Paraben resistance in bacteria from sewage treatment plant effluents in India
Krishna Kumar Selvaraj, Senthilkumari Sivakumar, Srimurali Sampath, Govindaraj Shanmugam, Umamaheswari Sundaresan and Babu Rajendran Ramaswamy

ABSTRACT
Parabens, the antimicrobial preservatives used in cosmetics, food and pharmaceuticals, are often detected in the aquatic environment. Generally, sewage treatment plants (STPs) receive community sewage containing parabens, which are ultimately released into streams/rivers. In this study, bacteria in STP effluents were evaluated for their resistance to parabens. The susceptibility was in the order of Staphylococcus aureus > Bacillus sp. > Escherichia coli > Pseudomonas aeruginosa. Gram-negative bacteria showed less susceptibility than their control and Gram-positive bacteria. Further, the bacteria were more sensitive towards butyl and ethyl parabens. Interestingly, the strains showed resistance to ≥5 mg of parabens, which is equivalent to or higher than reported environmental concentrations. The increase in paraben chain length did not enhance the susceptibility in all cases and it was understood that the activity may differ for each bacterium in the environment. This is the first profile on paraben resistance in common pathogens of Indian STPs. Paraben resistance may be developed due to continuous exposure even at sub-inhibitory and/or chronic levels in the environment and this resistance may be transferred to other pathogenic bacteria in receiving waters. Thus the study demonstrates the effectiveness of the disc diffusion method in environmental bacterial resistance assessment and addresses the risk involved in the use of parabens.

Key words | biocide, environment bacteria, paraben resistance, pathogens, preservative, sewage treatment plants

INTRODUCTION
Esters of p-hydroxybenzoic acid (parabens) are primarily antimicrobial preservatives or additives widely used for their effectiveness over a broad pH range. Four commonly used parabens, methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP), are added in combinations to extend the shelf life of the products and products’ activity against microbial growth in many personal care products (toothpastes, soaps, shampoos, etc.), food-stuffs (chocolates, sausages, condiments, jams, etc.), pharmaceutical products, etc. (Andersen 2008; Eriksson et al. 2008). Parabens are of concern for their health risks such as breast cancer in humans (Darbre & Harvey 2008) and toxic effects in aquatic organisms (fish, cladoceran, algae, etc.) (Kamaya et al. 2006; Ramaswamy et al. 2011). Their role in antimicrobial resistance is less studied (SCENIHR 2009). Recently, parabens have been detected in aquatic environments (Ramaswamy et al. 2011; Diaz-Alvarez et al. 2012).

Due to uncontrolled use of antimicrobial compounds, numerous antibiotic-resistant genes (ARGs) have been reported in hospital environments, sewage, and surface and drinking waters (Zhang et al. 2009). Very recently, bacteria resistant to multiple antibiotics have been found in clinical and environmental samples in India (Kristiansson et al. 2011; Walsh et al. 2011). Importantly, the New Delhi metallo-beta-lactamase-1 (NDM-1) gene, which confers multi-drug resistance (MDR) (even to carbapenem), has been detected in isolates of Escherichia coli and Klebsiella pneumoniae from patients in Indian hospitals, and subsequently the gene has been isolated in drinking water and wastewaters of New Delhi (India) (Kumarasamy et al. 2010; Walsh et al. 2011), confirming the severity of MDR.
prevalence in India. The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR 2009) points out that antibacterial resistance can develop rapidly following exposure to preservative(s). Although it is known that preservatives also play a key role in such ARGs, the studies reporting this relationship are scanty. So, the SCENIHR (2009) suggests the need for standardized methods for surveillance of resistance and cross-resistance, in conjunction with data on the use of biocides. Parabens are one such group of preservatives which needs to be assessed (Davin-Regli et al. 2006).

In India, wastewaters are not treated completely and the effluents from sewage treatment plants (STPs) are not fully compliant with wastewater standards (Hamner et al. 2006). Moreover, untreated wastewater and STP effluents are released into nearby rivers/environments which often serve drinking water or other household purposes. Around 37.7 million Indians are affected by waterborne diseases annually and there are reports to link their incidence with river pollution (Hamner et al. 2006). There are few studies reporting ARGs in Indian waters (Kristiansson et al. 2011; Walsh et al. 2011), and to our knowledge until now there is no report on the role of parabens in ARGs. Assessing the paraben resistance in wastewater bacteria might be helpful to monitor the flow of biocide resistance from wastewater bacteria to surface water bacteria. Thus, this preliminary study was designed to investigate the paraben resistance in bacteria of four STP effluents.

