DISSIMILATORY NITRATE AND SULPHATE REDUCTION IN MARINE SEDIMENTS

BY

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A THESIS

SUBMITTED IN FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

HERIOT-WATT UNIVERSITY

EDINBURGH

MAY 1983

"The pale-faced man peered down the microscope. He was evidently not accustomed to that kind of thing, and held a limp white hand over his disengaged eye. "I see very little," he said.

"Touch this screw," said the Bacteriologist; "perhaps the microscope is out of focus for you. Eyes vary so much. Just the fraction of a turn this way or that."

"Ah! now I see," said the visitor. "Not so very much to see after all ..."

"The Stolen Bacillus"

(H.G.Wells, 1905)

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ACKNOWLEDGEMENTS

I wish to thank Professors W.D.P. Stewart (University of Dundee), R.I. Currie (Scottish Marine Biological Association) and D.J. Manners (Heriot-Watt University) for the provision of research facilities. Thanks are also due to my supervisor Professor C.M. Brown for his advice during the course of this work.

I would like to extend my thanks to my friends, colleagues and fellow "glove-bag users" in the S.M.B.A. bio-geochemical cycling group - particularly Dr. S.J. Malcolm. John Douglas was as skilful as ever in the darkroom; as was Mrs. Christian Ranken who translated my manuscript into type-script.

Finally, I would like to express my gratitude to Clare, for her help and patience during the writing of this thesis.

ABSTRACT

The use of nitrate or sulphate as terminal electron acceptors for anaerobic respiration in marine sediments was investigated. The areas examined ranged in character from cellulose and alginate polluted sea-loch, unpolluted sea-loch and inshore to N.E.Atlantic stations on the continental shelf, slope and abyssal plain.

All the sediments examined had the potential for nitrate respiration which appeared to be of negligible importance in anaerobic mineralization due to low electron acceptor availability. Nitrite was not thought to be the end product of dissimilatory nitrate reduction. In both sea-loch and continental shelf sediments the end-product of this process appeared to be ammonia, although assimilation of this compound often resulted in variable ammonia levels. Counts of nitrate reducing bacteria recovered decreased with increasing water depth and were predominantly fermentative organisms of the *Aeromonas/Vibrio* genera which could reduce nitrate through to ammonia.

Sulphate respiration was not electron acceptor limited and appeared to be dependent on the organic content of the sediment (electron donor availability). Activity tended to increase with increased organic input into the sediment, with sulphate reducing bacteria in the cellulose polluted E-70 station being capable of the degradation of 49% of the total carbon mineralized by the sediment *per annum*. Counts of sulphate reducing bacteria did not reflect differences in activity between different sediments. Isolates of lactate oxidising sulphate reducing bacteria from E-70 were all

Desulfovibrio desulfuricans strains which were characteristically nutritionally limited. However, newly described cultural techniques demonstrated the presence of more versatile sulphate reducing bacteria in both this sediment and that on the continental shelf. These sediments utilized added substrates in the surface 5 cm of sediment in accordance with thermodynamic considerations.

The role of sulphate reducing bacteria in sedimentary nitrogen fixation was unclear, with activity probably being rate limited by high ammonia levels in the sediment pore water. Acetylene reduction rates measured tended to increase with increasing sediment organic content.

Growth of the *D.desulfuricans* E-70 isolates in a widely used medium was inhibited by citrate and a possible explanation for this is discussed together with the implications of this inhibition.

GENERAL INTRODUCTION

The marine sedimentary environment is determined by the complex relationship between several variables such as geographical location, the texture and major mineral matrix of the sediment and by the prevailing hydrographic conditions (see Malcolm and Stanley, 1982). However, in biological terms, it is the extent and biodegradability of organic deposition that exerts the most influence on the physical and chemical characteristics of the sediment (Stanley *et al*, 1978). This organic matter is mineralized within the sediment by aerobic and then anaerobic processes and results in the return of essential nutrients such as ammonia and phosphate to the overlying water (Stanley *et al*, 1981).

