-29.1	21.6 (4.6) 11.8-27.5
Salinity	36.3 (1.4) 33.3-38.4	38.5 (0.3) 38.1-39.0	41.5 (3.1) 34.6-46.9	37.8 (0.6) 36.7-39.0
pH	8.2 (0.4) 7.6-8.7	8.2 (0.06) 8.1-8.3	8.3 (0.4) 8.0-8.8	8.3 (0.1) 7.9-8.3
DO (mg L <sup>-1</sup> )	7.4 (0.8) 0.8-10.0	6.7 (1.5) 4.87-8	5.5 (2.1) 2.7-8.3	6.0 (0.8) 4.6-7.1
$\text{NO}_3$ (μM)	5.4 (7.6) 0-27	1.4 (1.1) 0.1-3.2	1.24 (1.8) 0.1-7.0	3.48 (4.22) 0.2-15
$\text{PO}_4$ (μM)	0.07 (0.1) 0.0-0.49	0.03 (0.04) 0.0-0.13	0.17 (0.37) 0-1.6	0.09 (0.09) 0.01-0.25
DOC (μM)	373 (84)*** 236-503	119 (12) 102-137	535 (208) 75-884	165 (80) 80-399.2
Chl <i>a</i> (μg L <sup>-1</sup> )	15.5 (12.2)*** 2.6-43.00	1.2 (1.2) 0.01-3.6	5.4 (7.1) 0.9-30.6	1.6 (3.1) 0.09-12.9
TBA (10 <sup>6</sup> cells mL <sup>-1</sup> )	6.5 (2.7)*** 2-11.0	0.6 (0.3) 0.1-1.2	4.2 (1.8) 1.5-7.8	0.6 (0.4) 0.2-1.4
beta-gal (nmol MUF L <sup>-1</sup> h <sup>-1</sup> )	33.6(20.8)*** 90.0-7.7	2.2(2.4) 7.5-0.2	53.3(39.6) 141.9-0.01	17.7(28.3) 100-0.43
beta-glu (nmol MUF L <sup>-1</sup> h <sup>-1</sup> )	18.1(19.0)*** 84.1-5.1	0.87(1.0) 2.7-0.1	7.9(12.3) 46.5-0.18	5.2(7.7) 28.1-0.2

$p < 0.001$ ) starting from June and reaching the maximum value in late September ( $11 \times 10^6$  cells mL<sup>-1</sup>, site S3). No temporal trends ( $p = 0.36$ ) were observed in the Orbetello lagoon sites in which the peak value was observed in October ( $8 \times 10^6$  cells mL<sup>-1</sup>, site O3). All seawater sites showed TBA values up to 10 folds lower than those observed inside the lagoons.

#### *Total coliforms and E. coli detection by ectoenzyme activities*

Significant TC (beta-gal) and *E. coli* (beta-glu) ectoenzyme activities were detected in both lagoon waters (Table 1) although different trends were observed. Sabaudia lagoon displayed a mean TC ectoenzyme activity of  $34 \pm 21$  nmol MUF L<sup>-1</sup> h<sup>-1</sup> with peaks (Fig. 2) occurred at the S1 site in

late July ( $90 \pm 2$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>) and late fall ( $64 \pm 3$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>). Overall, a decreasing trend of beta-gal activity was observed from S1 to S3 site. Moreover, in the seawater site (S4) a significantly lower TC activity was observed (Table 1). In the Orbetello lagoon, TC enzyme activity measurements showed a scattered trend (Table 1) and the highest rate in the O2 site (Fig. 2) in late fall sampling ( $142 \pm 1$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>). Large variability of the rates were observed also in the seawater (Table 1) with some exceptions (Fig. 2).

*E. coli* ectoenzyme activity in the Sabaudia lagoon (Table 1) followed a trend (Fig. 3) similar to that observed with beta-gal activity with the most elevated rates in late July (beta-glu activity peak  $84$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>, S1 site). In this period of time *E. coli* ectoenzyme activity followed an increasing trend from South (S3) to North (S1). The adjacent seawater site (S4) showed

significantly lower beta-glu rates (Table 1). Noteworthy to observe that the seawater site did not show significant increase of beta-glu activity in concomitance with the peaks observed within the lagoon (Fig. 3). Orbetello lagoon sites showed, similarly to Sabaudia, the highest *E. coli* ectoenzyme activity (Fig. 3) in the July samplings (max beta-glu activity  $47 \pm 1$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>, O2 sites) although the mean value was lower (Table 1). Overall, the adjacent seawater sites exhibited high variability of fecal contamination (Table 1). Beta-glu activity peaks were observed at the O4 and O1 sites in June and July (max beta-glu activity  $28 \pm 0.1$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>). In particular in the case of June sampling, beta-glu activity in the seawater sites was higher than in the nearby lagoon sites, testifying that these coastal waters received allochthonous inputs independently from the lagoon. The redundancy analysis (RDA) synthetically