**MATERIALS AND METHODS**

**Chemicals and bacterial strains**

Media and chemicals were procured from Himedia (Mumbai, India). MP, EP, PP and BP were obtained from Sigma Aldrich (MO, USA). Acetone was obtained from Qualigens Fine Chemicals (Mumbai, India). Hydrogen peroxide and sulphuric acid were from Merck Specialties Pvt. Ltd (Mumbai, India). Glassware was washed with 10% labolene, tap water and ultrapure water in sequence before sterilization.

Gram-negative controls, *E. coli* (MTCC-724) and *Pseudomonas aeruginosa* (MTCC-741) were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India.

**Sample collection**

Wastewater samples from STPs (Figure 1) were collected during May–June 2009 in two cities (Coimbatore – CBE and Tiruchirappalli – TPL) with a population of 10,61,447 and 8,46,915 and two towns (Tanjore – TAN and Mayiladuthurai – MAY) with a population of 2,22,619 and 84,505, respectively from Tamil Nadu State, southern India.

**Paraben analysis**

The parabens were extracted from STP effluents using solid phase extraction and quantified by gas chromatography–mass spectrometry. The extraction and analytical methodologies have been described in our previous paper (Ramaswamy et al. 2011).

**Isolation and identification of bacteria**

Initially 1 mL of STP effluent was separately mixed with 9 mL of sterile distilled water and then serially diluted up to $10^{-7}$. From the dilutions, 0.1 mL of sample was spread on nutrient agar plates and incubated for 24 h at 37°C. Then the distinct colonies were randomly isolated and pure isolates were obtained by the quadrant streaking method. After isolation, the bacteria were identified by Gram staining, plating in selective media and by performing biochemical tests as prescribed in *Bergey’s Manual of Determinative Bacteriology*. 

Figure 1 | STP sampling locations in Tamil Nadu, India.
Paraben susceptibility test

The antimicrobial susceptibility test was performed by a disc diffusion method. Paraben stock solutions were prepared individually in acetone at a concentration of 250 mg mL\(^{-1}\). Then using a sterile syringe, required working solutions were loaded on to sterile discs (Whatman No:1; 5 mm) at 1, 2, 3, 4 and 5 mg of paraben concentration per disc. Care was taken while loading the parabens not to spill any out of the disc. The control disc with an equal volume of acetone (solvent) was loaded in all the experiments. The saturation limit for any of the parabens was observed as 5 mg per disc; so further increased concentration in the disc was not performed. The bacterial strains were inoculated in nutrient broth and incubated in a shaker (Orbitek, India) at 37 °C for 18–24 h. The microbial swabs were made on sterile Muller Hinton agar plates and the paraben-impregnated discs were subsequently placed on the plates and incubated at 37 °C for 24 h. After incubation, the zone of inhibition (ZOI) was measured.

RESULTS AND DISCUSSION

Level of parabens in STPs

STPs distribute the pollutant load to receiving water bodies and to agricultural land through effluent and sludge disposal as manure. The parabens MP, EP, PP and BP were detected in the range of 0.19–667 ng L\(^{-1}\), ND (not detected) –73.1 ng L\(^{-1}\), ND–15.29 ng L\(^{-1}\) and 0.57–84.5 ng L\(^{-1}\), respectively in the effluents (Table 1). The varied levels of parabens show their regular usage. Even though the population of MAY is 84,505, the higher levels of EP and PP encountered may be due to the STP treatment conditions. Generally, efficiency of STPs in treating pharmaceutical and personal care products (PPCPs) varies according to design, conditions and capacity, and often lack the potency to removing PPCPs (including parabens) completely (Daughton & Ternes 1999). Ellis (2006) reported that chronic exposure to antibiotics has created a large pool of resistant genes which are difficult to treat in STPs. It is apparent that parabens dominate in household/municipal wastewater and their occurrence in sub-inhibitory concentration may even lead to biocide resistance.