The organic material (e.g. plant polysaccharides) which reaches the sediment is initially degraded by heterotrophic bacteria within an aerobic zone of variable depth with the concomitant utilization of oxygen. Assuming that the organic matter undergoing degradation has the average elemental composition of living marine plankton this reaction can be summarized by the equation (Richards *et al*, 1965):

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 106\ O_2 \rightarrow 106CO_2 + 106H_2O + 16NH_3 + H_3PO_4$$

The oxygen removed by this process is replenished by diffusion from the sediment-water interface through the sediment porewater. In course particle sediments such as those on the British continental shelf this process occurs at a higher rate than in the fine, silty sediments of the inner basins of many sea-lochs. This is a result of the larger interstitial spaces associated with the coarser sand particles of the shelf sediments. In shallow waters the amount of oxygen transport into the sediment can be increased by the burrowing of

polychaete worms such as *Capitella* and *Scolelepis* which irrigate the deeper sediment with oxygenated bottom water. This process is termed bioturbation and can occur at sediment depths of up to 40 cm in sea-loch (Pearson, 1982) and coastal sediments (Grundmanis and Murray, 1977).

Eventually a depth within the sediment is reached where the removal of oxygen by aerobic respiration exceeds replenishment. The depth where this occurs is dependent both on the levels of diffusion and bioturbation, and on microbial respiration rates, (which are organic content dependent), temperature, the oxygen tension of the overlying water and where applicable, tidal flushing (Nedwell, 1982). Within deep sea sediments the oxic zone is generally extensive and can extend to over 1 m sediment depth (Jørgensen, 1982). As the residence time of organic matter in the water column is so long such sediments generally receive a low input of refractory material. The overlying water is usually well oxygenated (Kester, 1975) which leads to the formation of well oxidised sediments. In contrast, inshore sediments generally receive higher sedimentation rates of more labile organic matter which result in a migration of the anoxic sediment zone upwards.

The degree of oxygenated or reduced conditions within the sediment is commonly expressed in terms of the redox potential (Eh) of the sediment. This is a measure of the tendency of a solution to give or take up electrons; that is, to be oxidised or reduced (Costilow, 1981). Eh is expressed in mV, with the higher the relative concentration of oxidant to reductant in solution being indicated by the more positive number of mV. Redox potential generally decreases in sediments

where organic degradation is occurring (Billen, 1982) and is determined by the various electrochemical couples formed from dissolved species such as O_2, NO_3^-, NH_4^+, SO_4^{2-}, HS^-, HCO_3^- and CH_4 (Breck, 1974). Eh measurements are usually made with a platinum electrode and a Ag/AgCl reference system and can be constructed at a microelectrode level (50 µm tip diam) facilitating the study of miroenvironments. "Bulk" sediment Eh measurements have been used to map the effects of pollution in a Scottish sea-loch (L.Eil) and proved superior to measurements of the sedimentary concentration of the pollutant (cellulose) as an indicator of the response of the sediment to organic input (Pearson and Stanley, 1979).

Within the anoxic zone of the sediment organic matter degradation continues either by the processes of anaerobic fermentation or by the use of alternate inorganic terminal electron acceptors to oxygen. Microbial fermentations can occur at any depth or redox potential within the sediment in the absence of oxygen (Jones, J.G., 1982). Anaerobic bacterial respirations however, show a vertical sequence down the sediment which is mainly determined by the decrease in energy yield as the electron acceptors gradually become less oxidizing (Jørgensen, 1980):

AVERAGE DEPTH IN SEDIMENT	RESPIRATION TYPE	CHANGE IN OXIDATION LEVEL
1 mm	$O_2 \longrightarrow 2H_2O$	O: $O \xrightarrow{x2} -2 = -4$
1 cm	$NO_3^- \longrightarrow N_2$	N: $+5 \longrightarrow 0 = -5$
10 cm	$SO_4^{2-} \longrightarrow H_2S$	S: $+6 \longrightarrow -2 = -8$
1 m	$CO_2 \longrightarrow CH_4$	C: $+4 \longrightarrow -4 = -8$

This vertical sequence is also dependant on the competition for electron donors (generally fermentative end-products), the distribution of electron acceptors and on requirements for specific redox levels of the sediment. This progression in the use of electron acceptors for organic mineralization had been predicted for some time from thermodynamic considerations but has only recently been demonstrated in fjordic and coastal sediments (Sørensen *et al*, 1979; Novitsky and Kepkay, 1981). These different types of metabolism can overlap (Marty, 1981), with bioturbation often resulting in elevated oxygen and nitrate concentrations in the deeper sediment layers (Grundmanis and Murray, 1977; Sørensen *et al*, 1979).

