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Characterization of sulphate-reducing bacterial populations within marine and estuarine sediments with different rates of sulphate reduction

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1. SUMMARY

Viable counts of sulphate-reducing bacteria, able to use a range of different growth substrates were determined in sediments from two Sea Lochs (Etive and Eil) and an estuarine site (Tay), in Scotland. The composition of the sulphate-reducing bacterial population, in terms of substrate utilization, broadly corresponded to the in situ substrates for sulphate reduction and concentration of substrates at each site. Addition of acetate, lactate, propionate, butyrate, hydrogen and glutamate/serine (20 mM) to replicate slurries from each site resulted in stimulation of the corresponding population of sulphate-reducing bacteria and the in situ rates of sulphate reduction. The metabolism of the added substrates and changes in bacterial phospholipid fatty acids (PLFA) were quantified. With the exception of acetate and hydrogen, added substrates were incompletely oxidised, producing a mixture of further substrates, which predominantly were sequentially oxidised, and resulted in the stimulation of a mixed population of sulphate-reducing bacteria. There were significant changes in the PLFA of slurries with added substrate compared to controls. Acetate was completely removed at all sites and the small increase in even chain PLFA together with the absence of stimulation of any other biomarker, indicated that acetate was oxidised by sulphate-reducing bacteria distinctly different from those using other substrates. A biomarker for *Desulfobacter*, 10 Methyl 16:0, was not stimulated in any of the acetate slurries or in slurries where acetate was produced. Biomarkers for the propionate utilizing *Desulfobulbus* sp (17:1w6, 15:1w6) were always stimulated in propionate slurries and also in lactate slurries, where

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partial lactate fermentation produced propionate and acetate. In lactate and glutamate/serine slurries from the Tay estuary and lactate and hydrogen slurries from Loch Etive the biomarker for *Desulfovibrio* sp (i17 : 1w7) as well as those for *Desulfobulbus* were stimulated. This provides direct evidence for the significance of *Desulfovibrio* sp. within sediment slurries and demonstrates the competitive interaction between members of this genus and *Desulfobulbus* sp. for lactate, hydrogen and amino acid metabolism. At the estuarine site, sulphate reduction was limited at higher sulphate concentrations (about 3.5 mM) than the Sea Loch sites (< 2 mM) and this had a significant effect on propionate and butyrate metabolism, as well as on methane production. These results demonstrate that although the sulphate-reducing bacterial population at each site could metabolise identical substrates, the types of sulphate-reducing bacteria involved and their sulphate thresholds were characteristically different.

2. INTRODUCTION

During the last decade major advances have been made in quantifying the ecological significance of dissimilatory sulphate reduction within marine, estuarine and freshwater sediments. This process has been shown to play an important, and often a dominant role in carbon flow within these environments [1–6]. There has also been a complementary increase in the metabolic diversity and flexibility of sulphate-reducing bacteria isolated [7]. Together, these have changed the perception of sulphate reduction. Initially it was thought to be an environmentally restricted activity, mediated by a limited and metabolically constrained group of specialist anaerobes. But the more recent perception is of a process of wide ecological significance, catalysed by a metabolically diverse and flexible group of bacteria, not solely restricted to anoxic environments [8]. Despite this progress there is still little direct evidence to confirm which, if any, of the increasing number of sulphate-reducing bacterial isolates are significantly active in the environment, or how sulphate-reducing bacterial populations vary between different sedimentary environments. Improvements have been made in culturing techniques for enumerating different types of sulphate-reducing bacteria, and this has resulted in significant correlation between numbers of sulphate-reducing bacteria and rates of sulphate reduction [9] and substantial increases in the recovery of sulphate-reducing bacteria from sediments [10,11]. Viable counts, however, still tend to underestimate the in situ population, especially in deeper sediments layers [11]. Also, they do not discriminate between different sulphate-reducing bacteria which utilize the same substrate and this is not resolved by subsequent microscopic analysis of enrichments or colonies as this has limited capacity for identification. The situation is further complicated if the growth substrate is incompletely oxidised or fermented, as a range of different types of sulphate-reducing bacteria develop, and the dominant type changes with increasing incubation time [12]. Direct analysis of bacterial cellular components, which do not require growth, such as gene probes [13,14] and phospholipid fatty acid analysis (PLFA, Refs. 12 and 15) have the potential to overcome many of the above problems and thus provide accurate characterization of the in situ bacterial community.

Bacteria contain characteristic PLFA in the C_{12}-C_{19} region [16,17] which distinguish them from eukaryotic organisms and often from each other. PLFA may therefore be used to characterise complex microbial communities in situ [12,15]. Several sulphate-reducing bacteria have specific PLFA [18–21] and these have been shown to be effective in characterising the sulphate-reducing bacterial community within marine sediment slurries [18]. The aim of this research was to use PLFA to characterise the sulphate-reducing bacteria in contrasting sites, and compare these data with both viable counts and the in situ substrates for sulphate reduction [22]. Selected populations of sulphate-reducing bacteria were obtained in sediment slurries, to which a range of individual substrates had been added, and the metabolism of these substrates was quantified. This allowed the PLFA of the viable sulphate-reducing bacterial population to be differentiated

from the PLFA of non-viable, dormant or dead biomass [12,23].

3. METHODS

3.1. Sampling

Three sampling sites were used, two sea lochs on the west coast of Scotland, Lochs Etive and Eil, and Kingoodie Bay in the Tay Estuary, on the east coast of Scotland [22]. Sediment cores approx. 30 cm in length were obtained in perspex tubes using a gravity corer [24] for the sea lochs, and by hand at low tide for the Tay estuary. Cores were brought back to the laboratory on ice and stored at 5°C until use, which was normally the next day.

