Simulation of Microbial Processes in Estuarine Sediments using Gel-stabilized Systems

By G. T. MACFARLANE,*† MOYA A. RUSS, S. M. KEITH
AND R. A. HERBERT

Department of Biological Sciences, The University of Dundee, Dundee DD1 4HN, UK

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Gel-stabilized model systems have been developed which simulate the physico-chemical gradients found in estuarine sediments of the River Tay, Scotland. Depth profiles of E_h and pH, and of concentrations of NH_4^+ , NO_2^- and NO_3^- and dissolved O_2 which developed within the gels were similar to those measured *in situ* in the sediments. The spatial distribution of populations of nitrifying, nitrate respiring and sulphate reducing bacteria in mature gels showed a good correlation with those recorded in the surface sediments of Kingoodie Bay in the Tay estuary. These data show that NH_4^+ oxidation by autotrophic nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) plays a significant role in the development of pH, NH_4^+ , NO_2^- and NO_3^- gradients within the gels, indicating that similar processes may also operate *in situ* in these sediments.

INTRODUCTION

Sediments are heterogeneous environments where rapid changes in physical and chemical gradients develop over extremely short vertical and horizontal distances. As a consequence, anaerobic processes, e.g. sulphate reduction, can occur in ostensibly aerobic regions of sediments (Jørgensen, 1977). The development of these micro-niches within sediments exerts a profound effect on the metabolic activities and therefore the mineralization processes which can be performed by the indigenous microflora. Estuarine sediments are areas of intense microbial activity (Wood, 1965; Jørgensen, 1980) and most authorities are of the opinion that surface sediments (0–5 cm depth) are the most microbiologically active (Sørensen, 1978; Billen, 1982; Macfarlane & Herbert, 1984). The vertical distribution of bacteria within sediments is largely dependent upon the presence or absence of oxygen and, generally, the distribution of the different physiological types exhibits a zonation similar to the chemical gradients which develop (Mechalias, 1974; Billen, 1982). Thus, a region of nitrate reduction immediately underlies the surface aerobic zone and is in turn succeeded by a zone of sulphate reduction, below which is a region of CO_2 reduction. The exchange of materials between the reduced and oxidized sediment horizons is facilitated by diffusion, bioperturbation and irrigation. Macfarlane & Herbert (1984) showed that the upper 3 cm of Tay estuary sediments were oxidized whereas below this depth they became progressively anaerobic and reduced. Autotrophic nitrification was restricted to the upper oxidized region and distinct gradients of pH, NH_4^+ , NO_2^- and NO_3^- were also recorded in this zone. Maximum rates of nitrate respiration were also found within the 0–3 cm horizon of the sediments reflecting the availability of NO_3^- as a terminal electron acceptor, generated, in part, as an end-product of nitrification. During the summer months significant rates of sulphate reduction were also recorded (Keith & Herbert, 1982).

In order to study the spatial distribution of these physiologically distinct groups of bacteria, we have used the gel-stabilized systems developed by Wimpenny *et al.* (1981) to model these microbial processes in the laboratory. The objectives of the present study were to use gel-stabilized

† Present address: Dunn Clinical Nutrition Centre, Addenbrookes Hospital, Cambridge, UK.

systems to generate physico-chemical gradients similar to those recorded *in situ* in Tay estuary sediments as a consequence of the metabolic activities of nitrifying, nitrate respiring, sulphate reducing and methanogenic bacteria.

METHODS

Measurement of physico-chemical characteristics of Kingoodie Bay sediments. Measurements of pH, E_h , temperature, dissolved O_2 tension, NO_3^- , NO_2^- and NH_4^+ in the sediments were determined at 1 cm increments from 0 to 5 cm depth as described by Macfarlane & Herbert (1984).

Enumeration of populations of nitrifying, nitrate respiring and sulphate reducing bacteria. Depth profiles of cell population densities of autotrophic nitrifying bacteria (NH_4^+ and NO_2^- oxidizers) and nitrate respiring bacteria were determined at 1 cm increments from 0 to 5 cm depth as described by Macfarlane & Herbert (1984). Sulphate reducing bacteria populations were enumerated by the most probable number (MPN) method (Alexander, 1965) using growth medium E of Postgate (1979). The tubes were incubated under a gas atmosphere of 95% (v/v) $H_2/15\%$ (v/v) CO_2 at 25 °C for 7–10 d. Tubes in which blackening of the culture medium occurred due to the production of FeS were counted as positives and the most probable number of sulphate reducing bacteria was estimated from the statistical tables of Alexander (1965).