Identification of bacteria

A total of 12 isolates were randomly isolated and identified from the four STP effluents. They belong to Gram-negative bacteria such as *E. coli* and *P. aeruginosa* and the Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus* sp. *P. aeruginosa* and *Bacillus* sp. were isolated in all four STPs, whereas *E. coli* and *S. aureus* were isolated only in two STPs (Table 1). With the exception of *Bacillus* sp., all bacteria are pathogenic forms and used in preservative/pharmacopeia efficacy tests (PET) (Tanner 2002). However, the inclusion of *Bacillus* sp. (ubiquitous genus in soil community) may provide clues regarding the paraben resistance prevalence other than PET representatives in the environment. Already such PET bacteria have been reported to occur ubiquitously in contaminated cosmetics (Amin et al. 2013), and in surface and wastewaters (Schwartz et al. 2003).

Paraben susceptibility in Gram-negative bacterial strains

The preliminary screening showed paraben resistance up to 0.5 mg, so the susceptibility tests were performed in the range of 1–5 mg paraben concentrations. Generally, efficiency of STPs in treating pharmaceutical and personal care products (PPCPs) varies according to design, conditions and capacity, and often lack the potency to removing PPCPs (including parabens) completely (Daughton & Ternes 1999). Ellis (2006) reported that chronic exposure to antibiotics has created a large pool of resistant genes which are difficult to treat in STPs. It is apparent that parabens dominate in household/municipal wastewater and their occurrence in sub-inhibitory concentration may even lead to biocide resistance.

### Table 1

<table>
<thead>
<tr>
<th>STP location (Code)</th>
<th>Population</th>
<th>Paraben (ng L(^{-1}))</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MP</td>
<td>EP</td>
</tr>
<tr>
<td>Coimbatore (CBE)</td>
<td>1,061,447</td>
<td>667</td>
<td>ND</td>
</tr>
<tr>
<td>Tiruchirappalli (TPL)</td>
<td>846,915</td>
<td>0.19</td>
<td>0.55</td>
</tr>
<tr>
<td>Tanjore (TAN)</td>
<td>222,619</td>
<td>26.76</td>
<td>ND</td>
</tr>
<tr>
<td>Mayiladuthurai (MAY)</td>
<td>84,505(^{b})</td>
<td>3.88</td>
<td>73.11</td>
</tr>
</tbody>
</table>

*India census data (2011).  
\(^{b}\)India census data (2001).  
ND – Not detected.  
+ – presence.  
– absence.  

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for resistance/susceptibility is predefined for antibiotics, but not for preservatives. Hence, in this study, the ZOI was compared with control bacteria to express the paraben sensitivity.

_**E. coli**_ was isolated only from CBE and MAY STP effluents. The MAY strain showed ZOI only at the maximum concentration (5 mg) whereas the CBE strain showed no ZOI (Figure 2) for MP. In the case of EP and BP, both CBE and MAY strains showed ZOI only at 5 mg. ZOI was not observed for any of the STP strains for PP. The control _E. coli_ (MTCC-724) showed ZOI for MP, PP and BP at 4 mg and for EP at 1 mg concentration itself.

_P. aeruginosa_ of all the STPs are less susceptible for MP, EP and BP, showing ZOI only at 5 mg or no ZOI. In the case of PP, the ZOI was observed at ≥4 mg (Figure 2). Particularly, the CBE strain showed no ZOI for any of the parabens even at maximum concentration (i.e. 5 mg). The control strain (_P. aeruginosa_ MTCC-741) showed ZOI at concentration ≥1 mg (Figure 2). _P. aeruginosa_ strains (except TAN) showed no susceptibility towards BP at 5 mg (maximum concentration), whereas the other STP Gram-negative _E. coli_ and Gram-positive bacteria showed ZOI at 5 and 1 mg, respectively. This explains that _P. aeruginosa_ isolated from a STP may metabolize/resist BP.

From Figure 2, it is apparent that Gram-negative strains showed ZOI only from 4 mg onwards (all parabens), and the control MTCC strains showed ZOI from ≥1 mg (i.e. up to four-fold higher susceptibility than STP strains) (Figures 2 and 3). So the study affirms that most of the characteristic resistant behaviour is observed in bacteria from areas contaminated with antimicrobial substances. This kind of resistance can be attributed to the lower permeability of the bacterium’s outer membrane with the multi-drug efflux system along with loss of porin (Perichon & Courvalin 2009). Rather than the often reported active efflux mechanism, _Enterobacter gergoviae_ showed a different mechanism for PP resistance and the same bacteria showed a positive correlation between increased minimum inhibitory concentration and frequent use of paraben (Davin-Regli et al. 2006).

