Phenotypic population characteristics of the enterococci in wastewater and animal faeces: implications for the new European directive on the quality of bathing waters

J.L. Wallis and H.D. Taylor
University of Brighton, School of the Environment, Cockcroft Building, Lewes Road, Brighton, East Sussex, UK (E-mail: H.D.Taylor@bton.ac.uk)

Abstract Faecal pollution of recreational bathing waters may derive from point sources of various wastewaters or from more diffuse sources such as run-off of agricultural wastes. The paper describes the application of population similarity studies to the enterococcal flora of various animal faeces and municipal wastewaters as a means of distinguishing human from animal faecal material. A simplified phenotypic testing technique (PhenePlate, PhP) was used to study the fermentation kinetics of eleven carbohydrates by all bacterial isolates. Enterococcal isolates (1,766) from six sources were investigated. Enterococcal population diversity (measured as Simpson’s Diversity Index) in wastewater samples was high (mean Di = 0.95) compared with those of non-human faeces. The mean diversity of isolates in seabird faeces was 0.72, in sheep and donkey faeces 0.44, in dog faeces 0.42 and in cattle faeces 0.32. Analysis of population similarity coefficients demonstrated that faeces from sheep and cattle showed the greatest similarity (Sp = 0.72). Sheep and cattle faeces demonstrated a low similarity to municipal wastewater samples. This would suggest that population similarity studies might be a useful tool for distinguishing the relative contributions of municipal wastewater and agricultural run-off to bathing water pollution. The PhP procedure identified a specific PhP type that appears to have high specificity to non-human faeces. It may, therefore, represent an important tool in source tracking. Additional phenotypic and genotypic analysis of PhP types that demonstrate a high degree of source specificity is required. The benefits and limitations of the use of population similarity studies to distinguish pollution sources are discussed in comparison with other source tracking approaches and the implications of these developments for future European Union legislation on the quality of bathing waters are discussed.

Keywords Diversity; faecal pollution; pollution; similarity; source

Introduction
The hygienic quality of bathing waters in the UK is measured by a number of tests prescribed by the European Union Directive on the quality of bathing waters (Anon., 1976). The principle parameters are two groups of faecal indicator bacteria, namely the faecal coliforms and the faecal streptococci. This second group is made up of the genus Enterococcus plus the species Streptococcus bovis and Streptococcus equinus. A revised directive on the quality of bathing waters is currently in preparation (Anon., 2000). It is envisaged that the new Directive will place less emphasis on regular monitoring of all beaches and place greater emphasis on investigating the causes of beach “failures”.

“…as well as monitoring water quality in bathing sites, the new Bathing Water Directive will tackle pollution sources, in particular wastewater discharges and agricultural run-off.” Anon. (2000)

The role of untreated or partially treated municipal wastewaters in the contamination of bathing waters is well documented. In recent years investment in coastal wastewater treatment works by the water and sewerage companies of England and Wales has led to an increase in compliance with the existing European Bathing Water Directive. Although bathing water quality in England and Wales is generally improving, there are specific
locations where improvements to wastewater treatment technology have failed to achieve adequate bathing water quality standards. There is increasing evidence that faecal bacteria of non-human origin may in some instances make a significant contribution to non-compliance with the EU Directive.

Clearly, an effective means of distinguishing human from non-human faecal contamination would be an important tool in recreational bathing water quality management. By the 1980s the idea that the ratio of faecal coliforms to faecal streptococci in a polluted water body gave a good indication of faecal origin was being questioned. It became apparent that markedly different die-off rates between the two bacterial groups limited this approach only to the analysis of recent pollution incidents. Other approaches to source tracking have included a range of chemical and microbiological techniques many of which are reviewed by Sinton et al. (1998).

Rapid phenotyping of bacterial isolates offers a low-cost means of studying mixed microbial communities from environmental samples. The PhenePlate technique (PhPlate Microplate Techniques AB, Stockholm, Sweden, PhP) is a simple screening system that can be used for comparing large numbers of isolates and is suitable for assaying the bacterial floras of polluted waters. The system is based on the kinetics of biochemical reactions performed in microplates. The discriminatory ability of the PhenePlate system has compared favourably with pulsed-field gel electrophoresis (PFGE) (Kühn et al., 1995) and they also used the system for comparison of coliform populations in polluted river water and factory effluents (Kühn et al., 1997). In our study, the PhP technique was used to study enterococcal isolates from animal faeces and wastewaters of predominantly human origin in an attempt to ascertain if phenotypic diversity may offer a method of distinguishing complex assemblages of faecal bacteria from different sources. The work constitutes part of a larger study (Taylor and Wallis, 2001) into sources of faecal pollution on the UK Fylde coast.