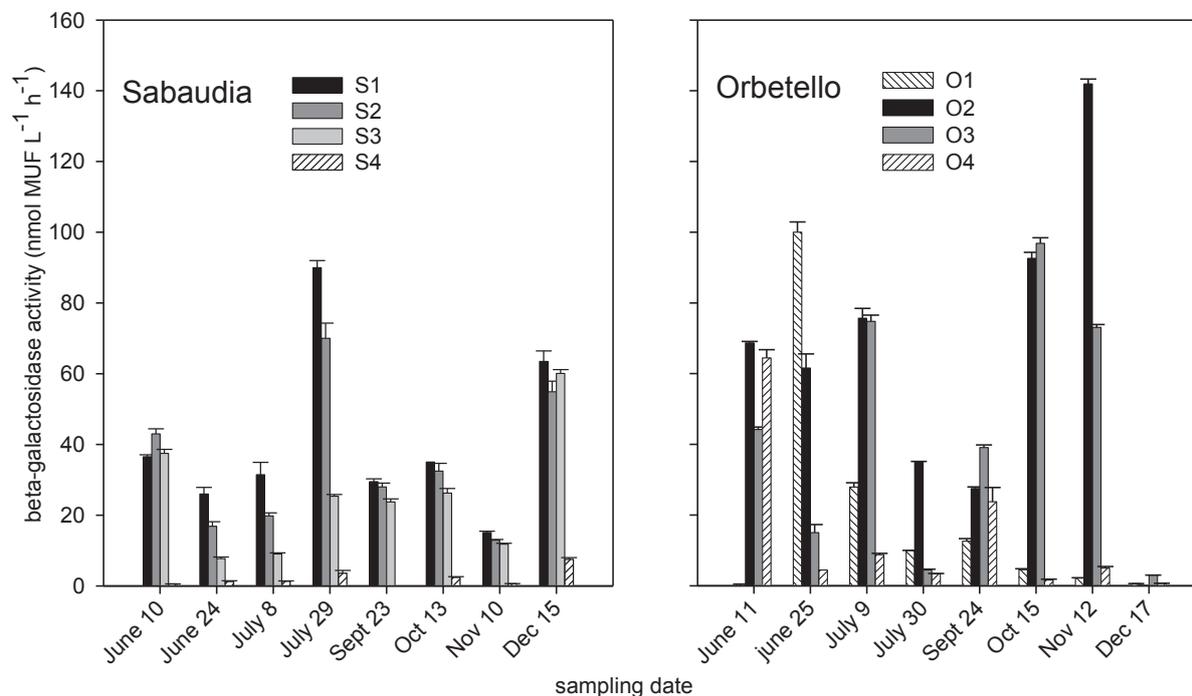


Figure 2. Changes in the total coliform ectoenzyme activity (beta-galactosidase) for Sabaudia and Orbetello coastal lagoons (greyscale bars) and seawater (pattern bars) during 2003 survey; see Fig. 1 for the location of sites. Means±standard deviation of four replicates.