Although this is the first report on paraben resistance of STP-isolated bacterial strains, such bacteria are known for their varied resistance behaviour. Scully _et al._ (1986) observed that _P. aeruginosa_ isolated from cosmetic and other products was resistant to several types of antibiotics. Valkova _et al._ (2002) reported that _E. coli_ has the ability to degrade parabens via esterases and can share the resistance with another genus. Although the exact resistance of the isolated Gram-negative bacteria is unknown, there is a possibility of the presence of species-specific efflux pumps such as MexA-OprM (_P. aeruginosa_), AcrAB-TolC (_E. coli_), etc. as mentioned in SCENIHR (2009). So, enzymes, efflux pumps and cell wall changes through adaptive or genetic linkage may be the prime factors determining bacterial susceptibility.

Kumarasamy _et al._ (2010) reported that _E. coli_ can exchange plasmid with coliforms readily in aquatic systems to develop MDR and R-plasmid outbreak. In India, _E. coli_ isolated from wastewater in Ujjain was reported with amikacin resistance in spite of the absence of amikacin in the wastewater (Diwan _et al._ 2013). A recent report by Walsh _et al._ (2013) on the emergence of NDM-1 MDR gene in _E. coli_ and _P. aeruginosa_ isolated from sewage and drinking waters of New Delhi is one study showing evidence of developing MDR resistance in Gram-negative bacteria.

**Figure 2** | Paraben susceptibility of bacterial strains from STP effluents and control bacteria (MTCC) (values 0–5 mg in y axis represent the susceptible concentration, and 6 mg represents no zone of inhibition at maximum concentration).
Paraben susceptibility in Gram-positive bacterial strains

*S. aureus* was isolated in STPs from TPL and CBE. For MP, the TPL strain showed ZOI at 4 mg whereas the CBE strain showed no ZOI even at 5 mg (Figure 2). However, strains from both STPs showed ZOI for EP and BP at lower concentration, ≥1 mg. For PP, ZOI in the TPL strain was at 5 mg and the CBE strain showed no ZOI even at maximum concentration (5 mg). The difference in sensitivity reflects its specific resistivity towards different lineages of parabens. The *S. aureus* strains were more susceptible to BP (ZOI up to 43 mm) and EP (ZOI up to 23 mm) than other isolates (Figure 3), confirming the parabens’ effectiveness against *S. aureus*.

In *Bacillus* sp., the TAN strain showed no ZOI for MP, at maximum concentration (5 mg) and strains from other STPs showed ZOI at ≥4 mg. TPL and TAN strains did not respond to EP, whereas CBE and MAY strains showed susceptibility at 5 and 4 mg, respectively (Figure 2). Most of the strains are less susceptible to PP, in which the TPL strain was not susceptible at maximum concentration (i.e. 5 mg), whereas TAN and CBE strains were susceptible at 5 mg. The MAY strain was susceptible at ≥5 mg concentration. Unlike other parabens, the BP is sensitive at the 1 mg level for all *Bacillus* strains. Such increased BP susceptibility may be due to the greater solubility of BP in the bacterial membrane, facilitating the movement towards cytoplasmic targets at a great concentration (Valkova et al. 2002). Therefore, it is inferred that environmental bacteria are gaining resistance to parabens selectively.

In the present study it was observed that Gram-positive strains are more susceptible than Gram-negative bacteria. However, Gram-positive *S. aureus* isolated from cosmetic formulations has been reported to resist parabens (Amin et al. 2010). Sasatsu et al. (1994) confirmed that plasmids in *S. aureus* confer resistance to antiseptics, dyes and preservatives (BP and MP). Even such plasmids are capable of resisting vancomycin by adopting *vanA* gene cluster synthesis and cell wall modifications (Sahlstrom et al. 2009). There are different possibilities for transfer of resistance between species. Even some efflux pumps are common in Gram-negative and Gram-positive MDR such as *S. aureus, B. subtilis* (Perichon & Courvalin 2009). Efflux-based quaternary ammonium compounds resistant gene (*qac*) and other resistant genes related to *qac* have been detected in the staphylococci, isolated from food samples (SCENIHR 2009). Sasatsu et al. (1994) reported that the endothelin-B receptor gene in *S. aureus* is quite well associated with resistance to both antiseptics and preservatives.