Determination of nitrification rates, nitrate respiration rates, sulphate reduction rates and methanogenesis. Nitrification rates in the sediments were determined using the [^{14}C]bicarbonate dark uptake method described by Billen (1976). Nitrate respiration to gaseous products (denitrification) and to NH_4^+ were determined using $Na^{15}NO_3$ (99 atom% ^{15}N) as described by Macfarlane & Herbert (1984). Sulphate reduction rates were determined according to the method described by Senior *et al.* (1982). Sulphate concentrations in the sediment pore water were determined by a barium sulphate turbidimetric method (American Public Health Association, 1974) after precipitation of sulphides by the addition of 1% (w/v) cadmium acetate. Sulphate reduction rates were calculated by the method of Sorokin (1962).

Methane production in Kingoodie Bay sediments was determined by extruding duplicate 5 g sub-samples of sediment from each depth horizon into 14 ml sterile serum bottles fitted with Suba-seals and then purging the vessels with oxygen-free nitrogen before incubation at 15 °C. Methane production was followed by removing 100 μ l headspace gas samples for analysis by gas chromatography with a Gow-Mac model 752 gas chromatograph fitted with a flame ionization detector. The 2 m stainless steel column (2 mm i.d.) was packed with 80–100 mesh Porapak T and operated isothermally at 100 °C and with a nitrogen carrier gas flow of 30 ml min^{-1} . Quantitative results were obtained by comparison of peak heights with those obtained by injection of CH_4 standards.

Construction of gel model systems. The gel model system described by Wimpenny *et al.* (1981) was modified and gels were constructed as follows. A basal mineral salts medium of the following composition was used to prepare both the solid and semi-solid agar layers (g l^{-1}): $MgSO_4 \cdot 7H_2O$, 0.15; $CaCl_2 \cdot 6H_2O$, 0.01; $NaHCO_3$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.014; KH_2PO_4 , 0.4; K_2HPO_4 , 4.2; cycloheximide, 0.05. The pH of the medium was adjusted to 7.6 before autoclaving at 121 °C for 15 min. The model system was set up by gelling 100 ml of the basal medium plus 10 mM- $(NH_4)_2SO_4$ with 1.0% (w/v) purified agar (Oxoid). This medium was then aseptically added to a sterile 1 litre Quickfit reaction vessel and allowed to gel. Approximately 100 ml of a freshly collected, well-mixed sediment sample from Kingoodie Bay (0–5 cm depth) was then evenly layered over the solid gel layer and a semi-solid agar layer (600 ml) containing the basal mineral salts medium plus 0.3% (w/v) purified agar was then aseptically poured down the side of the vessel to avoid disturbing the sediment layer. The gel model system was then covered with aluminium foil and incubated at 20 °C. The sole source of available carbon in these models was that derived from the sediment layer. In addition to the inoculated gels, controls to which no sediment addition had been made were run as well as gels which contained the nitrification inhibitor N-serve (2-chloro-6-trichloromethyl pyridine) at a concentration of 10 mg l^{-1} .

The semi-solid top layer of each gel system was sampled at 0, 4, 8, 12 and 13 weeks and the profiles of pH, O_2 tension, E_h , NO_3^- , NO_2^- and NH_4^+ were determined, together with the population densities of nitrifying, nitrate respiring and sulphate reducing bacteria using the methods described earlier. Samples were removed for analysis and cell enumeration using a sterile Pasteur pipette which was attached to a micro-manipulator.

Chemicals. N-serve (2-chloro-6-trichloromethyl pyridine) was a generous gift from the Dow Chemical Co., and $Na^{15}NO_3$ (99 atom% ^{15}N) was obtained from BOC Prochem, London, UK. $NaH^{14}CO_3$ [58 mCi $mmol^{-1}$ (2.15 GBq $mmol^{-1}$)] and $Na_2^{35}SO_4$ [60 mCi $mmol^{-1}$ (2.2 GBq $mmol^{-1}$)] were obtained from Amersham.

RESULTS

Microbial processes in Kingoodie Bay sediments

Seasonal rates of nitrification, nitrate respiration, sulphate reduction and methanogenesis have been determined (Keith & Herbert, 1982; Macfarlane & Herbert, 1984). Table 1

Table 1. Rates of nitrification, denitrification, NO_3^- dissimilation to NH_4^+ , dissimilatory sulphate reduction and methanogenesis in Kingoodie Bay sediments (June 1982)

Results are mean values of three samples; ND, not detected.