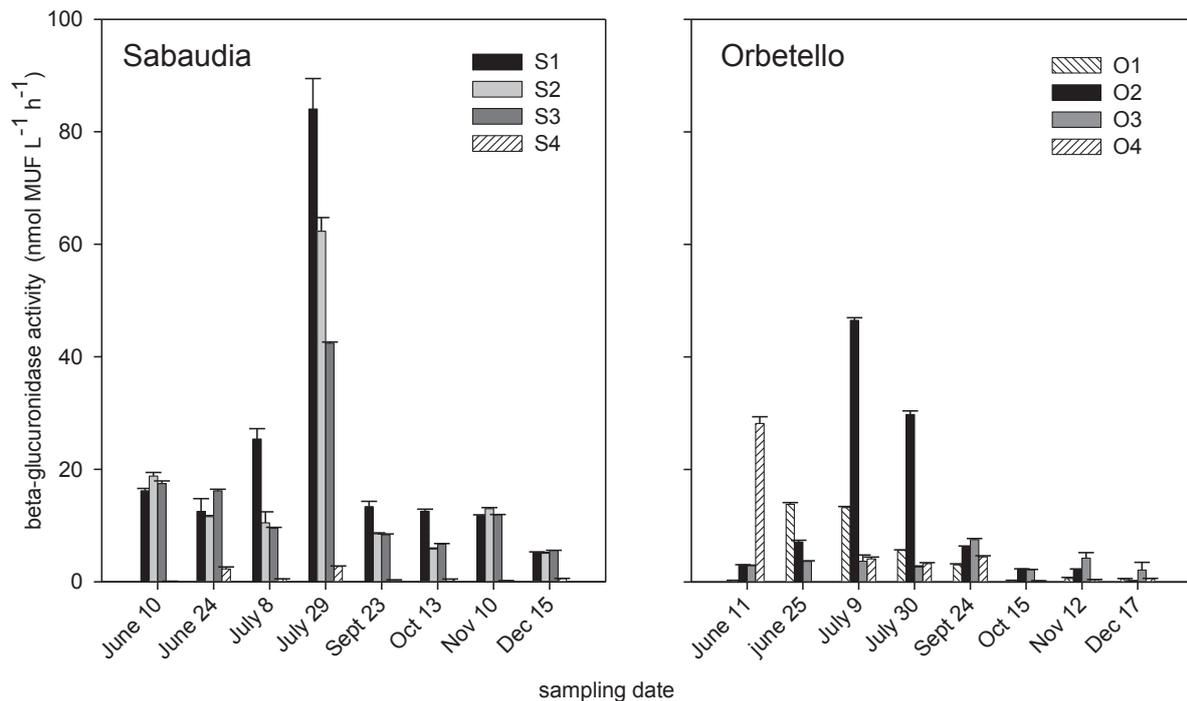


Figure 3. Changes in *E. coli* ectoenzyme activity (beta-glucuronidase) for Sabaudia and Orbetello coastal lagoons (greyscale bars) and seawater (pattern bars) during 2003 survey; see Fig. 1 for the location of sites. Means±standard deviation of four replicates.

represented the patterns of the environmental variability. In both lagoons, summer and autumn/winter conditions formed two separated clusters and were easily identifiable (Fig. 4).

All summer samples were characterised by high values of temperature, salinity, pH and low DO and NO<sub>3</sub> concentrations. This trend was associated to the increase of DOC concentration as well as of beta-glu activity, projected onto the factor space as supplementary variables.

#### *E. coli* live/dead cells enumeration

The application of immunofluorescence technique, combined with PI stain, allowed us to quantify the cell abundance of enteropathogenic *E. coli* strains in the range comprised between  $0.6 \pm 0.1 \times 10^3$  and  $7.0 \pm 0.2 \times 10^3$  cells mL<sup>-1</sup>, and the contribution

of dead cells from not detectable up to 22% (Fig. 5). In late July, we observed the highest beta-glu enzyme activity ( $12 \pm 0.3$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>) associated to relatively high *E. coli* cell abundance ( $4.6 \pm 0.2 \times 10^3$  cells mL<sup>-1</sup>) which contribution of dead cells was in the lower range of values (6%). The peaks of *E. coli* cell abundance, occurred in October in the lagoon ( $7.0 \pm 0.2 \times 10^3$  cells mL<sup>-1</sup>) and seawater ( $2.0 \pm 0.1 \times 10^3$  cells mL<sup>-1</sup>) sites, were characterised by the highest contribution of *E. coli* dead cells (22%). In this sample we also observed a relatively elevated ectoenzyme activity in the lagoon (beta-glu  $9.2 \pm 0.5$  nmol MUF L<sup>-1</sup> h<sup>-1</sup>). Afterwards *E. coli* cell abundance dropped to its minimum value observed in this survey. Seawater site (S4) exhibited a high degree of variability in *E. coli* cell abundance (mean  $0.43 \pm 0.74 \times 10^3$  *E. coli* cells mL<sup>-1</sup>).

Noteworthy to observe that beta-glu activity in the 2004 survey was in a lower range with

