

# Survival of faecal indicator bacteria in tropical estuarine waters (Bangpakong River, Thailand)

A.A. Bordalo<sup>1</sup>, R. Onrassami<sup>2</sup> and C. Dechsakulwatana<sup>3</sup>

<sup>1</sup>Laboratory of Hydrobiology, Institute of Biomedical Sciences, Porto, Portugal, <sup>2</sup>Faculty of Public Health and <sup>3</sup>Institute of Marine Science, Burapha University, Chonburi, Thailand

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**Aims:** To investigate the survival of cultivable bacteria in the tropical Bangpakong estuary (Eastern Thailand) under different salinities and light conditions.

**Methods and Results:** Dark and light microcosm experiments using membrane diffusion chambers were carried out under three different experimental conditions, namely (i) low salinity, (ii) progressive mixing with brackish water and (iii) fast mixing with high salinity water spiked with raw urban sewage. Faecal coliforms declined faster than faecal enterococci, as shown by survival  $T_{90}$  values ranging from  $82.2 \pm 4.2$  to  $14.5 \pm 0.8$  h and  $97.5 \pm 0.4$ – $20.6 \pm 1.2$  h, respectively. The survival of freshwater heterotrophic bacteria was higher but variable ( $121.2 \pm 5.0$ – $30.1 \pm 14.3$  h), whereas that of heterotrophic marine bacteria was rather stable ( $81.5 \pm 4.2$ – $44.6 \pm 2.5$  h).

**Conclusions:** Overall survival was higher in low salinities. Light had a further deleterious effect, since it accelerated the decay of faecal indicators, particularly in high salinities. Faecal enterococci had a higher resistance to environmental conditions compared with faecal coliforms.

**Significance and Impact of the Study:** This study is relevant to the understanding of the behaviour of different faecal indicator bacteria and the optimization of sewage treatment plants aimed at the reduction and/or elimination of faecal load discharged into estuarine waters submitted to salinity variations.

## INTRODUCTION

Faecal coliform (FC) bacteria and faecal enterococci (FE) have been widely used as indicators of water contamination by humans and other warm-blooded animals (APHA 1992; Bordalo 1993) and have been included in water quality standards in different parts of the world (European Union 75/44/EEC; USEPA 1986 Water Quality Criteria and Thailand NEB1984 Standard). Particular attention has been devoted to the survival of faecal indicator bacteria (FIB) for sanitary reasons (Colwell 1993). On the other hand, it is known that FE can survive longer in adverse conditions than FC due to the nature of their bacterial membrane (Evison 1989; Okpookwasili and Akujobi 1996). Light (particularly u.v. radiation), temperature, salinity, heavy metals, preda-

tion and competition have a deleterious effect on the integrity of FC and FE (El-Sharkawi *et al.* 1989; Evison 1989; Lim and Flint 1989; Gonzales *et al.* 1990; Bordalo 1993; Mezrioui *et al.* 1995; Baudisová 1997). However, little is known about the behaviour of FIB in tropical environments, particularly in estuaries, since most studies have been performed in temperate to cold locations.

The Bangpakong estuary is the end-member of the most important catchment basin in the eastern part of Thailand ( $14^\circ$  N). Due to a monsoon regime, two distinct seasons are well established. From December to May, the dry season (DS), the total rainfall presently averages 486 mm and the river discharge reaches its lowest values ( $21 \text{ m}^3 \text{ s}^{-1}$ ), in the vicinity of the river mouth. From June to November, the wet season (WS), rainfall and river flow increase dramatically to 939 mm and  $512 \text{ m}^3 \text{ s}^{-1}$ , respectively (Boonphakdee *et al.* 1999; Bordalo *et al.* 2001). During the DS the tidal estuarine boundaries are pushed about 150 km upstream of

Correspondence to: A.A. Bordalo, Laboratory of Hydrobiology, Institute of Biomedical Sciences, Lg. Abel Salazar, 2, 4099-003 Porto, Portugal (e-mail: bordalo@icbas.up.pt).

the river mouth (limit of salt intrusion by a dam) and during the rainiest period of the year the entire system is dominated by fresh to very low salinity waters (0–3 practical salinity units (psu)). The estuarine water temperature in the estuary averages 32 °C and is not significantly different between seasons (Bordalo *et al.* 2001).