Sediment depth (cm)	Rate of nitrification*	Rate of denitrification*	Rate of NO_3^- dissimilation to NH_4^+ *	Rate of sulphate reduction†	Methanogenesis‡
0-1	0.92	18.89	4.63	60.16	3.79
1-2	0.67	24.44	4.72	70.14	2.80
2-3	0.32	11.63	3.52	76.80	14.62
3-4	ND	4.71	2.42	58.40	16.52
4-5	ND	4.43	1.69	41.75	20.44

* $\mu\text{g N (g dry wt sediment)}^{-1} \text{ d}^{-1}$.† $\text{nmol SO}_4^{2-} \text{ reduced (ml sediment)}^{-1} \text{ d}^{-1}$.‡ $\text{nmol CH}_4 \text{ produced (g dry wt sediment)}^{-1} \text{ d}^{-1}$.Table 2. Distribution of nitrifying, NO_3^- respiring and sulphate reducing bacteria in Kingoodie Bay sediments (June 1982)

Results are given as cell number (g dry wt sediment) $^{-1}$.

Sediment depth (cm)	NH_4^+ oxidizing bacteria	NO_3^- oxidizing bacteria	Denitrifying bacteria	Bacteria dissimilating NO_3^- to NH_4^+	Sulphate reducing bacteria
0-1	7.0×10^3	1.6×10^4	1.0×10^5	5.0×10^7	3.8×10^3
1-2	No data	No data	1.5×10^5	6.3×10^6	4.2×10^4
2-3	6.0×10^3	7.8×10^3	8.9×10^4	1.2×10^6	6.1×10^5
3-4	No data	No data	9.5×10^4	8.8×10^5	4.3×10^4
4-5	3.0×10^3	3.0×10^3	4.5×10^4	2.1×10^5	3.3×10^4

summarizes these rates for the surface sediments of Kingoodie Bay (0-5 cm) in June 1982. Maximum rates of all the processes were recorded in the summer months when the sediment temperatures were highest (19 °C). The data also show that there is a spatial distribution of these processes within the surface sediments. Maximum nitrification rates were recorded in the 0-1 cm depth horizon of the sediments [$0.92 \mu\text{g N (g dry wt sediment)}^{-1} \text{ d}^{-1}$] whilst nitrate respiration rates (denitrification and NO_3^- dissimilation to NH_4^+) were maximal in the 1-2 cm horizon. Dissimilatory sulphate reduction occurred over a larger depth profile (1-3 cm) with maximum rates recorded at a depth of 2-3 cm. Methanogenesis was recorded at all depths but highest rates were recorded at 4-5 cm. Cell population densities of autotrophic nitrifying bacteria, nitrate respiring bacteria (denitrifiers and bacteria dissimilating NO_3^- to NH_4^+) and sulphate reducing bacteria followed a similar spatial distribution pattern in the sediments (Table 2) to those recorded for the rates of processes described previously. The progression of different physiological groups from the surface sediment downwards follows the general pattern of aerobes giving way to facultative and then strict anaerobes. Table 1 also shows that sulphate reduction was occurring within the oxic sub-surface sediments in Kingoodie Bay (see Fig. 1). Similar results have been reported by Jørgensen (1977) for inshore coastal sediments and have been attributed to the presence of anaerobic, reduced microsites within the predominantly aerobic surface sediments.

Development of physico-chemical gradients in gels

Comparison of the physico-chemical profiles of Tay estuary sediments (June 1982) with those that developed in the gel model systems after 12 weeks incubation at 20 °C (Fig. 1) shows that the gradients of pH, E_h and dissolved O_2 tension in both systems were similar. These gradients did not form in uninoculated gels, indicating the role of micro-organisms in these processes. Similarly, a comparison of the profiles of NH_4^+ , NO_2^- and NO_3^- in Kingoodie Bay sediments and those which developed in the gel system shows comparable gradients (Fig. 2). Concentrations of NH_4^+ increased with depth in both systems whereas the reverse occurred with NO_2^- and NO_3^- .

Fig. 1

Fig. 2

Fig. 1. Depth distributions of E_h , dissolved O_2 concentration and pH in Kingoodie Bay sediments (July 1982) and gel sediment systems after 12 weeks incubation at 20 °C. Kingoodie Bay sediments, filled symbols; gel sediments, open symbols; ●, ○, E_h or O_2 ; ▲, △, pH.