The process of anaerobic mineralization in marine sediments is complex due to the metabolic limitations of many of the individual bacteria. As a rule each organism utilises only a portion of the detrital energy - the remainder being supplied to the next reaction in the process in the form of excreted end-products. Detrital energy flow is therefore carried by small inorganic, extracellular molecules such as sulphur compounds, which may also function as electron acceptors. Comparatively little is known about these processes in marine sediments despite the importance of sedimentary bacteria as the main agents of nutrient recycling in shallow water ecosystems (Novitsky and Kepkay, 1981, Bell and Dutka, 1972). In an attempt to gain a greater understanding of anaerobic mineralization in marine sediments the two major types of anaerobic respiration in the surface layers were studied, namely nitrate and sulphate respiration (Jørgensen, 1980). A wide variety of deep sea, coastal and fjordic sediments

were examined, in tandem with representative members of the anaerobic nitrate and sulphate respiring (reducing) microflora.

AREAS OF STUDY

The geographical locations of the areas examined in this study are shown in figure i, together with the positions of the three north east Atlantic sampling stations. The detailed positions of the inshore sampling stations are indicated in figures ii, to iv, with their chemical composition summarized in table i.

N.E. ATLANTIC SAMPLING STATIONS

The positions of the three Atlantic sampling stations used in this study are indicated in figure i. Sediment from station A-1 was taken from the Porcupine Abyssal Plain (water depth 4,920 m) and consisted of Globigerina ooze - a soft, calcerous clay formed by the degradation of calcium carbonate shelled, planktonic organisms such as foraminiferan (*Globigerina*) and coccolithophores (Gage, 1978). Sediment from station A-2 also consisted of Globigerina ooze and was from a water depth of 2,880 m in the Rockall Trough. The sediments from both stations were typically low in organic carbon which together with the abundance of oxygen at all levels in the abyssal zone (*i.e.* below 2,000 m), resulted in well oxidised sediments (table i). A dominant feature of the abyssal environment is that there is little variation in salinity, oxygen or temperature either seasonally or otherwise (Menzies, 1965) and data published by Stanley *et al* (1978) for sampling stations A-1 and A-2 reported salinity measurements of 34.8-35.0‰ and temperatures of between 5° and 6°C which confirm this.

Sampling station A-3 was located in a water depth of 158m on the Malin Shelf region of the continental shelf of the British Isles. The sediment consisted of hard packed sand