The largest urban conglomerations are located by the main course of the river, particularly in the lower part of the water basin. The urban sewage/drainage system is generally of low efficiency. In addition, poultry and pig farms are widely spread along the banks of the river. Sewage treatment is limited to a few communities of the 1.3 million inhabitants within the catchment basin. The river is also a source of water for human consumption, irrigation, animal and fish/shrimp farming and for navigation. Phytoplankton and water hyacinth blooms and seasonal water pollution outbreaks are common, including in the estuarine and coastal areas (NEB 1986; Sojisporn 1995).

In this study, the survival of selected sewage bacterial populations was investigated in membrane diffusion chambers (MDCs) spiked with diluted raw urban sewage. Three different experiments were carried out in order to simulate the three major conditions prevailing in the Bangpakong estuary during the year: (i) low salinity during the WS; (ii) mixing with brackish water at the onset of the DS and (iii) fast mixing with high salinity coastal water during the DS.

## MATERIALS AND METHODS

### Inoculum preparation

Raw sewage was obtained at an urban sewage plant, 2 h prior to the initiation of the experiment. It was diluted fivefold with 0.2 µm filtered estuarine water of variable salinity, depending on the particular experiment, in order to obtain an initial FC concentration of 10<sup>6</sup> cfu 100 ml<sup>-1</sup>.

### Microcosm

Each microcosm consisted of a 5-l (25 × 20 × 10 cm) 3-mm thick clear Plexiglas MDC. The brand used was 90% u.v. transparent (Rhom & Hass GS, Philadelphia, PA, USA). Eighteen holes were drilled in each MDC and to each a 0.45-µm, 47-mm diameter polycarbonate membrane filter was fitted by means of an adapter. In this way, the passage of water but not of particles larger than the stated pore size, including Bangpakong-bond bacteria and protozoans, was allowed. The chambers had a tightly closed lid, large enough to allow the withdrawal of samples and introduction of probes. The MDCs were previously exposed to u.v. light for 24 h and filled with the appropriate sewage inoculum. The design was similar to that described in Mezrioui *et al.* (1995).

### Incubation

Triplicate MDCs were submerged in two 400-l tanks filled with natural estuarine water. One was exposed to natural sunlight and the second kept in the dark. The water inside the tank was thoroughly mixed by means of two submerged water pumps, thus preventing the settlement of particles and eventually hypoxia in the lower end of the tank water column. At the onset of the experiment and periodically thereafter (every 12–24 h) samples were withdrawn for bacterial counts. Incubations lasted between 93 and 127 h and were stopped when any of the FIB counts reached 0 cfu 100 ml<sup>-1</sup>. Temperature and salinity (expressed as psu) inside the chambers and in the tanks were measured twice a day with a probe (4200; Jenway, Felsted Dunmow, UK) in order to assess eventual diurnal variations. The experiments were carried out between August 2000 and March 2001.

### Bacterial counts

Typical FC colonies were counted after plating 0.1 ml decimal dilution or filtration through sterile 0.45-µm membrane filters onto mFC Agar (0677; Difco), depending on the expected final numbers, followed by 24 h incubation at 44.5 °C. The FE were assayed after dilution or filtration on KF Agar (0496; Difco) following a 24-h incubation of the plates at 35 °C. Nutrient agar (NA) colonies were enumerated on NA (0001; Difco) and marine agar (MA) bacteria on the same medium but supplemented with 35 g (w/v) of natural unpurified salt from the Bangpakong estuary. Counting was performed after a 4-d incubation at 25 °C. Regardless of the experiment, 89% of FC bacteria in raw sewage were *Escherichia coli* ( $n = 90$ ). Identification was carried out on the basis of ability to produce gas due to lactose fermentation and to produce indole in liquid peptone medium at 44.5 °C.

### Statistical analysis

The survival of FIB was analysed, by means of the  $T_{90}$  procedure, as the time necessary for a reduction of 1 log (90%) in the original count assayed by least square regression. Trend analysis was performed by least square regression using time as the independent variable. ANOVA was used to assess differences due to treatments and for comparison purposes. Differences in the data were considered statistically significant at the 95% confidence level.