**Materials and methods**

**Sample collection and treatment of isolates**

Samples were collected from four wastewater treatment works discharging to the Ribble estuary (Lancashire, UK) over approximately 18 months. Initial studies to compare enterococcal isolates from each of the four works showed high similarity levels between the four populations. Therefore, all 913 wastewater isolates were pooled for the purposes of further data analysis.

The faeces of five animal groups were collected and analysed over two years. The faeces of grazing cattle and sheep were collected from the salt marshes of the Ribble estuary. Seabird faeces were collected from various roosting sites on the Fylde coast in the vicinity of Lytham St Anne’s. Although it was not possible to relate faecal samples directly to bird species, evidence suggested that the primary sources were various species of gull. The faeces of donkeys that were used for children’s pleasure rides on Blackpool beach were analysed because of concerns that they might represent a source of indicator bacteria in the bathing water under certain conditions. The faeces of domestic dogs were obtained from various locations over approximately two years. Numbers of enterococcal isolates from such faecal sources (total 853) were 256 (sheep), 70 (cattle), 298 (bird), 72 (donkey) and 157 (dog). Samples of faeces and wastewaters were stored at 4°C and analysed for the presence of presumptive *Enterococcus* spp. within 24 h. Faecal samples were spread onto plates of Slanetz & Bartley agar (Oxoid) and incubated for 4 h at 37°C followed by 44 h at 44°C. Colonies were subcultured to Bile Aesculin agar (BAA; Oxoid) (18 h, 44°C) according to standard procedures (Anon., 1994). Colonies demonstrating a dark halo after this period were subcultured to PhenePlate microplates. Wastewater samples were membrane filtered and treated as above.
**PhP typing of Enterococcus spp isolates**

Pre-prepared PhP-RF PhenePlate microplates consisted of 96 wells containing eight rows of eleven dehydrated carbohydrates with each well being filled with a suspending medium (0.1% proteose peptone, 0.05% yeast extract, 0.01% bromothymol blue, pH 7.5). Enterococcal isolates were picked directly from BAA and inoculated into the first well of each row of the PhP-RF plates. A small volume of the bacterial suspension was transferred from the first well into each of the eleven other wells using a multi-channel pipette. The inoculated plates were incubated at 37°C and the absorbance value (620 nm) of each well was read using a microplate reader (Biotec ELX800, Instruments Inc. Vermont, U.S.A) after 16 h, 40 h and 64 h. After the final reading, the biochemical fingerprint was recorded as the mean absorbance value for each well over the three readings based on the reactions of the eleven carbohydrates (Kühn *et al.*, 1991).

In order to test the reproducibility of the PhP procedure, a number of reference strains were tested in duplicate during each assay and a pair-wise comparison of these duplicates was carried out using the PhP software. If a similarity of <0.97 was obtained the assay results were discarded. In addition, a number of rows in the PhP plates were not inoculated with bacteria, to ensure that the suspending medium had not become contaminated.

**Data analysis**

PhP software was used to perform the data processing and all of the calculations other than Brillouin’s Index. The biochemical fingerprint of each isolate was compared with that of all other isolates in a pair-wise fashion. The similarity between each pair of isolates was calculated as the correlation coefficient (r). Isolates showing a correlation coefficient identity level of >0.975 were assigned to the same phenotype (PhP type). Isolates with correlation coefficients lower than the identity level were assigned to a single phenotype (S_p). The similarity between different bacterial populations, (S_p) was calculated (Kühn *et al.*, 1991) being obtained by calculating the occurrence of matching PhP types within different bacterial populations. Correlation coefficients and population similarities were clustered using the unweighted-pair group method using an average linkages (UPGMA) method (Sneath and Sokal, 1973). Simpson’s Diversity Index (D_i) (Atlas, 1984) was used to calculate the diversity of each of the bacterial populations. The Brillouin Index (Brillouin, 1962) was used to estimate the representative sample size for each population. The method for determining minimum sample size has been outlined by Pielou (1974).

**Results**

**Population diversity (D_i)**

Table 1 shows the mean diversity of each source examined. This figure is obtained by calculating the mean of the diversity values for each individual wastewater source or individual animal within each source group. The range represented the highest and lowest values for individual animals or wastewater sources. The mean diversity among bacteria from wastewaters was generally high (mean D_i = 0.95) compared with the non-human potential faecal sources examined. The diversity among different wastewaters did not vary greatly. However, the diversity within other potential sources varied considerably.