3.2. Sediment slurries

For each site a known volume of sediment was sectioned from the anaerobic zone [4] of several different cores, into a beaker continually flushed with oxygen-free-nitrogen (OFN). This sediment was then transferred into an anaerobic cabinet (Forma Scientific), and thoroughly mixed. An equivalent volume of deoxygenated 50% (v/v) seawater, containing 1 mM Na_2S, was mixed with the sediment and then passed through a 1 mm sieve. Whilst mixing, the slurry was distributed in 150 ml amounts into screw cap conical flasks (250 ml) which were sealed with a septum. The flasks were removed from the anaerobic cabinet, with subsequent additions or removals being made via the septum, with OFN flushed syringes. Deoxygenated solutions of the sodium salts of acetate, propionate, lactate and butyrate were separately added, in duplicate, to provide a final concentration of 20 mM. For the Tay estuary site a mixture of 10 mM glutamate and 10 mM serine was also added. Flasks were flushed for 5 min with OFN, leaving a slight positive pressure. Flasks without additions were either used as controls or flushed with H_2/CO_2 (80/20, v/v). A slurry sample (5 ml) from each treatment was taken for time zero analysis and then the flasks checked for gas leaks. Slurries were then incubated on an orbital shaker (100 rpm) at 28°C in the dark. Incubations were terminated when the initial substrate or immediate fermentation product had been removed which was generally after 14 days, or when there was no substrate change after 4–5 days. For the Tay estuary only, the effect of longer incubation (28–32 days) and further sulphate additions (4–15 mM) on propionate and butyrate metabolism were investigated. After incubation, slurries were frozen and then freeze-dried prior to extraction for PLFA analysis.

3.3. Analysis of slurries during incubation

Samples were removed approximately daily and filtered through a pre-combusted glass fibre filter paper (GFF, Whatman) to remove particulate matter. Samples for sulphide and sulphate determination were added to zinc acetate (4 ml sample to 1 ml 10% zinc acetate) to prevent sulphide oxidation and aliquots taken for sulphide [25] and sulphate [3] analysis. Volatile fatty acids and lactate were determined by ion chromatography (Dionex, Sunnyvale, USA). Samples with high concentrations of volatile fatty acids (> 1 mM) were diluted (1 : 250) with double-distilled water before analysis, whilst samples with lower concentrations were vacuum distilled [22] to remove interfering salts. Headspace gas analysis (H_2, CH_4, CO_2) was conducted on a Pye Unicam Model 104 gas chromatograph with a katharometer detector [22]. At the end of the experiment the flask headspace was adjusted to atmospheric pressure with a nitrogen filled manometer prior to gas analysis. Amino acid concentrations were determined by HPLC (Gilson, Ltd.) as *O*-pthaladehyde derivatives on a reverse-phase column [26].

3.4. Viable counts of sulphate-reducing bacteria

Viable counts of sulphate-reducing bacteria were determined in sediment from each site and incubated slurries, using the agar shake technique [27] and different growth substrates. In order to optimize viable counts two different media were used [10], adjusted for the salinity at each site. Postgate's medium 'E' was used for lactate, butyrate and H_2 utilizing sulphate-reducing bacteria, whilst Widdel's medium was used for acetate, propionate, glutamate and serine utilizers. One ml of sample was initially diluted in 9 ml of 0.9% (w/v) NaCl containing 1 mM sulphide and

0.00001% (w/v) cetyl trimethylammonium bromide (CTAB, Ref. 10) and then serial tenfold dilutions made using the agar shake technique. Additional iron (66 μg $FeSO_47H_2O$ in 9 ml complete medium) was added to Widdel's medium so that sulphate-reducing colonies would be characterized by black iron sulphide formation [18]. Agar shake tubes were incubated at 28°C for 28 days and black colonies counted.

3.5. Lipid analysis

Lipid extraction of approx. 15 g of freeze-dried material was performed in a chloroform/methanol/3% aqueous NaCl solution as described by Dowling *et al.* [30]. After silicic acid separation of the phospholipid fraction, methanolysis of the fatty acid methyl esters was conducted in a solution of toluene/methanol/KOH [30]. The pure methyl esters were analysed initially in their original state on a Shimadzu GC9A capillary gas chromatograph, using both polar and non-polar columns. Subsequent analysis of the monoenoic and cyclopropyl fractions was by comparison with hydrogenated samples [31]. Final confirmation of fatty acid identity was obtained by capillary-mass spectrometry (Hewlett Packard 5995A), as previously described [30]. Quantitative data were normalised to 100 and expressed as percentages.

4. RESULTS

4.1. Distribution of sulphate-reducing bacteria in sediments

Sulphate-reducing bacteria were present in the three sites at all depths, but their distribution in the estuarine site was different from those in the Sea Loch sediments (Fig. 1). At all sites total numbers of sulphate-reducing bacteria were highest in the 5–10 cm interval (2.2 to 11.4 $\times$ 10^4/ml). In the Tay Estuary numbers subsequently decreased markedly (3.2 $\times$ 10^2/ml at 28 cm), whilst in the Sea Loch sediments there was only a slow decrease with sediment depth (4.2 $\times$ 10^3/ml at 28 cm). Despite this, the total population of sulphate-reducing bacteria in the top 28 cm of sediment was still higher (approx. $\times$3) in the Tay Estuary compared to the other sites. The distribution of various types of sulphate-reducing bacteria in the Tay Estuary was also very different, as lactate utilizers represented over 70% of the total viable population (Table 1). The sulphate-reducing bacterial population in the Sea Loch sites was much more uniform with acetate, propionate, butyrate, and lactate-utilizers all comprising significant proportions of the total population. In Loch Eil, lactate was again the dominant substrate but in Loch Etive the percentage of acetate and lactate-utilizers were similar. Loch Etive was also the only site where hydrogen-utilizers were present at similar concentrations to the other bacterial types.

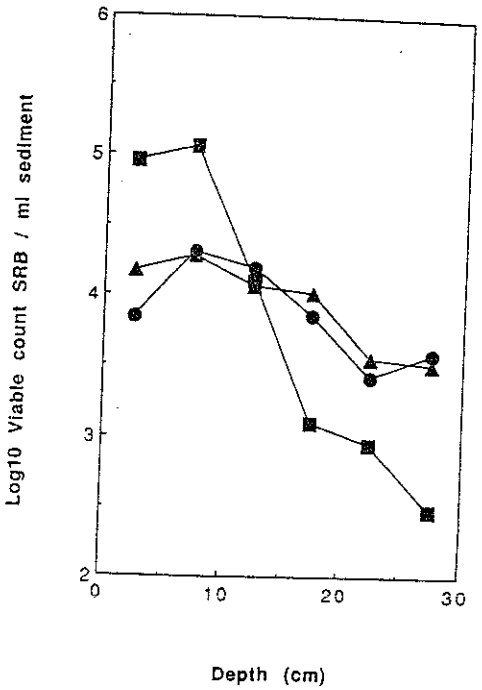

Fig. 1. Distribution of total viable sulphate-reducing bacteria with sediment depth, Loch Etive (●), Loch Eil (▲) and the Tay Estuary (■).