## RESULTS

The characteristics of raw sewage used for the preparation of the inocula in the MDC experiments are presented in Table 1. Although the experiments were phased in time,

**Table 1** Characteristics of raw sewage used for the preparation of the inocula

Experiment	Conductivity ( $\mu\text{S cm}^{-1}$ )	pH	N-NO <sub>3</sub> (mg l <sup>-1</sup> )	N-NH <sub>4</sub> (mg l <sup>-1</sup> )	N-NO <sub>2</sub> (mg l <sup>-1</sup> )	PO <sub>4</sub> (mg l <sup>-1</sup> )	BOD <sub>5</sub> (mg l <sup>-1</sup> )	NA (log <sub>10</sub> cfu ml <sup>-1</sup> )	MA (log <sub>10</sub> cfu ml <sup>-1</sup> )	FC (log <sub>10</sub> cfu 100 ml <sup>-1</sup> )	FS (log <sub>10</sub> cfu 100 ml <sup>-1</sup> )
Low salinity	573	6.65	11.81	84.59	0.023	5.36	126	6.80	5.89	6.87	6.68
Progressive mixing	576	6.71	13.35	89.15	0.037	8.30	195	6.75	5.76	6.75	6.72
Fast mixing	573	6.78	14.16	87.20	0.036	9.37	200	6.80	5.81	7.00	6.70

NA, Nutrient agar bacteria; MA, marine agar bacteria; FC, faecal coliforms; FE, faecal enterococci; cfu, colony forming units.

according to the season, the sewage characteristics were rather similar for all the variables studied (ANOVA,  $P > 0.25$ ).

### Low salinity experiment

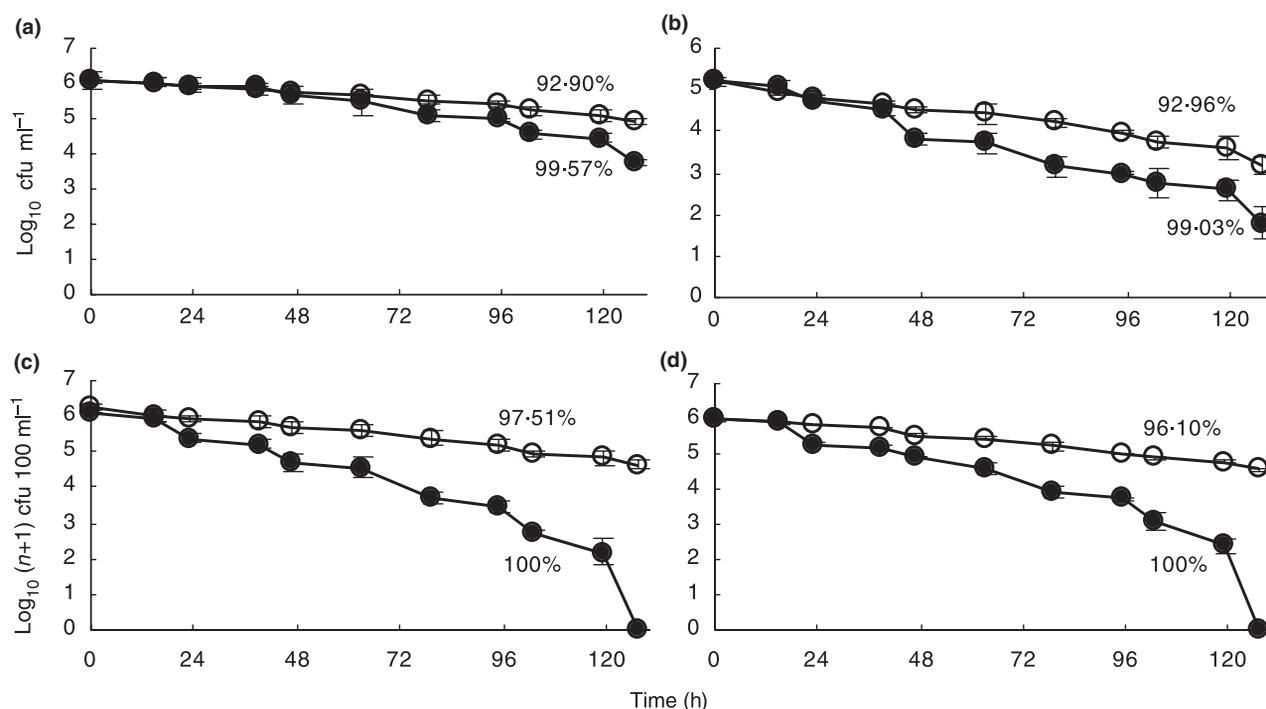
This experiment was conducted during the WS and was designed to simulate the mixing of sewage with very low salinity estuarine water (0.8 psu). The salinity inside the MDCs (0.7 psu) was similar to that in the tank and remained constant throughout the 127 h experiment. No differences were found between light and dark treatments (ANOVA,  $P > 0.25$ ). The temperature ranged from  $28.6 \pm 0.0$  °C at the onset of the experiment to  $33.8 \pm 0.1$  °C in the light-exposed MDCs and  $34.6 \pm 0.1$  °C in the dark MDCs by the end of the experiment. The temperature rise throughout the experiment was only statistically significant in the light microcosms ( $R^2 = 0.661$ ,  $P < 0.005$ ). In the dark microcosms, although temperature increased over time, no statistically significant trend was established.