Fig. 2. Depth distributions of NH_4^+ , NO_3^- and NO_2^- in Kingoodie Bay sediments (July 1982) and gel sediment systems after 12 weeks incubation at 20 °C. Kingoodie Bay sediments, filled symbols; gel sediments, open symbols; ●, ○, NH_4^+ ; ▲, △, NO_3^- ; ■, □, NO_2^- .

These chemical gradients failed to develop in control gels which contained no sediment inoculum, providing further evidence for the involvement of micro-organisms in these processes. In the gel model system, NH_4^+ was the sole inorganic nitrogen species present in the medium and so the appearance of NO_2^- and NO_3^- in the surface layers of the gel is indicative of nitrification. In mature gels, populations of autotrophic nitrifying bacteria were only detected in the top 1 cm of the gels (Table 3) and these data correlate well with the observed concentrations of NO_2^- and NO_3^- (Fig. 2). To confirm that nitrifying bacteria were essential for the production of NO_2^- and NO_3^- from NH_4^+ , inoculated gels were treated with the nitrification inhibitor N-serve ($10\ mg\ l^{-1}$) at zero time and examined at periodic intervals for the production of NO_2^- and NO_3^- . NO_2^- and NO_3^- production could not be detected under these conditions and no nitrifying bacteria could be isolated. The addition of $10\ mg\ N\text{-serve}\ l^{-1}$ to mature gels resulted in the disappearance of the NO_2^- and NO_3^- gradients within 7 d and the NH_4^+ and NO_2^- oxidizing populations declined sharply. These results further support the conclusion that autotrophic bacteria play a central role in the generation and maintenance of NO_2^- and NO_3^- gradients.

The spatial distribution of the individual groups of bacteria in the gels after 12 weeks incubation at 20 °C followed a similar pattern to those recorded in Kingoodie Bay sediments (Tables 2 and 3). Nitrifying and potential nitrate respiring bacteria were dominant in the oxic 0–1 cm horizons of the gels and cell populations decreased with depth, although the decline in the nitrate respiring populations was less rapid than for the nitrifiers, which could not be detected at depths greater than 1 cm. Maximum populations of sulphate reducing bacteria were recorded in the 2–3 cm horizon of the gels and the spatial distribution was similar to that observed in Kingoodie Bay sediments. These results demonstrate that the physico-chemical gradients and spatial

Table 3. Distribution of nitrifying, NO_3^- respiring and sulphate reducing bacteria in a stabilized gel after 12 weeks incubation at 20 °C

Results are given in cell number ml^{-1} ; ND, not detected.

Sediment depth (cm)	NH_4^+ oxidizing bacteria	NO_3^- oxidizing bacteria	Denitrifying bacteria	Bacteria dissimilating NO_3^- to NH_4^+	Sulphate reducing bacteria
0-0.5	8.2×10^4	1.0×10^4	3.0×10^5	6.2×10^6	1.4×10^2
0.5-1	8.0×10^3	6.2×10^3	2.0×10^6	3.5×10^6	2.1×10^3
1-2	ND	ND	2.0×10^6	1.2×10^6	4.3×10^4
2-3	ND	ND	2.5×10^5	2.2×10^6	3.8×10^6
3-4	ND	ND	1.8×10^5	5.0×10^5	1.2×10^6
4-5	ND	ND	3.1×10^4	2.0×10^5	1.8×10^5

distribution of bacterial populations which developed within the gel systems were similar to those found in the surface sediments of Kingoodie Bay, and provide the basis of a model system for determining the parameters governing the distribution of bacteria in these environments.