Paraben levels in STP effluents vs bacterial susceptibility

At CBE, a higher level (667 ng L⁻¹) of MP was quantified and the Gram-negative bacteria showed no ZOI (Table 1 and Figure 2). However on considering the rest of the parabens, their concentrations were lower and the susceptibility pattern does not seem to be affected. For example, the BP level was lower in MAY and TPL, but the respective *P. aeruginosa* strains showed no ZOI. Regardless of the paraben level in STP, the Gram-positive strains were more susceptible than the Gram-negative strains. Hence, the present study implies that the short-term variance in the environmental concentration of parabens was not directly related to the susceptibility. The strains from CBE were less susceptible than those from TPL, TAN and MAY. This order of susceptibility coincides with the human population and urbanization, which determines the contaminant load in wastewaters (Table 1).

The property of chain length on susceptibility was elucidated based on ZOI (Figure 3). The overall observation showed no clear indication of increase in susceptibility with respect to paraben chain length. It indicates that the paraben chain length may not play any effective role in its resistance. This variation clearly states that individual paraben effect varies within strains and between species.
Potential risk of paraben resistance

The presence of paraben resistance in wastewater bacteria may be associated with any of the MDR mechanisms. There is strong evidence for several patterns of cross-resistance in environmental microbes, and their resistance genes can be easily localized in the environment and resident organisms (Al-bahry et al. 2009). In India, ~93 million people inhabiting the slums lack proper sanitation and housing facilities (Ministry of Housing and Urban Poverty Alleviation 2011), and most of them are dwelling close to sewage or wastewater wetlands. Antibiotic resistance in the Indian environment is so prevalent that pharmaceutical wastewater contaminated river sediment contained a plethora of resistance genes with various mobile genetic elements (integrons, transposons and plasmids) which are capable of mobilization towards human pathogens (Kristiansson et al. 2011). Recently, in Delhi (India), the MDR NDM-1 gene isolated in hospital patients was also identified in environmental carrier microbes such as E. coli (Walsh et al. 2011). According to Abigail et al. (2004), once the resistant bacteria enter the human internal environment, the intestinal Bacillus and Staphylococcus sp. can acquire the resistance gene from bacteria passing by (Abigail et al. 2004). Such infectious resistant bacteria are capable of transforming PPCPs into toxic compounds, making the treatment challenging (Amin et al. 2010). This phenomenon will lead to increased prescribing and use of antibiotics and biocides, respectively. Hence this study brings out the urgent need for MDR assessment in STPs and linked surface waters.

CONCLUSIONS

This is the first study which reports the paraben resistance in STP effluent bacteria in India. Out of 12 isolates, nine showed resistance (no ZOI) at maximum concentration (5 mg) to at least one of the parabens. P. aeruginosa from CBE showed no zone formation for all four parabens. In general, Gram-positive isolates are more sensitive than Gram-negative isolates. Further, we could not find any correlation in the activity with chain length, because some bacteria are resistant to long chain length parabens while others are not. Also, the STP’s paraben concentration does not reflect its bacterial susceptibility pattern. Such differential susceptibility suggests that bacteria do not respond to all the parabens alike and the metabolism/resistance to parabens is expected to differ in each strain. Environmental isolates representing PET bacteria have developed resistance against some parabens beyond 5 mg, which is ~1,000-fold higher than reported environmental concentrations. Although the paraben levels are at sub-inhibitory concentrations, the bacterial resistance may be retained. Since the wastewaters are often drained (without treatment) into drinking water sources (rivers, lakes, etc.), human exposure to biocide-linked MDR bacteria is likely. Also, the increase in paraben resistance in environmental isolates may decrease the broad spectrum activity of parabens. This baseline study will be helpful in further surveillance of paraben resistance in surface waters and other water resources.

ACKNOWLEDGEMENTS

One of the authors, K. K. S., is grateful to the Department of Science and Technology (DST), India, for providing a Junior Research Fellow under the DST-PURSE programme. The co-author, Srimurali Sampath, would like to thank the University Grants Commission, India, for providing research fellowship.

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First received 19 December 2012; accepted in revised form 27 June 2013. Available online 19 October 2013.