At  $T_0$  the concentration of heterotrophic bacteria isolated on NA was one order of magnitude higher than MA counts (Fig. 1). The numbers were comparable during the initial 63 h (for NA) and 23 h (for MA); after this period, the abundance of bacteria in light-exposed MDCs declined more rapidly than in dark chambers. The differences between treatments were highly significant (ANOVA,  $P < 0.0001$ ). The removal rates for both NA and MA bacteria were in excess of 99% in the light treatment and ~92% in the dark. Thus, the survival of bacteria protected from the influence of sunlight was higher.  $T_{90}$  values ranged from  $70.6 \pm 2.9$  h for MA and  $121.2 \pm 0.5$  h for NA in the dark to  $45.0 \pm 0.8$  h for MA and  $72.7 \pm 6.3$  h for NA in the light-exposed MDCs (Table 2).

No appreciable lag period ( $> 18$  h) could be found for FIB (Fig. 1c and d). Their numbers steadily declined from an initial concentration of  $6.09 \pm 0.03$  log<sub>10</sub> cfu 100 ml<sup>-1</sup> for FC and  $6.00 \pm 0.01$  log<sub>10</sub> cfu 100 ml<sup>-1</sup> for FE to zero per 100 ml in the light-exposed MDCs after a 127-h incubation (100% reduction). However, in the dark treatment, not only was the decline less abrupt, but the removal rates were less dramatic (97.5% for FC and 96.1% for FE). Thus, a strong light effect on the survival of FIB in very low salinity water was detected. Averaged  $T_{90}$  survival values in the dark ( $82.0 \pm 4.2$  h for FC and  $97.5 \pm 0.4$  h for FE) were more than double those in the light-exposed MDCs (Table 2).

### Progressive mixing brackish water experiment

This experiment was designed to simulate the progressive mixing of sewage in brackish water during the onset of the DS. Raw sewage was mixed with 0.2  $\mu\text{m}$  filtered low salinity



**Fig. 1** Effect of time on the abundance of bacteria inside 5-l membrane diffusion chambers in the slow mixing experiment. (a) Nutrient agar bacteria; (b) marine agar bacteria; (c) faecal coliforms; (d) faecal enterococci. ●, Exposure to natural sunlight; ○, dark treatment. The bars represent 95% confidence level for triplicates