**Clustering of bacterial populations from potential faecal sources**

All enterococcal isolates from each of the six examined sources were compared. The resulting population similarity coefficients were clustered and expressed as a dendrogram (Figure 1). The dendrogram showed that the similarity between sheep and cattle faeces was high (0.72) and that all animal sources tended to cluster together at around 0.35 whereas wastewater isolates clustered with the animal sources at around 0.2. The mean population
similarity between faeces of agricultural animals and wastewaters was low at 0.13. However, the mean similarity between the other non-human sources and wastewaters was slightly higher at 0.26. This pattern could be accounted for by the fact that a specific phenotype was detected that appeared to be predominantly non-human in origin. The greatest numbers of this phenotypic type were found in sheep and cattle faeces (52% and 63% of isolates respectively) while >30% of enterococci from donkey and dog faeces and 14% of enterococci from birds were found to belong to this PhP type. Wastewaters contained a low percentage of this phenotype (<3%).

**Discussion**
Calculating the phenotypic diversity of enterococcal bacteria in environmental samples may offer a useful source-tracking tool. In this study the enterococcal populations of wastewaters demonstrated much higher levels of diversity than those of grazing agricultural animals. Since the high diversity in wastewaters was consistent (range 0.93–0.98), a contaminated bathing water demonstrating low diversity would be more likely to have a predominantly non-wastewater source. A high diversity would suggest that a variety of sources are responsible or that municipal wastewater was a significant component.

The use of a population similarity coefficient appears to be a useful and rapid tool for distinguishing between human and animal faecal pollution in bathing waters. However, in this study the technique did not demonstrate an ability to distinguish accurately between different animals. One of the weaknesses of population similarity analysis is that it puts equal weighting on the presence of all phenotypes. The detection of a phenotype with high specificity to non-human faeces in this study was significant. However, population similarity calculations did not take account of the presence or absence of a particular phenotype.
The results suggest that the PhenePlate technique could usefully be employed to screen for phenotypes demonstrating high source specificity.

If the methods outlined above were to be used to compare faecal sources with recreational waters it would, at this stage, have to be assumed that the viability of all enterococcal phenotypes in the natural environment was broadly similar. Therefore, the proportions of each phenotype within a given population would remain the same with time. However, if a particular phenotype dominated in a polluted bathing water, it could be either because it survived for longer in the natural environment or because the source of faecal pollution was dominated by this particular phenotype. The proposed revised European Union Directive on the quality of bathing water (Anon., 2000) results will take account of research into the health impacts of faecal pollution since the original Directive (Anon., 1976). Epidemiological data suggests that the existing EU standards quantify inappropriate indicators and are insufficiently stringent to prevent significant levels of minor illness acquisition in bathers (Kay and Rees, 1997). Intestinal enterococci appear to be a better indicator of the risk of gastroenteritis to water users and so this group is likely to become the main parameter in the Directive. Therefore, a better understanding of the faecal sources of members of this group is needed. Other areas requiring further research include studies into differential die-off rates among members of the group and the possibility of re-growth in protected, nutrient rich environments.

The revised Directive should mark a shift in emphasis from bathing water monitoring to bathing water quality management in line with the principles enshrined in the Water Framework Directive (Anon., 2000). “Beach profiles” will be required that indicate all potential sources of pollution in the vicinity of the bathing area. In many cases effective faecal source tracking studies will have a role to play in the development of such profiles.

Conclusions
The use of an automated and rapid phenotyping technique was shown to be a useful tool for studying the population characteristics of Enterococcus spp. in wastewaters and animal faeces. Diversity was highest in bacteria found in municipal wastewaters and bird faeces. Although it was assumed that the faeces found in the wastewaters were mostly of human origin, the contribution of animal faeces from urban run-off to the sewers could not be assessed. The high phenotypic diversity of the enterococci in bird faeces complicated any attempt to assess the contribution of this source to the contamination of bathing waters. Population similarity studies demonstrated a high correlation between the enterococcal populations in cattle and sheep faeces. Conversely, the similarity between these populations and those found in wastewaters was low. Therefore, rapid phenotypic screening of enterococcal bacteria may be a useful tool in distinguishing the impacts on bathing waters of agricultural run-off from municipal wastewaters.

To date no single technique has been shown to consistently distinguish human faecal contamination from that of other animals in all situations. Limitations of using the phenotypic characteristics of faecal bacterial populations exist while questions remain as to the spatial and temporal stability of phenotypic characteristics and differential rates of viability loss within the population. In complex aquatic environments with multiple sources of faecal bacteria, it is probable that satisfactory source tracking will only be achieved using a “basket of determinants”. However, despite its limitations, the method described here may offer a relatively low-cost and rapid contribution to source tracking studies. The PhenePlate technique may, in the future, play an additional role in screening for potential source specific phenotypes of faecal bacteria.
Acknowledgements

The authors would like to acknowledge the financial support of the former UK Dept for the Environment, Transport & the Regions and would like to thank colleagues at the DETR and the Environment Agency Northwest Region for their contributions.

References