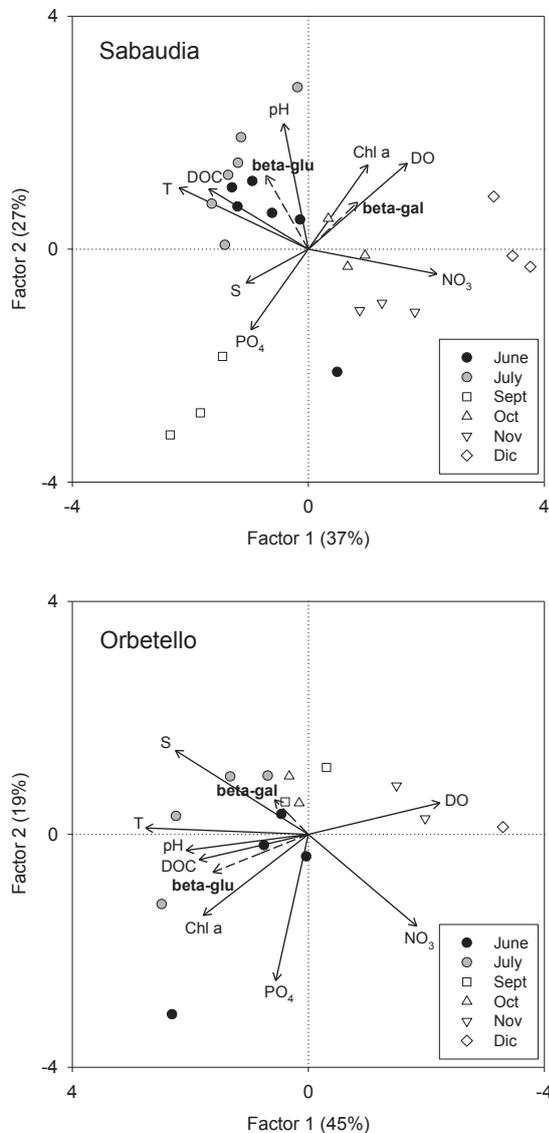


Figure 4. Biplots from a redundancy analysis (RDA) of ectoenzymatic activities as a function of the physical and chemical parameters (active variables, solid line). Beta-galactosidase and beta-glucuronidase activities were projected onto the factor space as supplementary variables (dashed lines). The angle between arrow pairs is a measure of the correlation between the variables that they represent. The length of each arrow is proportional to the contribution of the corresponding variable to the analysis.

respect that measured in the 2003 (Figs 3 and 5), when also significantly lower (paired t-test  $p < 0.01$ ) mean DOC concentration ( $336 \pm 44$  and  $420 \pm 57$   $\mu\text{M}$  DOC respectively, S1 site) was observed.

### Discussion

Incidence of coliform enzyme activities was constantly observed in both lagoons while, overall, contamination in coastal water was very low and even undetectable.

Elevated concentrations of nutrients and biomass found in these lagoons describe a trophic level much over that observed in the adjacent coastal waters. These findings let infer that particular hydromorphological conditions associated to anthropogenic pressure, allow Sabaudia and Orbetello to act as sink for allochthonous material including microbial contaminants.

Notwithstanding, both lagoons were affected by fecal coliform pollution, they significantly differed (t-test,  $p < 0.001$ ) for the *E. coli* ectoenzyme activity. In these terms, Sabaudia resulted the most polluted and our investigations found abundant live *E. coli* cells belonging to enteropathogenic serotypes. This contamination (range  $0.6\text{-}5.4 \times 10^3$  *E. coli* cells  $\text{mL}^{-1}$ ) can be compared to that obtained by Caruso et al. (2002) in coastal waters defined as affected by high levels of fecal pollution. Coliform contaminations up to 180000 and 27000 MPN  $100 \text{ mL}^{-1}$  total and fecal coliforms respectively, were previously reported in the Sabaudia lagoon (Boccia et al. 1985). Recent data from the local environmental agency (ARPA-Lazio, 2007) on *E. coli* contamination report a mean concentration value of 373 CFU  $100 \text{ mL}^{-1}$  in the period December 2006 - August 2007. The discrepancy observed between cell abundances obtained by immunofluorescence (Fig. 5) and the traditional methods can be due to methodological constrains, as plate counts fails to detect the fraction of the viable but non culturable (VBNC) bacteria (Caruso

et al. 2002). The statistical analysis of log transformed *E. coli* total cell abundance (live+dead) vs log transformed ectoenzyme activity showed significant correlation between these two parameters (Log beta-glu = 0.72 Log *E. coli* total cells -1.82,  $r^2= 0.56$ ;  $n=14$ ,  $p=0.005$ ). Even a more significant correlation was obtained when only viable

*E. coli* cell numbers were considered (Log beta-glu = 0.85 Log viable *E. coli* cells-2.13,  $r^2= 0.96$ ,  $n=14$ ,  $p<0.001$ ), indicating the high specificity of the enzyme reaction for viable *E. coli* cells. The slope of the regression line below 1 was similar to that reported in previous studies where the abundances were determined by plate counts (George et al.