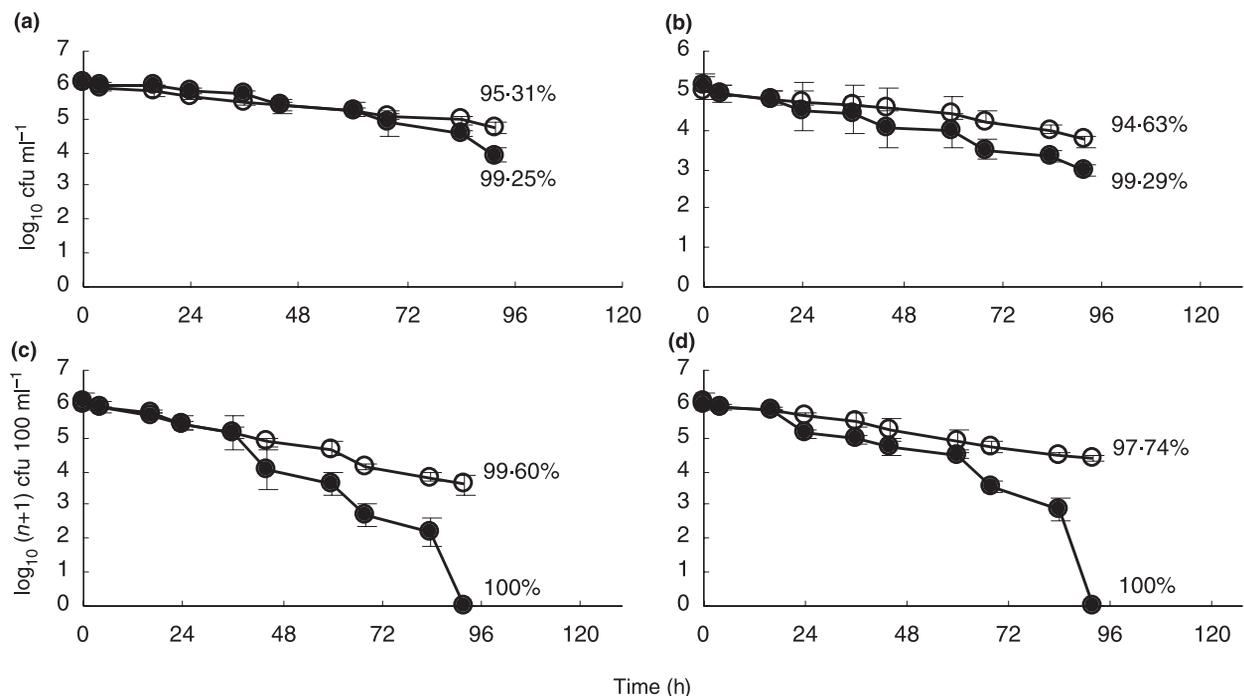
**Table 2** Averaged  $T_{90}$  survival rates for nutrient agar (NA), marine agar (MA), faecal coliform (FC) and faecal enterococci (FE) bacteria ( $h \pm 95\%$  confidence level)

Experiment	Treatment	NA	MA	FC	FE
Low salinity	Light	72.7 $\pm$ 6.3	45.0 $\pm$ 0.8	37.1 $\pm$ 2.9	40.9 $\pm$ 0.4
	Dark	121.2 $\pm$ 5.0	70.6 $\pm$ 2.9	82.0 $\pm$ 4.2	97.5 $\pm$ 0.4
Progressive mixing	Light	61.1 $\pm$ 3.8	44.6 $\pm$ 2.5	27.4 $\pm$ 2.5	33.7 $\pm$ 2.1
	Dark	71.0 $\pm$ 1.3	81.5 $\pm$ 4.2	54.5 $\pm$ 3.8	73.0 $\pm$ 8.8
Fast mixing	Light	30.1 $\pm$ 14.3	52.5 $\pm$ 6.7	14.5 $\pm$ 0.8	20.6 $\pm$ 1.2
	Dark	60.7 $\pm$ 7.1	70.6 $\pm$ 15.5	21.3 $\pm$ 1.3	31.6 $\pm$ 2.5

estuarine water with an initial salt content of 1.9 psu. All MDCs were submerged in high salinity natural estuarine water (31.0 psu). Throughout the experiment, the salinity inside the microcosms slowly increased linearly ( $R^2 = 0.974$ ,  $P < 0.001$ ) up to 14.2 psu in the light-exposed chambers and 18.8 psu in the dark chambers. The increasing rate in the latter treatment reached 4.4 psu  $d^{-1}$  whereas in the former it was lower at 3.2 psu  $d^{-1}$ . The temperature ranged from  $34.1 \pm 0.9$  °C at the beginning of the experiment in the light MDCs ( $33.0 \pm 0.2$  °C in the dark) to  $34.4 \pm 0.4$  °C ( $33.3 \pm 0.6$  °C in the dark) after a 92-h incubation. Although no increase in temperature occurred during the experiment, important diel fluctuations were

found with daily  $\Delta T$  up to 4 °C in the dark microcosms and 6 °C in the light-exposed MDCs.

An initial 36 h lag phase for NA bacteria was observed (12 h for MA bacteria) followed by a sharp decrease (Fig. 2a and b). At the end of the experiment, the loss of NA bacteria reached 99.25% in the light-exposed chambers but only 95.31% in the dark; for MA bacteria the rates were 99.29 and 99.63%, respectively. Thus, the  $T_{90}$  survival rate was higher in the dark treatment and reached 71.0  $\pm$  0.1 h for NA and 81.5  $\pm$  4.2 h for MA bacteria. On the other hand, the  $T_{90}$  values for chambers exposed to sunlight averaged 61.1  $\pm$  3.8 h and 44.6  $\pm$  2.5 h, respectively, for nutrient and MA heterotrophs.