4.2. Metabolism in the sediment slurries

In almost all the slurries there was a small increase in the concentration of added substrates by day one. The reason for this is unclear, but presumably it was due to equilibration processes within the slurry. The sediment slurries were anoxic and remained so throughout the incuba-

tion period with sulphide being produced, although in the controls the amount produced was small (approx. 1 mM). Addition of substrates to the slurries resulted in marked decreases in sulphate (between 3.6 & 19.6 mM) and increases in sulphide concentrations (between 2 & 10 mM) at all sites (Table 2). The relationship between sulphate removed and sulphide produced varied considerably from the expected stoichiometric relationship of 1 : 1, and hence a significant amount of the sulphide produced must have precipitated as iron sulphides. This was most marked in the Tay estuary slurries where the sulphate/sulphide ratio was about 5 : 1 compared to 2 : 1 for the Sea Loch sites (Table 2) and reflects the relatively high iron concentrations in the Tay estuary [28]. Sulphide at high concentrations (above 8–12 mM, Ref. 29) is toxic to sulphate-reducing bacteria and its precipitation by iron in these experiments generally kept concentrations below this level, ensuring the optimal development of sulphate-reducing bacteria.

In Lochs Etive and Eil all substrates, with the exception of butyrate, were completely removed during the incubation period, with concomitant sulphate reduction. Butyrate was only partially degraded (9 & 49% Loch Etive & Eil, respectively, Table 2). Tay estuary slurries were similar, except both propionate (34%) and butyrate (77%) were incompletely utilized, despite being incubated for 28 days, compared to 14 days for the Sea Loch slurries. At all sites acetate was completely removed within 14 days, presumably to carbon dioxide, and propionate was incompletely oxidised to acetate (Figs. 2 and 3). Lactate metabolism was more complicated, being partly fermented to acetate and propionate and partly oxidised to acetate. The propionate produced was subsequently oxidised to acetate (Fig. 2). Butyrate in the Sea Loch slurries was oxidised to acetate but in the Tay Estuary slurries there was partial oxidation to acetate and partial fermentation as indicated by the production of propionate. The concentration of propionate was relatively small (< 3 mM) and varied markedly with time in a cyclical manner (Fig. 3). In replicate Tay slurries with propionate and butyrate, plus elevated sulphate (35 mM), both substrates were completely metabolised within 10 days (Fig. 4). Butyrate metabolism again occurred with intermediate propionate production. The effect of sulphate in determining propionate and butyrate metabolism in Tay slurries was further investigated in slurries with normal sulphate concentrations to which was added additional sulphate (4 mM) when substrate removal had ceased. This addition resulted in a rapid and complete decomposition of butyrate and renewed removal of propionate, but not complete decomposition (Fig. 4). Further sulphate additions to the propionate slurry also stimulated decomposition but again degradation was incomplete (Fig. 4).

In Tay slurries with glutamate and serine (10 mM each) glutamine (3.5 mM) was present, a few

Table 1

Distribution of sulphate-reducing bacteria (SRB) in sediments and slurries compared to natural substrates for sulphate reduction

Substrate	% SRB in sediment			% SRB in control slurry			% substrate for sulphate reduction **		
	Etive	Eil	Tay	Etive *	Eil *	Tay *	Etive	Eil	Tay
Acetate	28	16	10	25	29	9	100	64	35
Lactate	32	46	77	54	38	73	–	–	43
Propionate	19	19	6	10	16	7	–	13	6
Butyrate	14	14	6	6	13	6	–	5	7
Hydrogen	13	6	0	5	4	4	2	0	–
Glutamate	n.d.	n.d.	n.d.	n.d.	n.d.	1	–	2	1
Serine	n.d.	n.d.	n.d.	n.d.	n.d.	1	–	2	1

n.d., Not determined.

* SRB composition in sediment and control not significantly different ($P > 0.05$), Mann & Whitney U-test.

** Data from Parkes et al., 1989.

Table 2

Effect of addition of potential substrates for sulphate reduction to anaerobic slurries

Sampling site	Start of incubation period				End of incubation period						
	Treatment (20 mM)	Sulphate (mM)	Sulphide (mM)	Incubation time (days)	Sulphate (mM)	Sulphide (mM)	Substrate (mM)	Acetate (mM)	Propionate	headspace % composition	
										CH_4	CO_2
Loch Etive	Control	19.0	0.1	14	18.0	1.7	–	0.01	0	–	0.4
	Acetate	19.0	0.1	14	2.0	9.1	0	0	0	–	1.6
	Lactate	18.7	0.1	14	1.6	7.7	0	20	0	–	2.1
	Propionate	19.3	0.1	14	2.3	8.0	0	19.9	0	–	1.8
	Butyrate	19.2	0.1	14	15.6	2.3	17.5	2.0	0	–	1.0
	*H_2/CO_2	19.5	0.1	14	2.3	7.7	0	0.01	0	–	5.3
Loch Eil	Control	20.9	0.1	14	19.2	1.2	–	0.01	0	–	0.6
	Acetate	20.3	0.1	14	1.8	10.2	0	0	0	–	2.6
	Lactate	21.1	0.1	14	1.5	10.0	0	16.3	0	–	2.8
	Propionate	20.0	0.2	14	1.8	9.9	0	17.5	0	–	2.8
	Butyrate	19.7	0.2	14	8.8	6.1	10.1	4.1	0	–	2.1
	*H_2/CO_2	20.6	0.2	14	2.8	8.6	0	0.01	0	–	4.0
Tay Estuary	Control	19.1	0.1	28	18.5	0.6	–	0.01	0	–	2.6
	Acetate	19.2	0.1	14	3.2	3.3	0	0		–	4.5
	Lactate	19.5	0.1	14	2.7	3.4	0	13.5	0	–	7.2
	Propionate	19.2	0.1	28	3.9	3.1	12.6	14	12.6	0.7	3.6
	Prop. + sulphate (15 mM)	35.1	0.2	14	22.0	3.1	0	21.3	0	–	1.8
	Butyrate	19.4	0.1	28	3.3	3.2	4.5	25.1	1	0.6	3.2
	But + sulphate (15 mM)	35.1	0.1	14	21.9	2.9	0	19.5	0	–	1.4
	*H_2/CO_2	19.1	0.1	28	9.5	2.3	0	0.1	0	17.4	2.8
	Glut/Ser	19.0	0.2	10	5.1	2.1	0	0	0	–	9.8

* Headspace (80/20, v/v).