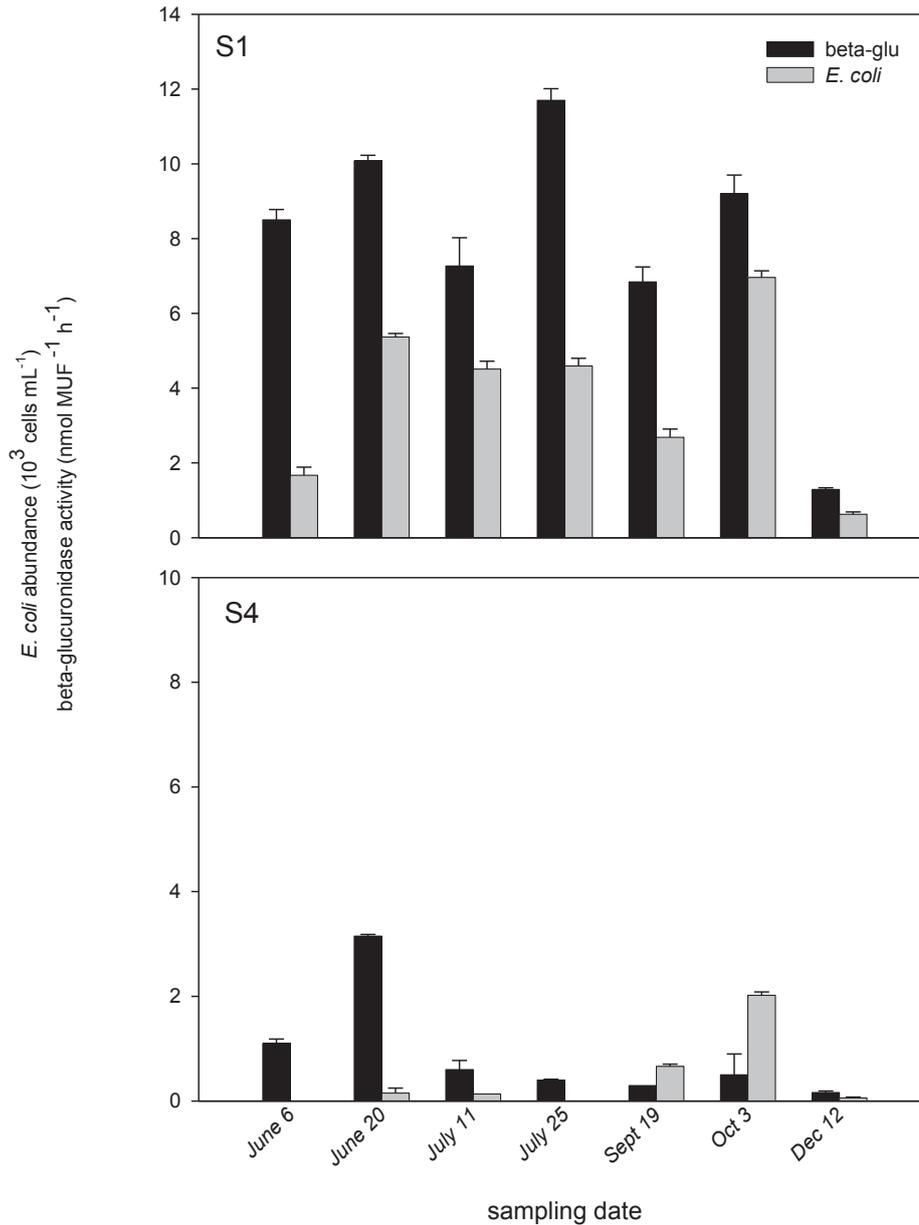


Figure 5. Changes in *E. coli* ectoenzyme activity (beta-glucuronidase) and *E. coli* total cell abundances (live+dead) of enteropathogenic strains for Sabaudia lagoon (S1) and seawater (S4) sites during 2004 survey. See Fig. 1 for the location of sites. Means  $\pm$  sd of 3 replicates.

2000; Farnleitner et al. 2001). Analysing only data gathered from lagoon samples we obtained a linear regression ( $r^2=0.94$ ,  $p<0.01$ ) which slope was equal to 1, indicating a rather constant beta-glu ectoenzyme activity and *E. coli* cell abundance ratio ( $2.3\pm 0.5$  pmol MUF h<sup>-1</sup>cell<sup>-1</sup>). Interestingly, this ratio was lower and highly variable when analysing seawater samples only ( $1.1\pm 1.3$  pmol MUF h<sup>-1</sup> cell<sup>-1</sup>). Hence specific beta-glu ectoenzyme activity can vary as function of the environmental conditions up to reach significantly lower ratios in the oligotrophic coastal waters where stressing factors (i.e. severe nutritional limitation and light) might result in a reduction of cell viability (Fiksdal et al. 1994; Petit et al. 2000; An et al. 2002). Immunofluorescence technique provides a spectrum of pathogenic serotypes including EIEC and EPEC, which strains O111, O26, O55 and O86 were recently isolated in Italian patients affected by Shiga Toxin-*Escherichia coli* infection, hemorrhagic colitis and hemolytic uremic syndrome (Tozzi et al. 2003). These findings let us infer that the

contamination observed in the Sabaudia lagoon by *E. coli* (up to  $5.4\pm 0.3 \times 10^3$  live cells mL<sup>-1</sup>) might threaten the health of exposed persons.