**Fig. 2** Effect of time on the abundance of bacteria inside 5-l membrane diffusion chambers in the progressive mixing experiment. (a) Nutrient agar bacteria; (b) marine agar bacteria; (c) faecal coliforms; (d) faecal enterococci. ●, Exposure to natural sunlight; ○, dark treatment. The bars represent 95% confidence level for triplicates

The FIB in the progressive mixing experiment decreased in a similar fashion as in the previous experiment. From an initial concentration of  $6.08 \pm 0.24 \log_{10} \text{ cfu } 100 \text{ ml}^{-1}$  for FC and  $5.98 \pm 0.06 \log_{10} \text{ cfu } 100 \text{ ml}^{-1}$  for FE, the 100% elimination in the light treatment was reached earlier than in the dark, at 92 h (Figs 2c and d). Concomitantly, the removal of faecal bacteria was slower in the dark experiment, particularly for FE (97.74%). As regards FC survival, averaged values in dark MDCs were exactly double those of light MDCs (Table 2), whereas for FE the differences were more than double.

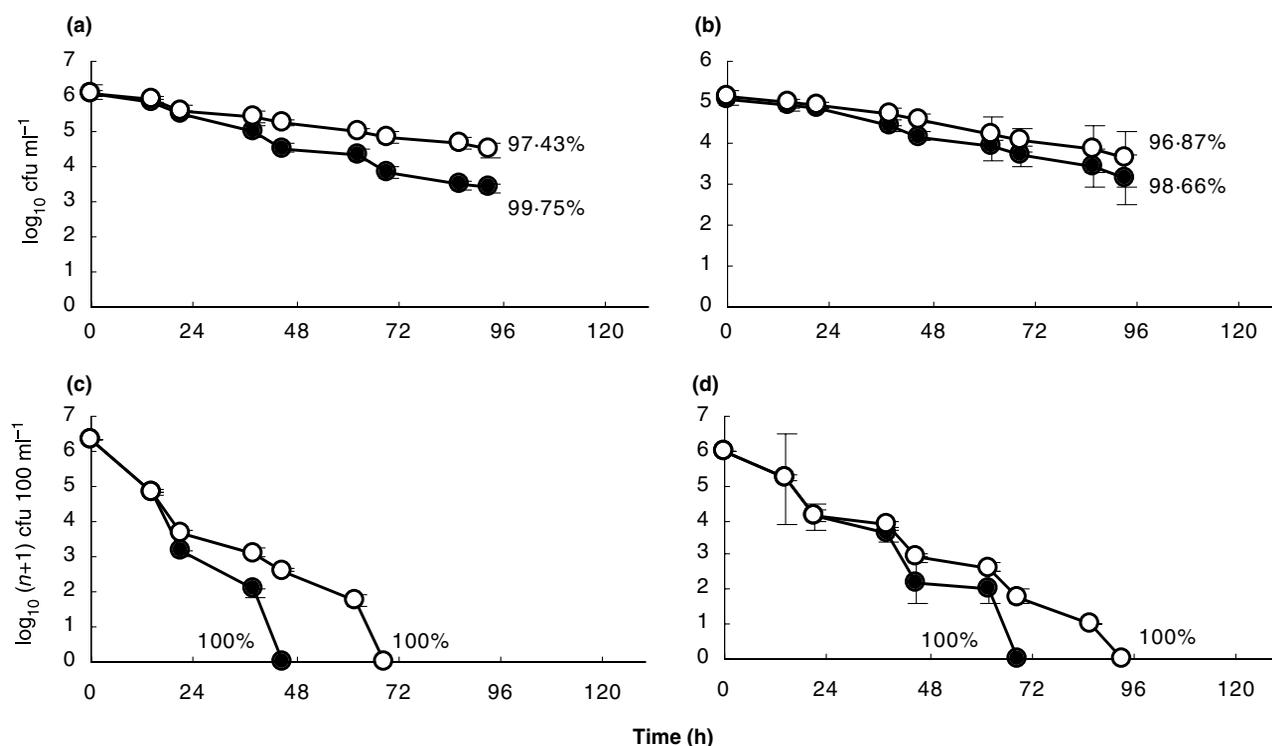
### Fast mixing saline experiment

This experiment was designed to simulate the fast mixing of sewage in high salinity water during the DS. The  $T_0$  salinity inside the chambers averaged  $25.2 \pm 0.0 \text{ psu}$ . The surrounding salt content was  $33.2 \text{ psu}$  throughout the experiment. During the 93 h incubation, salinity levels rose to  $31.1 \pm 0.1 \text{ psu}$  inside the light-exposed chambers and to  $31.5 \pm 0.3 \text{ psu}$  in the dark chambers. The increase was linear ( $R^2 = 0.973$ ,  $P < 0.0001$  and  $R^2 = 0.991$ ,  $P < 0.0001$ , light and dark MDCs, respectively) at a rate of  $1.27\text{--}1.62 \text{ psu d}^{-1}$  depending on the treatment. The temperature at the onset of the experiment was  $27.6 \pm 0.0 \text{ }^\circ\text{C}$  and evolved in a rather curvilinear fashion during the incubation,

in spite of diel fluctuations. Maximal temperatures were found in the first 20–40 h. The final temperature ranged between  $27.9 \pm 0.0 \text{ }^\circ\text{C}$  in the light chambers and  $26.5 \pm 0.0 \text{ }^\circ\text{C}$  in the dark chambers and the maximum daily  $\Delta T$  were  $5.7$  and  $4.0 \text{ }^\circ\text{C}$ , respectively.

