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THE DISPERSAL OF NITROGEN-FIXING ENTEROBACTERIACEAE FROM SEWAGE INTO THE WATERS AND SEDIMENTS OF MORECAMBE BAY,UK.

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ABSTRACT - The sediments of Morecambe Bay contain large numbers of salinity-tolerant, nitrogen-fixing Enterobacteriaceae (NFEs) (mainly *Klebsiella pneumoniae)* which appear to be derived from sewage. Sewage effluents, regardless as to whether they have received primary or secondary treatment, contain huge numbers of NFEs with salinity-tolerant and freshwater strains present in roughly equal quantities. The bacteria are discharged into the rivers and estuaries and carried out to sea. Once in the sea the salinity-tolerant strains retain their viability better than the freshwater strains so that the incoming tide, which doses the sediments, contains mainly salinity-tolerant NFEs. It is probable that similar processes occur all round the UK. coast as inshore coastal waters in the North Sea and English Channel have also been shown to contain salinity-tolerant NFEs.

Key words: nitrogen-fixing Enterobacteriaceae (NFEs).

RÉSUMÉ - Les sédiments de la baie de Morecambe présentent un grand nombre d'enterobacteries halotolérantes et fixatrices d'azote (NFEs) (principalement *Klebsiella pneumoniae*) qui semblent provenir des eaux d'égouts. Les effluents domestiques, indépendamment de leur traitement primaire ou secondaire, sont très riches en NFEs dont les proportions en souches halotolérantes et en souches d'eau douce sont rigoureusement égales. Les bactéries sont déversées dans les rivières et estuaires, puis sont transportées vers la mer. Une fois en mer, les souches halotolérantes sont plus viables que les souches d'eau douce. Ainsi dans les prélèvements de sédiment, effectués à marée montante, la flore bactérienne est principalement constituée de NFEs halotolérantes. Il est probable que les mêmes phénomènes se produisent tout au long des côtes de la Grande Bretagne. En effet, les eaux côtières intérieures de la Mer du Nord et de la Manche contiennent également des NFEs halotolérantes.

Mots clés: entérobactéries fixatrices d'azote (NFEs).

INTRODUCTION

It has been shown that salinity-tolerant, nitrogen-fixing strains of bacteria in the family Enterobacteriaceae are present in the estuary and sediments of the river Lune and that they may contribute to nitrogen fixation in the sediments (Jones, 1982a and 1982b). However, it is not clear whether the presence of such bacteria is a local phenomenom caused by the discharge of only partially treated sewage into the Lune estuary, or whether similar bacteria are also present in treated sewage effluents, rivers, other estuaries and seawater.

This paper presents evidence that both freshwater and salinity-tolerant strains of nitrogen-fixing Enterobacteriaceae are widespread in UK. rivers and estuaries and that salinity-tolerant strains are selected for in coastal waters.

METHODS

Bacterial counts

Nitrogen-fixing Enterobacteriaceae (NFEs) were enumerated and isolated using Most Probable Number (MPN) methodology. This technique, although less accurate than other viable counts, is particularly suitable for nitrogen fixation studies as it enables the use of acetylene reduction activity (ARA) as the «end point» for counting and does not require the picking off of individual colonies for subsequent tests for nitrogen fixation. It also serves as an excellent method for selecting and isolating nitrogen-fixing bacteria from both aquatic and terrestrial samples.

Water samples were collected in sterile bottles and analysed the same day. 1 cm^3 samples from decade dilutions (up to 10^{-8}) in Ringers solution were inoculated into 6 screwcapped Bijou bottles (7 cm^3) containing nitrogen-free glucose medium to a final salinity of just above 0‰ in 3 bottles and 35‰ into the other 3. The atmosphere in the bottles was replaced with nitrogen and they were incubated anaerobically at 25°C. After 5 days 1 cm^3 acetylene was injected into each of the bottles and, after a further 24 h incubation, 1 cm^3 samples of the bottles' atmosphere were removed and analysed for ethylene production. Positive results for ARA were taken as positive for nitrogen fixation (Stewart *et al.*, 1967) and inocula from these bottles were grown aerobically on McConkey agar plates prior to re-testing for ARA in nitrogen-free glucose broth under anaerobic conditions. MPN tables were consulted (Collins, 1967) and the results expressed as the number of freshwater (for those positive at 0‰) or salinity-tolerant (for those positive at 35‰) NFEs 100 cm^{-3} water).

Sediment samples were analysed in a similar way except that they (10 g wet weight) were mixed with Ringers solution and the volume made up to 100 cm^3. The resulting suspension was stirred vigorously for 20 minutes prior to being serially diluted and inoculated into nitrogen-free glucose broth. The results are expressed as the number of NFEs per gram dry weight of sediments.

Deposition experiments were carried out by placing sterile filter papers (3 sheets thick) onto the surface of the sediment and fastening them to the sediments with pins. After tidal cover the top filter paper was placed in a sterile petri dish for transport to the laboratory. 5 cm^2 portions were shaken in bottles containing 10 cm^3 Ringers solution and then serially diluted for MPN estimations.

Identification

NFEs were isolated from positive ARA assay bottles and cultured aerobically on McConkey bile salts agar. Pure cultures were identified using the API 20E analytical profile index (API Laboratory Products Ltd., Basingstoke, UK.).