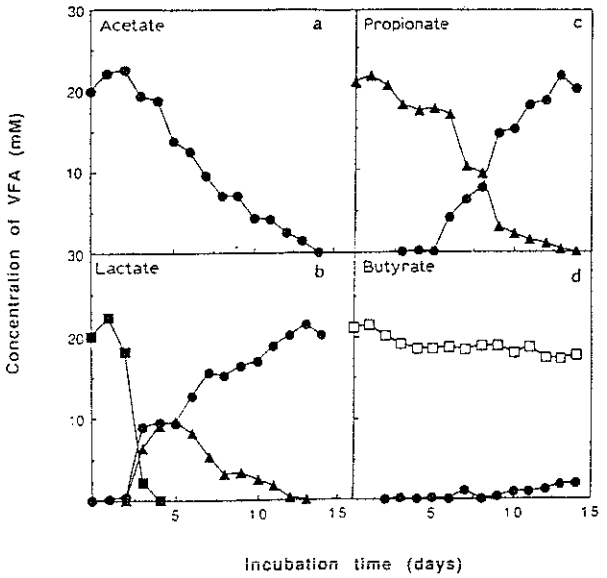

Fig. 2. Anaerobic metabolism of acetate, lactate, propionate, and butyrate (20 mM) added to Loch Etive slurries: acetate (●), propionate (▲), lactate (■), butyrate (□). The patterns of metabolism in Loch Eil slurries were identical to those for Loch Etive and hence summary data only is presented (Table 2).

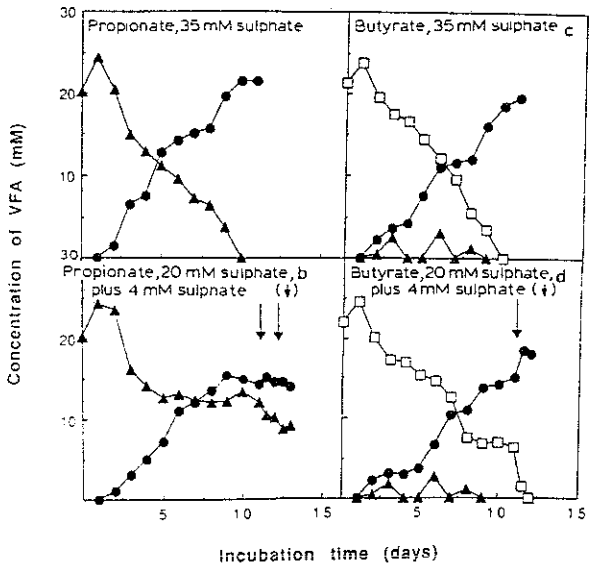

Fig. 4. The effect of elevated sulphate concentrations on the metabolism of propionate and butyrate in Tay Estuary slurries: acetate (●), propionate (▲), butyrate (□).

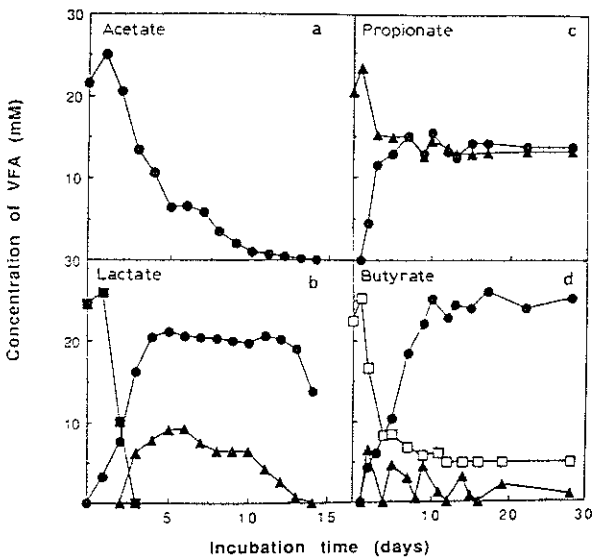

Fig. 3. Anaerobic metabolism of acetate, lactate, propionate and butyrate (20 mM) added to Tay Estuary slurries: acetate (●), propionate (▲), lactate (■), butyrate (□).

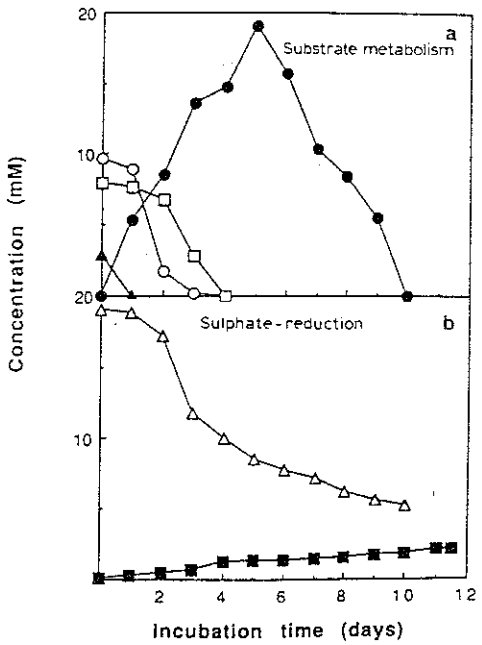

Fig. 5. Anaerobic metabolism of glutamate and serine in Tay Estuary slurries: glutamate (□), serine (○), glutamine (▲), acetate (●), sulphate (△), sulphide (■).