Freshwater heterotrophic bacterial numbers decreased steadily throughout the experiment, whereas the marine heterotrophic bacterial abundance was rather stable during the first 21 h before declining (Fig. 3a and b). The NA  $T_{90}$  survival rates were particularly short, reaching  $30.1 \pm 3.4 \text{ h}$ , whereas MA values averaged  $52.5 \pm 1.4 \text{ h}$ . Light had a negative impact on the survival of both heterotrophic populations. The reduction was faster (99.75% for NA and 98.66% for MA against 97.43% for NA and 96.87% for MA) in the dark chambers. Consequently, survival rates increased in the dark treatment to  $60.7 \pm 1.7 \text{ h}$  for NA and  $70.6 \pm 3.7 \text{ h}$  for MA bacteria.

High salinity had a clear deleterious effect on FIB, especially on coliforms. The FC numbers decreased from  $6.30 \pm 0.00 \log_{10} \text{ cfu } 100 \text{ ml}^{-1}$  ( $6.00 \pm 0.00 \log_{10} \text{ cfu } 100 \text{ ml}^{-1}$  for FE) at the onset of the experiment to zero in just 45 h (69 h for FE) in the light treatment. In the dark experiment there was a considerable delay in the removal, up to 69 h for FC and 93 h for FE. Independent of the treatment, the survival of FE was higher than that of FC. The  $T_{90}$  values reached  $14.5 \pm 0.2 \text{ h}$  in the light for



**Fig. 3** Effect of time on the abundance of bacteria inside 5-l membrane diffusion chambers in the fast mixing experiment. (a) Nutrient agar bacteria; (b) marine agar bacteria; (c) faecal coliforms; (d) faecal enterococci. ●, Exposure to natural sunlight; ○, dark treatment. The bars represent 95% confidence level for triplicates

FC bacteria ( $21.3 \pm 0.3$  h in the dark) and  $20.6 \pm 0.3$  h ( $31.6 \pm 0.6$  h in the dark) for FE bacteria. The decay of FIB proceeded in a two-step fashion, regardless of the treatment. During the first 21 h for FC and 45 h for FE, the decrease was faster than in the second period (Fig. 1c).

## DISCUSSION

The experimental approach taken in this study was designed specifically to simulate three dominant mixing regimes for sewage introduced into the tropical Bangpakong River estuary, in order to evaluate, in a total sense, the temporal fate (such as growth, survival and die-off) of FC, FE and other allochthonous bacteria. It is recognized that this approach, in a strict sense, can only evaluate the 'fate' and not the causation, e.g. differences in results in light and dark treatments may be due to light directly, selective die-off due to protozoan activity and temperature changes. The protocol, however, does put important constraints on interpreting causation, as will be discussed below.