In both lagoon-seawater systems TC enzyme activity showed to be negatively correlated to salinity (Table 2). Rainfall events can influence this result by flushing into the lagoon coliforms associated to terrigenous material. Analysing all data set of this survey only 56% of the variance in the log-transformed beta-gal activity is explained by the variation in log-transformed beta-glu activity ( $p<0.001$ ), reflecting the wide number of environmental genera included in the TC group. Unlike the coliform group, *E. coli* are almost exclusively of fecal origin. Hence in these lagoons monitoring fecal contamination by TC activity may not provide an ultimate proof of fecal pollution. *E. coli* ectoenzyme activity was correlated to DOC concentration ( $p<0.01$ ) in both systems (Table 2) as well to variables such as the temperature, Chl *a* and TBA. After the release of *E. coli* into the environment,

Table 2 - Correlation coefficients (Pearson's) between TC (beta-gal) and *E. coli* (beta-glu) ectoenzyme activities, and the characteristics of lagoon and seawater sites (2003 survey, number of samples 32; ns=not significance; \*  $p<0.05$ ; \*\* $p<0.01$ ). See tab 1 for symbols.

	Sabaudia		Orbetello	
	beta-gal	beta-glu	beta-gal	beta-glu
T	ns	0.51**	ns	0.47*
salinity	-0.53**	ns	-0.32*	ns
pH	ns	ns	ns	ns
NO <sub>3</sub>	0.40*	ns	ns	ns
PO <sub>4</sub>	ns	ns	ns	ns
DOC	0.54**	0.59**	0.50**	0.70**
Chl <i>a</i>	0.87**	0.69**	ns	0.41*
TBA	ns	0.34*	0.42*	0.39*
beta-gal	-	0.89**	-	0.70**

a long survival of the coliform cells is a function of interacting biological and physical factors (Noble et al. 2004; Bergholz et al. 2011). Large amounts of suspended matter may contribute to transform lagoons in suitable harbours able to protect *E. coli* cells from death. As matter of fact in this study changes in dissolved organic carbon represent a predicting variable to detect *E. coli* activity as 60-70% of it depends on DOC concentration (Table 2). It is known that organic matter enhances the survival of these microorganisms (Lyons et al. 2010) by reducing osmotic pressure and enhancing the attenuation of solar radiation (Troussellier and Legendre, 1989; Pommepuy et al. 1996). Other Authors described also as *E. coli* die-off rate increases with increasing of the temperature, a high level of dissolved oxygen and an elevated pH (Noble et al. 2004; Foppen and Schijven 2006). In addition solar radiation, predators, lack of nutrients and salinity can affect coliform viability (An et al. 2002). According to these reports, in summer we would expect low *E. coli* abundances and low viability because the occurrence of concomitant adverse environmental conditions (the most elevated value of salinity, temperature, solar radiation and the lowest pH). In this survey, the high occurrence of *E. coli* cell abundance in summer, in concomitance with elevated concentrations of DOC and nutrients (e.g.  $PO_4$ ) may be rather the signal of direct discharges into the lagoon, when the highest record of tourists is usually found (Latina APT source; Innamorati and Melillo 2004). The impact of the overpopulation can be relevant at Sabaudia, where the municipality does not have yet completed the sewage system, and at Orbetello, where the local sewage treatment plant has an insufficient depuration capacity (Corsi and Focardi 2002; Specchiulli et al. 2008; ARPA-Lazio 2007). However episodes of water contamination can also occur away from summer period

(Fig. 5). In these shallow basins we can hypothesise that the resuspension of sediment can be another pattern of contamination for the water column. The adsorption and sedimentation are known processes by which fecal bacteria can accumulate and survive in the sediments at levels 100-1000 times higher than in overlying waters (Irvine and Pettibone, 1993; Cabrill et al. 1999; Steets and Holden 2003; Evanson and Ambrose 2006).

### Conclusions

The application of enzyme and immunofluorescence techniques greatly improved the information with respect to existing data on microbial pollution in these areas. We could provide a precise information on the presence of vital cells of *E. coli* enteropathogenic strains which preserve metabolic activity and potential for virulence. This knowledge is particular important for lagoon systems which features (i.e. low water circulation, reduced depths and the elevated trophic level) allow the preservation of microbial contaminants at living state for long time from their release into the environment. The presence of live cells belonging to pathogenic serotypes can pose a serious human health risk in these systems potentially signalling the presence of other human pathogens (Orskov and Orskov, 1981; Guzewich and Mores, 1986). These results suggest that much attention has to be paid in monitoring water systems for microbial contamination by supporting standard methods by more advanced methodologies.

### Acknowledgments

This study has been carried out as part of the research program "Development of Criteria to evaluate the environmental risk associated with pathogens in aquatic environments" funded by the National Institute for Occupational Safety and Prevention (ISPESL).

Special thanks to Maura Manganelli for her effort to promote and coordinate this Project. We gratefully acknowledge Alberto Puddu for the helpful revising of the manuscript; Rosaria Arnone for the *E. coli* immunofluorescence cell counts; Raffaele Scenati for chlorophyll *a* analysis and Orbetello sampling.