Table 3

Changes in populations of viable sulphate-reducing bacteria (colony counts/ml sediment) during slurry incubations

Site	Treatment	AcSRB mean	% Incr c.f.Ctl	LacSRB mean	% Incr c.f.Ctl	PropSRB mean	% Incr c.f.Ctl	ButSRB mean	% Incr c.f.Ctl	H_2SRB mean	% Incr c.f.Ctl	Total	% Incr c.f.Ctl	Glut/Ser SRB mean	% Incr c.f.Ctl
Loch	Control	4000	–	8500	–	1500	–	950	–	750	–	15700	–		
Etive	Acetate	**9000** *	**225**	8500	–	2500	167	1000	105	850	113	21850	139		
	Lactate	5500	137	**19000**	**224**	**3500**	**233**	850	–	700	–	29550	188		
	Propionate	4500	112	8500	–	**4500**	**300**	950	–	450	–	18900	120		
	Butyrate	**6000**	150	7500	–	1000	–	**2500** *	**263**	400	–	17400	111		
	Hydrogen	4500	112	9000	106	1500	–	700	–	**2500**	**333**	18200	116		
Loch	Control	7000	–	9000	–	3900	–	3000	–	900	–	23800	–		
Eil	Acetate	**60000**	**857**	10000	111	3500	–	5000	167	1500	167	80000	336		
	Lactate	**44000**	**629**	**50000**	**556**	**28500**	**731**	4000	133	800	–	127300	535		
	Propionate	9500	136	9000	–	**14500**	**372**	5000	167	400	–	38400	161		
	Butyrate	**20000**	**286**	20000	222	**40000**	**1026**	**60000** *	**2000**	500	–	140500	590		
	Hydrogen	**24500**	**350**	9000	–	3500	–	5500	183	**4000**	**444**	46500	195		
Estu-	Control	8000	–	65000	–	6500	–	5000	–	3500	–	88500	–	500	–
ary	Acetate	**60000** *	**750**	90000	130	9000	138	6000	120	2000	–	168000	190	**1000**	200
Tay	Lactate	9000	112	**400000** *	**615**	9000	138	7000	140	0	–	425200	480	200	–
	Propionate	8667	108	**296667**	**456**	**33333** *	**513**	5667	113	3000	–	347567	393	233	–
	Butyrate	4667	–	46667	–	6333	–	**20000** *	**400**	3667	105	81500	92	167	–
	Hydrogen	5000	–	43333	–	6667	103	2633	–	**12333**	**352**	70333	79	367	–
	Glut/Ser	4000	–	60000	–	2000	–	900	–	0	–	68400	77	**1500** *	**300**
										mean% Increase c.f. Ctl.			238		

* $P < 0.05$ compared to control, analysis of variance after log transformation.

Bold, major changes in sulphate-reducing bacteria.

– no change or a decrease.

Table 4

Summary of changes in PLFA in anaerobic slurries

Site	Treatment	PLFA (%)												
		14:0	i15:0	15:1w6	15:0	16:1w7	16:0	i17:1w7	10Me16:0	17:1w6	cy17:0a	17:0	18:1w7	18:0
Loch Etive	Control	4.33	4.93	0.00	1.44	7.36	19.02	0.79	3.64	0.66	0.81	1.29	9.14	5.10
	Acetate	4.26	4.46	0.00	1.31	7.02	**22.62**	0.74	3.36	0.71	0.48	1.30	12.58	5.18
	Lactate	3.17	4.25	**0.46**	**3.59**	7.77	18.44	**2.60** *	3.11	**2.27**	0.85	2.09	12.39	3.75
	Propionate	2.93	3.87	**0.30**	**6.28** *	7.24	15.41	0.59	2.50	**4.03**	0.45	**4.10**	13.94	3.58
	Butyrate	4.42	3.48	0.07	1.06	**13.68**	**21.24**	0.41	2.67	0.52	0.57	0.91	12.01	4.04
	Hydrogen	3.06	4.23	**0.58**	1.94	9.19	19.45	**1.20**	2.83	**1.76**	0.78	1.87	**15.83**	3.46
Loch Eil	Control	7.62	3.62	0.41	1.70	14.21	19.11	0.35	1.47	0.98	0.71	1.70	10.55	1.98
	Acetate	6.71	3.92	0.39	1.81	11.12	**21.14**	0.40	1.52	1.01	0.76	0.91	10.79	2.26
	Lactate	6.39	4.19	**2.26** *	**5.12** *	8.66	17.35	0.37	1.27	5.51 *	0.58	1.46	8.67	1.94
	Propionate	6.69	4.05	**0.62**	3.28 *	8.57	19.53	0.36	1.73	**1.90**	0.67	1.35	9.43	2.84
	Butyrate	**9.29** *	**5.31** *	0.00	**2.74**	2.80	**27.37** *	0.00	1.68	0.22	0.76	1.46	2.40	**4.73** *
	Hydrogen	**8.56**	**6.24** *	0.19	**2.58**	4.34	**26.06** *	**0.50** *	1.68	0.47	0.68	1.30	3.49	**3.92**
Estuary Tay	Control	2.58	3.25	0.28	1.29	10.14	24.44	0.51	2.57	0.95	1.03	1.40	11.05	4.48
	Acetate	3.36	3.16	0.41	1.24	**15.10** *	22.11	0.91	2.57	1.31	**2.23** *	1.10	10.32	3.09
	Lactate	3.24	3.53	**0.82** *	**3.00** *	10.39	20.68	**1.21**	2.29	**2.71** *	**1.28** *	**1.78**	8.31	3.25
	Propionate	2.66	3.09	**1.15**	**3.63** *	9.47	20.73	0.43	2.08	**5.77** *	0.77	**1.93** *	9.43	3.69
	Butyrate	2.90	2.67	0.29	1.07	**11.80**	24.98	0.63	1.97	0.78	0.72	0.99	**13.78** *	3.70
	Hydrogen	←					n.d.							→
	Glut/Sur	1.02	2.90	**0.50**	**2.55** *	10.97	22.39	**2.03** *	1.90	**1.50**	**1.39** *	**3.96** *	11.64	4.53

* $P < 0.05$ compared to control, analysis of variance after Arc-Sin transformation.

Bold, major increases.

n.d., No data.