Membrane diffusion chambers of different designs have been widely used in survival studies of bacteria of faecal origin (e.g. Vasconcelos and Swartz 1976; Terzieva and

McFeters 1991; Mezrioui *et al.* 1995; Wet *et al.* 1995; Barcina *et al.* 1997; Quang and Button 1998). The chamber model used in this study (see Materials and methods) was made of clear Plexiglas material in order to allow light and 90% u.v. penetration. Some authors consider that the use of MDCs to determine the decay rates in water may be jeopardized because of slow diffusion of molecules across the membrane (Springthorpe *et al.* 1993). Therefore, the set-up of the experiments simulated either a slow mixing (progressive mixing with low salinity/brackish water) or a fast mixing with preincorporation with high salinity water.

The water temperature pattern inside the MDCs during the experiments reflected the expected natural warming that occurs during daytime and the cooling during the night in tropical waters. The experimental temperature range,  $25.1\text{--}34.4$  °C, was in agreement with that expected in the Bangpakong estuary (Bordalo *et al.* 2001) during the WS/DS cycle. Such high naturally occurring temperatures may be deleterious for faecal bacteria in contact with saline waters. For instance, in earlier reports Vasconcelos and Swartz (1976) reported that the viability of faecal and coliform bacteria is inversely proportional to temperature, since high temperatures enhance the death of such bacteria in seawater.

Several authors have mentioned nutrient starvation as a factor influencing the maintenance of allochthonous bacteria in water (Mitchell 1968; Munro *et al.* 1987; Lim and Flint 1989; Rippey and Cabelli 1990; Bogosian *et al.* 1996). Given the high concentration of inorganic but also organic matter, the latter estimated from BOD<sub>5</sub> values, at the onset of the experiment (Table 1) nutrient limitation is unlikely to have occurred. Predation, either by viruses, bacteria or protozoans, is another ecological factor that may contribute to the removal of non-indigenous bacteria from the environment (Enziger and Cooper 1976; Scheuerman *et al.* 1988; Gonzales *et al.* 1990; Davies *et al.* 1995; Sherr and Sherr 2000). The passage of protozoans and bacteria of > 0.45 µm from the tank estuarine water into the MDCs was prevented by the pore size of the membranes. However, freshwater protozoans may not survive at all in brackish water and/or seawater (Bordalo 1993).

None of the colony-forming bacteria showed a net growth in estuarine water under any given condition. In fact, the numbers of sewage-borne heterotrophic bacteria, FC and FE decreased sharply on contact with natural estuarine water of variable salinity. The lowest salt content gave the highest survival of all four bacterial assemblages in the tropical Bangpakong estuarine waters (Table 2). Earlier research on the survival of FIB in temperate environments found that, before bacterial counts decreased to 90% of the initial concentration in 72–120 h in seawater, there was a period of abundance stabilization (Carlucci and Pramer 1960; Mitchell 1968). In our experiments, the die-off pattern of FC and FE almost never followed such a curve, but rather a single line (Fig. 1). The FIB decreased almost linearly, except in the dark treatment of the very low salinity experiment when an initial phase, that lasted about 36 h for FC and 16 h for FE, was noticed (Fig. 1a). Only heterotrophic bacteria showed a systematic initial phase of 21–63 h, increasing with decreasing salinity in the case of NA bacteria, and around 23 h for MA, independent of the experiment salinity. This clearly shows the inability of allochthonous bacteria of faecal origin to survive independently, or at least to form colonies, in warm, tropical estuarine environments. For instance, some authors stated that cells may be injured by solar light or salinity but remain viable although unable to form colonies (Dave and Penrose 1978; Colwell 1993; Gourmelon *et al.* 1994).

This study confirms the higher resistance of FE to different environmental conditions typically found in the tropical Bangpakong estuary. Regardless of the experimental conditions and treatments, the survival of FE was always higher than that of FC (Table 2), although the initial numbers of the latter bacterial group in raw sewage were higher, as expected. However, the abundance of FE was of the same order of magnitude as FC, representing between 6.5 and 49.9% (30.4% average) of the latter numbers. In

temperate environments, typical FC/FE ratios in raw sewage are >10 (Geldreich and Kenner 1969), i.e. at least one order of magnitude higher. These findings suggest that FE may be used as a better sanitary indicator for monitoring faecal pollution in Bangpakong tropical estuarine waters than FC, due to high initial numbers but especially to the lower rate of decrease, giving a more satisfactory indication of the pathogenic potential of these waters.

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