DISCUSSION

The significance of spatial and temporal heterogeneity of natural environments on the activities of microbial communities is widely recognized (Jørgensen, 1980; Wimpenny, 1981; Bull & Slater, 1982; Jones, 1982; Wimpenny *et al.*, 1983). However, the experimental methodology currently available to study these activities *in situ* is either insufficiently sensitive or discrete to probe these micro-environments without destroying their structural integrity, although considerable advances have been made recently in the development and application of micro-electrodes for measuring physico-chemical gradients in marine sediments (Revsbach *et al.*, 1980). In order to study how microbial communities function in natural environments, it is often necessary to use model systems to identify the key processes involved. In operating simple model systems it is essential to establish how closely the model simulates the environmental conditions under investigation. The results presented in this paper show that the physico-chemical gradients (Figs 1 and 2) and spatial distribution of the microbial populations which develop in the gel systems were similar to those found *in situ* in Kingoodie Bay sediments. In this study we have investigated the effects of opposing gradients of O_2 and NH_4^+ on the development and spatial distribution of nitrifying, nitrate respiring, sulphate reducing and methanogenic bacteria in the absence of exogenous carbon sources, except for those introduced with the sediment inoculum. In estuarine and marine sediments NH_4^+ concentrations generally increase with depth as a consequence of ammonification (Sørensen, 1978; Owens *et al.*, 1979; Stanley *et al.*, 1981). NH_4^+ generated in smaller quantities as an end-product of NO_3^- respiration supplements that derived by ammonification (Macfarlane & Herbert, 1984). In the gel model system the only inorganic nitrogen source present was 10 mM- $(\text{NH}_4)_2\text{SO}_4$ and this was introduced in the source layer in order to simulate the NH_4^+ pool. The upward diffusion and NH_4^+ gradient which subsequently developed followed a profile similar to that recorded in the sediments albeit an order of magnitude greater. NO_3^- and NO_2^- production in the aerobic 0-1 cm horizon of the gels resulted from the activities of autotrophic nitrifying bacteria which could only be detected in this zone. Maximum populations of potential NO_3^- respiring bacteria in the gels were also present in the oxic 0-1 cm horizon, although it is debatable whether these bacteria were respiring NO_3^- *in situ*, since at the recorded dissolved O_2 tensions synthesis of dissimilatory NO_3^- and NO_2^- reductase would be repressed (Dunn *et al.*, 1979). These results indicate that autotrophic nitrifying bacteria play a central role in establishing and maintaining NO_3^- and NO_2^- gradients in the gel system. The addition of N-serve, a specific inhibitor of NH_4^+ oxidation (Shattuck & Alexander, 1963), to inoculated gels at zero time totally suppressed the production of NO_2^- and NO_3^- gradients whilst the addition of this inhibitor to established gels resulted in the rapid disappearance of the NO_3^- and NO_2^- gradients. These results substantiate the findings of Vanderborght & Billen (1975) that nitrification was responsible for NO_3^- and NO_2^- gradients which are found in an artificial lagoon at Ostend, and indicate a similar role for nitrifying bacteria in Kingoodie Bay sediments.

The relationship between the aerobic nitrifying bacteria and the facultatively anaerobic NO_3^- respiring bacteria is probably complex and tightly coupled. The data presented in this paper show that not only are maximum populations of both groups of bacteria present in the same depth horizons but that this zone is also the site of maximum activity (Table 1). These results indicate that both processes are occurring simultaneously in the oxidized surface sediments in Kingoodie Bay and corroborate the field experiments of Sørensen (1978) and Koike & Hattori (1978). Field data (Table 1) show that denitrification was the principal route of NO_3^- respiration in Kingoodie Bay sediments and NO_3^- dissimilation to NH_4^+ was a minor, but nevertheless not inconsequential process. The latter process in terms of intermediates is the reverse of nitrification and it is interesting to speculate that an internal nitrogen cycle may operate between the nitrifying bacteria and the nitrate respiring bacteria. Experimental evidence to support this hypothesis has been obtained using a multi-stage diffusion chemostat in which a NO_3^- respiring *Vibrio* sp. supported the growth of *Nitrosomonas* sp. and *Nitrobacter* sp. (Macfarlane, 1983).

In addition to aerobes and facultative anaerobes, populations of strict anaerobes such as sulphate reducing bacteria and methanogens also developed in the deeper regions of the gels, where the redox potential was sufficiently low to initiate growth. Methanogenesis and sulphate reduction occurred in the same depth horizons in Kingoodie Bay sediments but the latter process was dominant. These data are in agreement with those obtained by Senior *et al.* (1982) for salt-marsh sediments. A similar distribution of sulphate reducing bacteria and methanogens developed within the gel model sediment system and CH_4 , identified by gas chromatography, was trapped in the agar source layer.

In summary, the gel model system described offers considerable promise as a research tool for the investigation of microbial interactions in estuarine sediments. The system is reproducible and, whilst it does suffer some disadvantages such as the lack of bioperturbation and absence of either gas or solute exchange, it nonetheless permits the analysis of microbial processes which are presently unobtainable by other means.

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