RESULTS AND DISCUSSION

NFEs in Rivers

The main rivers flowing into Morecambe Bay and the Gipping, which flows *via* the Orwell into the North Sea, were analysed for the occurrence of NFEs (table 1). In all of the rivers substantial numbers of both freshwater and salinity-tolerant strains were detected with the freshwater strains, on average, twice as plentiful. Similar data were obtained for the Lune and Orwell estuaries (Tab. 1) with the freshwater strains either exceeding or equal in number to the salinity-tolerant strains.

River	Sampling site	Number of bacteria (MPN 100 cm^{-3} x 10^2) At 0 %	At 35 %
FRESHWATER			
Ribble	Tickled Trout	680	150
Wyre	Garstang	430	430
Lune	Halton	590	511
Bela	Milnthorpe	2400	1950
Kent	LevensBridge	1415	34
Leven	Haverthwaite	34	24
Gipping	Bramford	540	93
ESTUARINE			
Lune	GlassonDock	670	670
Orwell	Pin Mill	580	150

Table 1 : Freshwater and salinity-tolerant nitrogen-fixing Enterobacteriaceae in the freshwater and estuarine sections of rivers.

NFEs in sewage effluents

It has been suggested that the large numbers of NFEs in the sediments of the river Lune (where this study began) are the result of the discharge of Lancaster's sewage, which is subject only to primary sedimentation, into the Lune estuary at each high tide (Jones, 1982a and 1982b). However, the freshwater reaches of the Lune and other rivers in the Morecambe Bay area also contain large numbers of NFEs and they are exposed only to sewage effluent which has been treated. Therefore we tested the effluents from sewage treatment plants where secondary treatment takes place. The results (Tab. 2) show that effluents from activated sludge and trickle filter treatment plants also contain huge numbers of NFEs with salinity-tolerant and freshwater strains present in similar amounts.

Location	Treatment	Number of bacteria (MPN 100 cm^{-3} x 10^6) At 0 ‰	At 35 ‰
Hawkshead	Activated sludge	110	78
Little Langdale		67	28
Caton	Trickle filter	110	110
Crag Bank		110	110
Stodday	Sedimentation	383	352

Table 2 : Nitrogen-fixing Enterobacteriaceae in sewage effluents

NFEs in the sea

The results from Morcambe Bay (Fig. 1) show that once in the sea the numbers of NFEs decline due to dilution and death. In seawater the ratio of freshwater NFEs to salinity-tolerant NFEs is reversed and there are approximately ten times more salinity- tolerant strains. It seems probable, therefore, that salinity- tolerance conveys survivability for NFEs at seawater salinities. The result for 4 miles west of Heysham, which differs from the others, may be due to contamination with untreated sewage from Morecambe which is discharged directly into the sea 3 miles to the North.

NFEs in the sediments

Salinity-tolerant NFEs are found in sediments throughout Morecambe Bay where they are thought to contribute as nitrogen fixers in both uncolonised sediments (Jones, 1982a and 1982b) and those colonised by *Spartina anglica* C.E. Hubbard (Wolfenden, 1984). It has also been proposed that the source of these bacteria is the sewage effluent released into the rivers, which after mixing with seawater, inoculates the sediments with the incoming tide. This is corroborated by NFEs estimations of intertidal sediments taken before and after tidal cover which show an increase from 57,290 to 95,900 NFEs g^{-1} dry weight sediments. These results are confirmed by the use of sterile filter papers as sediment traps (Tab. 4) in which twice as many salinity-tolerant NFEs were deposited as freshwater strains. Some of the bacteria will have originated from resuspended surface sediments, especially in rough seas, and some will have settled out of the water column, as illustrated by the results obtained in calm weather.

	Number of bacteria (MPN NFEs 5 cm^{-2})	
Sea conditions	Freshwater strains	Salinity-tolerant strains
Calm	884	1858
Rough	197 x 10^3	430 x 10^3

Table 4. Deposition of nitrogen-fixing Enterobacteriaceae on filter paper sediment traps during one tidal cover of intertidal sediments.

(each result is the average of 5 samples)

Identity of NFEs

Several nitrogen-fixing strains of bacteria from the Enterobacteriaceae have been isolated from the waters and the sediments. They include *Citrobacter freundii, Enterobacter agglomerans,* and *Klebsiella pneumoniae* with *K. pneumoniae* the most frequent isolate and the main species capable of nitrogen fixation at seawater salinities.

It is common practice in the UK. to release partially treated sewage into the sea and into estuaries. From our studies it is apparent that members of the Enterobacteriaceae can survive in seawater and marine sediments and that there is selection of salinity-tolerant strains. The results are confirmed by Neilson (1980) who showed several members of the Enterobacteriaceae isolated from the Baltic Sea to be capable of aerobic growth at sea water salinities. Since it is known that pathogenic Enterobacteriaceae are able to survive for quite long periods in agricultural soils (Platz, 1981) and that they too can form salinity-tolerant strains (Rudulier *et al.,* 1981), it is likely that these less benign Enterobacteriaceae are also able to survive in marine habitats. We are left, therefore, to agree with Neilson (1980) and Xu *et al.,* (1982) who suggest that it is time for the survival of pathogens in the aquatic environment to be reassessed.

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