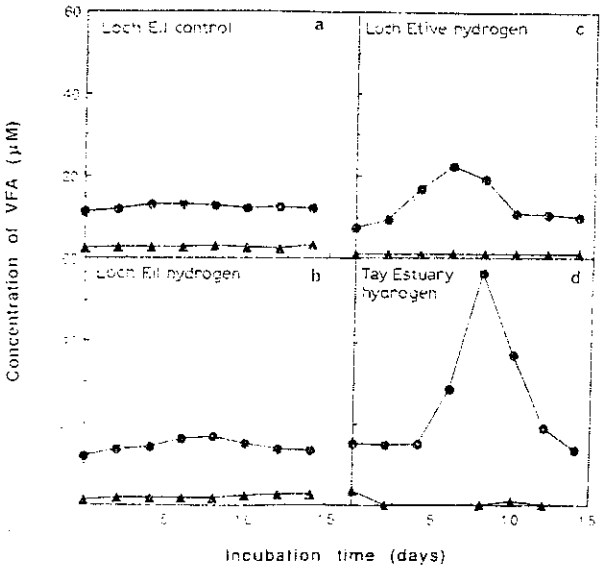

Fig. 6. Anaerobic metabolism in control and hydrogen slurries; acetate (●), propionate (▲).

hours after set up (Fig. 5), with a corresponding reduction in glutamate (3 mM). This glutamine was rapidly removed (day 1) followed by serine (day 3) and then glutamate (day 4) with the production of acetate. Acetate concentration increased until day 5 and then was rapidly and completely removed by day 10.

Concentrations of volatile fatty acids in the hydrogen supplemented slurries and controls were low, approx. 10 and 2 μM for acetate and propionate respectively. In the hydrogen slurries, however, there was a peak in acetate about day eight which was most marked in Tay (60 μM) and Etive (22 μM) slurries (Fig. 6). In the Tay slurry a very small propionate peak coincided with the acetate peak and this was the only hydrogen slurry to produce methane (Table 2). Trace amounts of methane were also present in Tay slurries with propionate and butyrate but methane was absent when elevated sulphate concentrations were used (Table 2).

4.3. Stimulation of sulphate-reducing bacteria in the slurries

The addition of specific substrates always resulted in a stimulation of the corresponding population of sulphate-reducing bacteria, ranging from 224–2,000% compared to the control and many of these increases were highly significant ($p < 0.05$). In addition, other sulphate-reducing bacteria were also stimulated. Thus substrate additions resulted in a marked increase in the total sulphate-reducing bacterial population compared to the controls (Table 3). The average stimulation for all substrates and sites was 240% with the largest stimulation in Loch Eil (363%) and the lowest in Loch Etive (135%).

4.4. Stimulation of bacterial lipid fatty acids in slurries

Marked differences in the distribution of PLFA in the substrate added slurries, compared to the controls, were found over the C12–C19 range where bacterial contributions would be expected [16]. There were both increases and decreases in individual PLFA, but as increases in PLFA should be specifically associated with the stimulated sulphate-reducing bacterial populations (Table 3), only increases will be considered (Table 4). These increases were usually substantial (average 240%) and the majority were significant ($P < 0.05$). Acetate addition resulted in only minor changes in PLFA, these were in the even chain fatty acids 16:0 (Etive and Eil) and 16:1w7 (Tay). Addition of the other substrates produced marked increases in a range of PLFA and there were clear differences between the three sites. In general, lactate, propionate, hydrogen and glutamate/serine addition resulted in increase of odd numbered PLFA, both saturated and unsaturated (15:1w6, 15:0, i17:1w7 and 17:1w6), whilst butyrate addition stimulated both even and odd chain PLFA (16:0, 16:1w7, 15:0). At all sites 17:1w6 was consistently increased by propionate and lactate addition. In Etive slurries with hydrogen and Tay slurries with glutamate and serine both 17:1w6 and i17:1w7 increased, but in Eil slurries plus hydrogen only i17:1w7 was stimulated. There was no increase in 10 Me16:0 in any of the slurries. In addition to these substrates related changes cy17:0 and 17:0 appeared to increase in Tay slurries whilst 14:0, i15:0 and 18:0 were often stimulated in Loch Eil slurries. No site related increase in PLFA was apparent for Etive slurries.

5. DISCUSSION

The distribution of different types of sulphate-reducing bacteria at each of the three sites broadly corresponded to the contribution of different substrates to sulphate-reduction (Table 1) and the concentration of substrates present [22]. For example, lactate-utilizing sulphate-reducing bacteria were numerically dominant in the Tay Estuary, lactate was the major substrate for sulphate reduction and lactate concentrations were high at this site. Conversely, acetate was a more important substrate for sulphate reduction in the Sea Loch sites, especially Loch Etive, and the percentages of acetate-utilizing sulphate-reducing bacteria were correspondingly higher at these sites than the Tay Estuary. Loch Etive was the only site where hydrogen was a substrate for sulphate reduction and this was also reflected in the viable counts (Table 1). These differences in the distribution of sulphate-reducing bacteria were largely maintained during incubation in the control slurries (Table 1). Also numbers of total sulphate-reducing bacteria at each site reflected the measured rates of sulphate reduction; with the Tay Estuary, having much larger viable sulphate-reducing populations (Fig. 1) and rates of sulphate reduction [4] than the Sea Loch sites. Therefore, viable counts of sulphate-reducing bacteria do reflect the substrates utilized by natural populations and the intensity of sulphate reduction. A similar conclusion was recently made by Jørgensen and Bak [11]. In addition, as these characteristic populations were approximately maintained within subsequent control sediment slurries, stimulation of specific types of sulphate-reducing bacteria within substrate supplemented slurries should provide a useful approach to further characterise sulphate-reducing bacterial populations from different environments and to study their metabolism.

All substrate additions to the slurries resulted in increases in sulphate reduction (Table 2), the sulphate-reducing bacterial population (Table 3) and PLFA compared to controls (Table 4). Although the addition of specific substrates always resulted in enhancement of corresponding population of sulphate-reducing bacteria, other types were also stimulated, but usually to a much lesser extent (Table 3). This reflected the incomplete oxidation of substrates other than acetate, which provides acetate and sometimes propionate in addition to the added substrate (Figs. 2–5). The acetate and propionate were subsequently utilized by other sulphate-reducing bacteria resulting in an increase in their numbers (Table 3) and changes in PLFA (Table 4). In addition to this indirect enhancement of acetate and propionate utilizers, there was some increase in other types of sulphate-reducing bacteria (e.g., lactate and butyrate utilizers in Loch Eil and Tay Estuary, Table 3), which may reflect substrate flexibility by the enriched bacteria.