## References

- Amann RJ, Ludwig W, Schleifer KH 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* 59: 143-169.
- An YJ, Kampbell DH, Breidenbach GP 2002. *Escherichia coli* and total coliforms in water and sediments at lake marinas. *Environmental Pollution* 120: 771-778
- APHA, AWWA, WEF 2005. Standard methods for the examination of water and wastewater 21st edition, Washington DC.
- ARPA-Lazio, 2007. Relazione sullo stato del lago di Sabaudia. Risultati del monitoraggio di indagine Dicembre 2006- Agosto 2007. Technical Report, Sezione Provinciale di Latina.
- Bergholz PW, Jesse DN, Buckley DH 2011. Environmental patterns are imposed on the population structure of *Escherichia coli* after Fecal Deposition. *Applied and Environmental Microbiology* 77, 211-219
- Boccia A, De Giusti M, Arpaia F 1985. Aspetti batteriologici e valutazioni igienico-sanitarie del sistema costiero. Proceedings Seminario Informativo sui risultati del progetto 'Laghi Costieri. Provincia di Latina, University Sapienza (Ed), Roma, Italy 1-23
- Cabrill C, Donald R, Snelling J, Foust R, Southam G 1999. The impact of sediments fecal coliform reservoir on seasonal water quality in Oak Creek, Arizona. *Water Research* 33: 2163-2171
- Caruso G, Crisafi E, Mancuso M 2002. Development of an enzyme assay for rapid assessment of *Escherichia coli* in seawaters. *Journal of Applied Microbiology* 93: 1-9.
- Caruso G, Mancuso M, Crisafi E. 2003. Combined fluorescent antibody assay and viability staining for the assessment of the physiological states of *Escherichia coli* in seawaters. *Journal of Applied Microbiology* 95: 225-233.
- Corsi I, Focardi S 2002. Nonylphenol in a lagoon environment: 4-nonylphenol and nonylphenol ethoxylates in fish tissues. *Bulletin of Environmental Contamination and Toxicology* 68: 908-914.
- Evanson M, Ambrose RF 2006. Sources and growth dynamics of fecal indicator bacteria in a coastal wetland system and potential impacts to adjacent waters. *Water Research* 40: 475-486.
- Farnleitner AH, Hocke L, Beiwil C, Kavka GG, Zechmeister T, Kirschner AKT, Mach RL, 2001. Rapid enzymatic detection of *Escherichia coli* contamination in polluted river water. *Letters in Applied Microbiology* 33: 246-250.
- Fiksdal L, Pommepuy M, Caprais MP, Midttun I 1994. Monitoring of faecal pollution in coastal waters by use of rapid enzymatic techniques. *Applied and Environmental Microbiology* 60: 1581-1584.
- Foppen JWA, Schijven JF 2006. Evaluation of data from the literature on the transport and survival of *Escherichia coli* and thermotolerant coliforms in aquifers under saturated conditions. *Water Research* 40: 401-426
- Garcia-Armisen T, Lebaron P, Seravis P 2005. Beta-D-glucuronidase activity assay to assess viable *Escherichia coli* abundance in freshwaters. *Letters in Applied Microbiology* 40: 278-282.
- George I, Petit M, Servais P 2000. Use of enzymatic methods for rapid enumeration of coliforms in freshwaters. *Journal of Applied Microbiology* 88: 404-413.
- Guzewich, J. J and D. L. Morse. 1986. Sources of shellfish in outbreaks of probable viral gastroenteritis: implications for control. *Journal of Food Protection* 49:389-394.
- Haile RW, White JS, Gold MR, Cressey C, McGee RC, Millikan A, Glasser N, Harawa C, Ervin P, Harmon J, Harper J, Dermand J, Alamillo K, Barrett M, Nides G, Wang Y 1999. The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology*, 10: 355-363.
- Hoppe H 1993. Use of fluorogenic model substrates for extracellular enzyme activity measurement of bacteria. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (Eds), *Handbook of Methods in Aquatic Microbial Ecology*. Lewis Publisher, Boca Raton, FL, 423-431.
- Innamorati M, Melillo C 2004. Studio della Laguna di Orbetello: ecologia ed aspetti economici. Laboratorio di Ecologia, Dipartimento di Biologia Vegetale Università di Firenze, <http://eprints.unifi.it/archive/00001260/>
- Irvine KN, Pettibone GW 1993. Dynamics of indicator bacteria populations in sediment