Although acetate addition clearly increased acetate-utilizing sulphate-reducing bacteria at all sites (between 225–857%), there were only very limited increases in PLFA (e.g., 16:0 and 16:1w7, Table 4) and only the increase in 16:1w7 in the Tay Estuary was significant ($P < 0.05$). The results for Loch Etive and Eil are identical to previous results from Loch Eil [18]. Acetate-utilizing sulphate-reducing bacterial belonging to the genus *Desulfobacter*, are dominated by even chain fatty acids, particularly 16:0 which represents approx. 30% of the total PLFA [21,32] The increase in even chain fatty acids in the slurries, however, is difficult to interpret as it is small compared to the control. This is because acetate is the dominant substrate for sulphate-reduction at these sites and hence the biomass of *Desulfobacter* type sulphate-reducing bacteria (Table 1) and concentration of even chain fatty acids are already high (Table 4). In addition even chain fatty acids are common bacterial fatty acids. The absence of a stimulation of any other potential PLFA biomarker for sulphate-reducing bacteria [18], however, suggests that the increase in 16:0 is either due to *Desulfobacter* or other some other acetate utilizing sulphate-reducing bacteria with comparable PLFA. A similar situation is possible in the Tay Estuary, as 16:1w7 is also an important PLFA in all acetate-oxidising sulphate-reducing bacteria examined [21]. Alternatively, the increase in 16:1w7 may be due to other acetate utilizers such as the *Desulfobacter*-like 'fat vibrio' and *Desulfotomaculum acetoxidans*

which both contain high concentrations (30%) of this fatty acid [21]. *Desulfotomaculum* sp. have been regularly isolated from the Tay Estuary [34,35] but not from the Sea Loch sites.

10 Me 16:0 is an unusual PLFA characteristic of *Desulfobacter*-type sulphate-reducing bacteria and hence has been proposed as a biomarker for these bacteria [21,30]. This PLFA, however, was not stimulated in any of the acetate slurries or in those where acetate was produced, which is consistent with previous results [18]. Thus the use of 10 Me 16:0 as a biomarker for *Desulfobacter* sp., under these conditions, needs to be carefully considered.

The stimulation of odd chain PLFA in the slurries with lactate, propionate, hydrogen and glutamate/serine included potential biomarkers for *Desulfovibrio* sp., i17:1w7 and *Desulfobulbus* sp. 15:1 and 17:1w6 [18,19,20] and demonstrated that these compounds were degraded by different sulphate-reducing bacteria to those that degrade acetate. Both *Desulfovibrio* sp. and *Desulfobulbus* sp. utilize lactate and hydrogen, and in addition *Desulfobulbus* can incompletely oxidize propionate to acetate [7]. Propionate slurries at all sites had a marked increase in 17:1w6 and to a lesser extent 15:1 which, together with the incomplete oxidation of propionate (Figs 2, 3, 4), strongly suggested that *Desulfobulbus* spp. were the dominant propionate-utilizing sulphate-reducing bacteria at these sites. The same PLFA were also stimulated in Loch Eil slurries with lactate and it seems likely that *Desulfobulbus* was responsible for the oxidation of propionate produced from the partial fermentation of lactate, as previously observed [18]. As approx. 30% of the added lactate was oxidized at this site *Desulfobulbus* could also have been responsible for or involved in this oxidation, although previous results showed no stimulation of *Desulfobulbus* biomarkers during lactate degradation [18]. What is clear, however, is that known *Desulfovibrio* sp. were not responsible for lactate oxidation as there was no stimulation in its biomarker (i17:w7). This is consistent with previous results from this site [18]. In contrast, in the lactate slurries from the Tay estuary and Loch Etive biomarkers for both *Desulfobulbus* sp. and *Desulfovibrio* sp. were stimulated (Table 4). As these PLFA also increased in hydrogen slurries from Loch Etive, it would suggest that both these sulphate-reducing bacteria were directly involved in lactate and hydrogen oxidation. The involvement of propionate-utilizing sulphate-reducing bacteria in lactate metabolism was supported by a marked increase in their population in lactate slurries from all sites (Table 3). This is the first direct evidence (i.e. not based on viable counts) that *Desulfovibrio* type sulphate-reducing bacteria are involved in the utilization of hydrogen and lactate within sediments, however, *Desulfobulbus* sp.competes, often successfully, for the same role (Table 4). The high proportion of lactate oxidation (70%) to fermentation in the Tay estuary slurries, compared with the Sea Loch slurries (average 25%) is consistent with the lactate being a dominant substrate for sulphate reduction at this site [22].

Surprisingly, Loch Eil hydrogen slurries resulted in a stimulation of i17:1w7 and not 17:1w6, which means that although *Desulfovibrio.* sp were not responsible for lactate metabolism at this site they were involved in hydrogen metabolism (Table 4). This contrasts with previous results from this site [18].

The addition of glutamate and serine to Tay slurries resulted in enhanced sulphate reduction which was quantitatively similar to the other substrate supplemented slurries (Table 2). The rapid appearance of glutamine must have been produced from the added glutamate in combination with ammonia in the slurries and possibly glutamine synthetase, common in bacteria. The amino acids were then rapidly oxidized in sequence: glutamine, serine and glutamate, with production of acetate, which was oxidized once all the amino acids had been removed (Fig. 5). During the period of rapid amino acid metabolism, and prior to acetate oxidation (0–6 days, Fig. 5), there was rapid sulphate removal and sulphide production (85 and 70% respectively of final concentration changes). Therefore, sulphate-reduction was directly involved in amino acid metabolism, including glutamate, which in other sediments is solely fermented (Burdige, 1991). A number of PLFA were stimulated (Table 4) including the biomarkers for *Desulfobulbus*