- and water near a combined sewer outfall. *Environmental Technology* 14: 531-542
- Lenzi M, Palmieri R, Porrello S 2003. Restoration of the eutrophic Orbetello lagoon (Tyrrhenian Sea, Italy): water quality management. *Marine Pollution Bulletin* 46:1540-1548.
- Lorenzen CJ 1967. Determination of chlorophyll and phaeopigments: spectrophotometric equation. *Limnology Oceanography* 12: 343-346.
- Lyons M.M, Ward JE, Gaff H, Hicks RE, Drake JM, Dobbs FC 2010. Theory of island biogeography on a microscopic scale: organic aggregates as islands for aquatic pathogens. *Aquatic Microbial Ecology* 60: 1–13
- Miller WA, Atwill ER, Gardner IA, Miller MA, Fritz HM 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. *International Journal of Parasitology* 35: 673–684
- Müller-Niklas C, Herndl G 1992. Activity of fecal coliform bacteria measured by 4-methylumbelliferyl-beta-D-glucuronide substrate in the northern Adriatic sea with special reference to marine snow. *Marine Ecology Progress Series* 89: 305-309.
- Noble RT, Lee IM, Schiff KC 2004. Inactivation of indicator micro-organisms from various sources of faecal contamination in seawater and freshwater. *Journal of Applied Microbiology* 96: 464-472.
- Nocciolini S, Spadafina L, Vacri MR, Bacci E 2000. Simple bacterial index for relative water quality: preliminary application in the Orbetello lagoon (Tuscany, Italy). *Chemosphere*, 41: 1065-1069.
- Orskov F, Orskov I. 1981. Enterobacteriaceae, p. 340–352. In A. I. Broude (ed.), *Medical microbiology and infectious diseases*. The W. B. Saunders Co., Philadelphia, Pa.
- Perdicaro R 1985. Effetti della riduzione del carico eutrofizzante sulle condizioni chimico-fisiche del lago di Sabaudia. (Campagna di rilevamenti del 1984). *Nova Thalassia*, 7: 343-348
- Petit M, George I, Servais P 2000. Survival of *Escherichia coli* in freshwater: b-D-Glucuronidase activity measurements and characterization of cellular states. *Canadian Journal of Microbiology* 46: 679–684
- Pettine M, Capri S, Manganelli M, Patrolecco L, Puddu A, Zoppini A 2001. The Dynamics of DOM in the Northern Adriatic Sea. *Estuarine Coastal Shelf Science* 52: 471–489
- Pettine M, Casentini B, Fazi, S, Giovanardi F, Pagnotta R 2007. A revisitation of TRIX for trophic status assessment in the light of the European Water Framework Directive: Application to Italian coastal waters. *Marine Pollution Bulletin* 54: 1413-1426.
- Pommepuy M., Butin M, Derrien A, Gourmelon M, Colwell RR, Cormier M 1996. Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. *Applied Environmental Microbiology* 62: 4621-4626.
- Porter J, Deere J, Pickup R, Edwards C 1996. Fluorescent probes and flow cytometry: new insights into environmental bacteriology. *Cytometry* 23: 91-96.
- Specchiulli A, Focardi S, Renzi M, Scirocco T, Cilenti L, Breber P, Bastianoni S 2008. Environmental heterogeneity patterns and assesment of trophic levels in two Mediterranean lagoons: Orbetello and Varano, Italy. *Science of Total Environment* 402: 285-298
- Steets BM, Holden PA 2003. A mechanistic model of runoff-associated fecal coliform fate and transport through a coastal lagoon. *Water Research* 37: 589-608.
- Troussellier M, Legendre P 1989. Dynamics of fecal coliform and culturable heterotroph densities in an eutrophic ecosystem: stability of models and evolution of these bacterial groups. *Microbial Ecology* 17: 227-235.
- Tozzi A, Caprioli EA, Minelli F, Gianviti A, De Petris L, Edefonti A, Montini G, Ferretti A, De Palo T, Gaido M, Tizzoni G 2003. Shigatoxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988–2000. *Emerging Infectious Diseases* 9: 106–108.
- Tryland I, Fiksdale L 1998. Enzyme characteristics of beta-D-galactosidase and beta-D-glucuronidase positive bacteria and their interference in rapid methods for detection of water borne coliforms and *Escherichia coli*. *Applied Environmental Microbiology* 64: 1018-1023.
- Vollenweider RA, Rinaldi A, Viviani R, Todini E 1996. Assessment of the state of eutrophication in the Mediterranean sea. Technical Reports Series No. 106 Food and Agriculture Organization of the United Nations, Mediterranean Action Plan Med Pol, United Nations Environment Programme. Athens, MAP

- WHO 2003. Guidelines for safe recreational waters. Volume 1, *Coastal and fresh waters*. World Health Organization Ed., Geneva. pp. 220
- Zaccone R, Crisafi E, Caruso G 1995. Evaluation of fecal pollution in coastal Italian waters by immunofluorescence. *Aquatic Microbial Ecology* 9: 79-85.
- Zoppini A, Puddu A, Fazi S, Rosati M, Sist P 2005. Extracellular enzyme activity and dynamics of bacterial community in mucilaginous aggregates of the northern Adriatic Sea. *Science of Total Environment* 353: 270-286.