sp. (17:1w6 & 15:1) and *Desulfovibrio* sp. (i17:1w7). As neither of these sulphate-reducing bacteria can utilize acetate this further confirms that sulphate-reducing bacteria were directly involved in amino acid metabolism. Although the degradation of amino acids by sulphate reduction has been previously demonstrated at this [22] and other sites [36,37] this is the first direct indication of the types of sulphate-reducing bacteria involved. The involvement of *Desulfovibrio* sp. in amino acid metabolism is consistent with results of pure culture studies [38], but to our knowledge amino acid metabolism in *Desulfobulbus* sp. has not been demonstrated in pure culture [7]. Alternatively, amino acid metabolism may be due to fermentative bacteria coupled with *Desulfovibrio* [30], or *Desulfobulbus* sp. in which propionate is a major product [39] and *Desulfobulbus* would then, in addition, be responsible for propionate removal, as no propionate was present in the slurry (Fig. 5). The precise bacterial interactions involved will require further study but the results clearly show the close involvement of both *Desulfovibrio* and *Desulfobulbus* sp. in amino acid metabolism, and also demonstrates the potential of bacterial biomarker analysis to unravel interactions of mixed microbial populations [30].

In the Sea Loch slurries butyrate was incompletely oxidized to acetate, and as only about 50% of the theoretical acetate production (butyrate : acetate, 1 : 1) was present (Fig. 2) there was also significant acetate consumption. This is consistent with the stimulation of acetate-utilizing sulphate-reducing bacteria (Table 3) and would account for the stimulation of even chain PLFA characteristic of *Desulfobacter* sp. (e.g., 16:0, 16:1w7). Changes in PLFA tended to be more limited in Loch Etive slurries, as relatively little butyrate was degraded (Fig. 2), but the stimulation of odd numbered PLFA in Loch Eil slurries (15:0, i15:0) may be due to butyrate-utilizing sulphate-reducing bacteria. Conversely, they may also be due to *Desulfobacter* sp., as the addition of volatile fatty acids to pure cultures of *Desulfobacter* sp. results in an increase in odd numbered and branched PLFA [21]. The absence of stimulation of the biomarker for *Desulfovibrio* sp. demonstrates that these sulphate-reducing bacteria were not responsible for butyrate oxidation in any of the slurries, despite the ability of some species to incompletely oxidize butyrate in pure culture [7]. Butyrate metabolism in the Tay slurries was different from that in the Sea Loch slurries: (1) partial butyrate fermentation produced small amounts of propionate, and (2) very little of the acetate produced was consumed, especially in the slurry without added sulphate (Figs. 3 and 4). Except for a small stimulation of 16:1w7, which was also found in the acetate slurry and may reflect acetate oxidation (see previous discussion), the only PLFA stimulated was 18:1w7 which is a common PLFA.

The marked peak in concentrations of acetate in the Tay and Etive hydrogen slurries and to a much lesser extent in Loch Eil (Fig. 6) may have reflected the activity of acetogenic bacteria, but the amounts of acetate produced were probably too small and transient to have an affect on the bacterial composition and thus on the distribution of PLFA. The same would apply to the small amount of methane produced in the Tay slurries. However, methane production in the Tay and its absence in the Sea Loch slurries, with identical hydrogen and sulphate concentrations, does demonstrate that in addition to differences in the composition of the sulphate-reducing bacterial populations, there are some physiological differences between the bacteria from these sites. This is clearly shown in the marked difference in propionate and butyrate oxidation. Propionate was all removed within 14 days (Fig. 2) and sulphate reduced to 2 mM in the Sea Loch slurries (Table 2), whereas in the Tay slurries, propionate degradation was incomplete, even in the presence of 4 mM sulphate and traces of methane were produced (Fig. 3, Table 2). When excess sulphate was added (35 mM), all the propionate was removed and there was no methane production (Fig. 4, Table 2). Propionate degradation in the Tay, therefore, seemed to be sulphate limited and this was confirmed when addition of extra sulphate allowed propionate metabolism to continue (Fig. 4). A similar situation was observed for butyrate degradation (Figs. 2, 3 and 4). Therefore, the threshold at which sulphate becomes limiting is higher in the Tay slurries (about 3.5

mM) than in the Sea Loch slurries (< 2 mM) and as a result of this other anaerobic bacteria can more effectively compete for common substrates. This may be a result of the much higher amount of available organic matter [4] and higher substrate concentrations [22] at the Tay site compared to the Sea Lochs. A similar difference in sulphate thresholds for sulphate-reduction has been found between surface and deeper sediments [40].

Although there was a diverse population of sulphate-reducing bacteria at all three sites, capable of degrading all the substrates added to the slurries, there was a clear order in which substrates were utilized, despite these substrates being utilized by different types of sulphate-reducing bacteria. For example, propionate and acetate were utilized by *Desulfobulbus* and *Desulfobacter* type sp. respectively, but acetate was not utilized until all the propionate was removed (Fig. 2). This may reflect the poor ability of *Desulfobacter* sp to compete for limiting sulphate [41] or some other factor (as sulphate was probably not limiting for most of the experiment, see previous discussion). Sequential substrate utilization does not occur in slurries without added substrate [22], but it does in enrichments for sulphate-reducing bacteria [12], together with sequential changes in the dominant sulphate-reducing bacterial population. Therefore, considerable caution has to be exercised in interpreting the results of viable counts of sulphate-reducing bacteria on substrates which are incompletely oxidized, as the dominant types of bacteria will change with incubation time. Bacterial PLFA analysis of such enrichments would be an invaluable tool for characterising the resulting populations, especially as many of the sulphate-reducing bacteria cannot be readily distinguished by microscopy.

These results demonstrate that the composition of the sulphate-reducing bacterial population in slurries from the three sites studied were quite distinct. This is consistent with previous data showing that the in situ substrates for sulphate reducing bacteria at these sites were also different [22]. In addition, the sulphate threshold characteristics of the sulphate-reducing bacteria, from the estuarine site varied considerably from those from the Sea Loch sites. Further research is required to determine how results from slurries relate to populations and metabolism of sulphate-reducing bacteria within undisturbed sediments and to ascertain which environmental characteristics (e.g., type and quality of organic matter input, availability of sulphate, iron and other nutrients etc.) determine the characteristic populations at each site. PLFA analysis, however, in combination with measurements of the activity and metabolism of the bacterial population, seem invaluable tools for such studies.

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