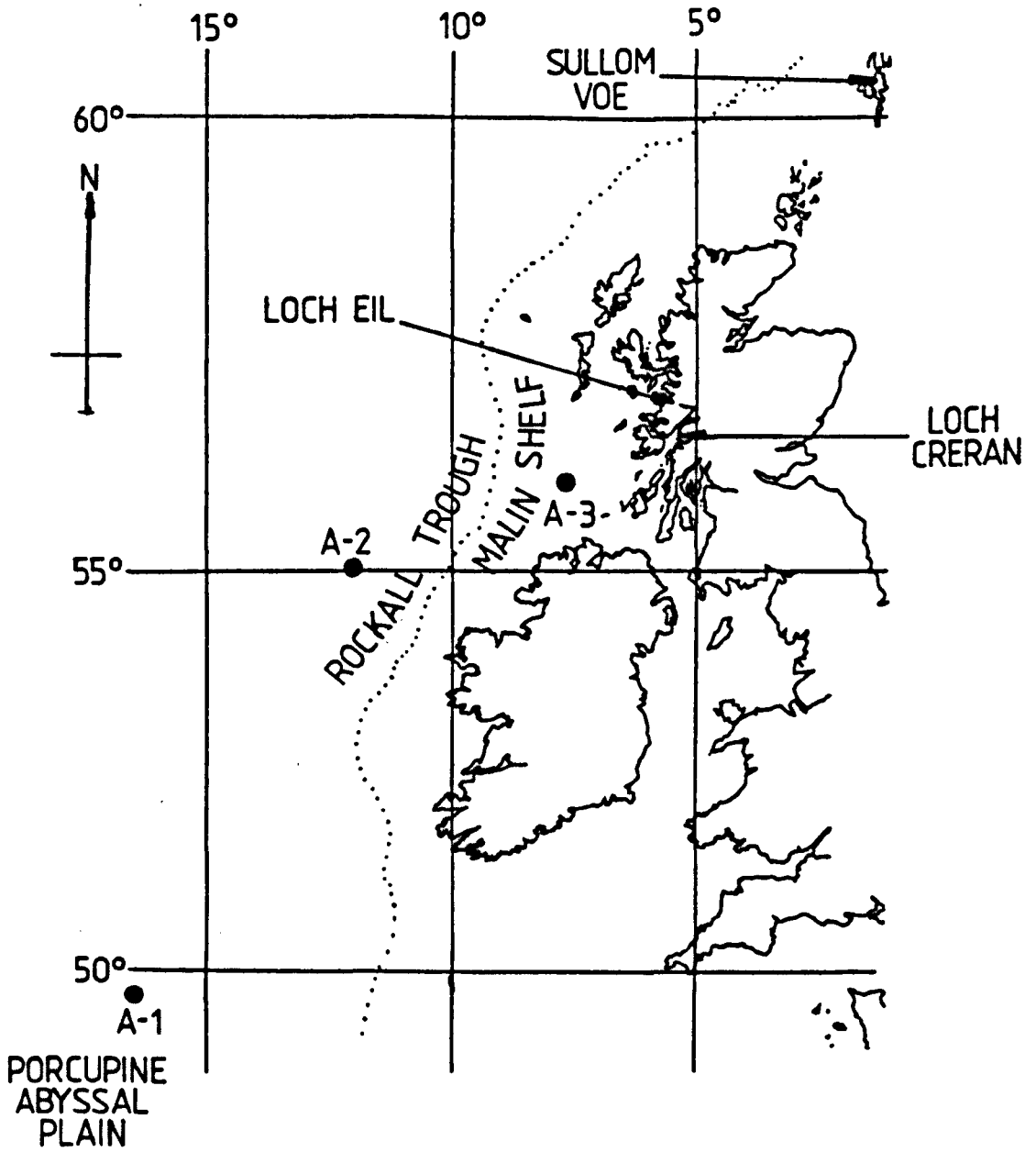


Fig.i: Areas of study and the positions of the N.E. Atlantic Sampling Station.

TABLE i: TYPICAL PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE SAMPLING STATIONS USED IN THIS STUDY (after Stanley *et al*, 1978, 1981, and Pearson and Stanley, 1977)

AREA AND STATION		WATER DEPTH (m)	AVERAGE TEMPERATURE (^{o}C)	Eh (mV) SURFACE	Eh (mV) 4 cm	% CARBON
N.E.ATLANTIC	A-1	4,920	5-6	-	+525	0.3
	A-2	2,880	5-6	-	+341	0.3
	A-3	158	5-6	-	+365	0.5
LOCH EIL	E-24	30	6-13	+100	-150	5.3
	E-70	49	6-13	+197	-158	6.2
LYNN OF LORNE	LY-1	47	6-12	-	+100	2.5
LOCH CRERAN	CR-1	13	6-13	-100	-150	20.0
	CR-2	18	6-13	+175	+50	2.0
SULLOM VOE	D-4	44	6-11	+30	-177	9.6
COLLA FIRTH	CF-1	24	6-12	+50	-150	8.0

- data not available.

and although low in organic carbon was organically richer than either A-1 or A-2 sediment (table i).

LOCH EIL AND THE LYNN OF LORNE

This area is shown in figure ii. Loch Eil is an enclosed sea-loch (fjord) forming part of an extensive fjordic system on the west coast of Scotland. The loch is approximately 10 km long and 1.5 km wide and is separated from Loch Linnhe by a 10 m deep sill at the Annat Narrows, which encloses the Loch Eil basin (maximum depth 70m). Since 1966 a pulp and paper complex at Annat Point has discharged effluent into the loch through a diffuser pipe in the Annat Narrows during periods of tidal flood and ebb (when strong currents flow in the shallow narrows). The rate of discharge of the effluent has varied considerably over the years with the mean rate being of the order of 12-18 tonnes suspended solids d^{-1} (Pearson, 1981). The effluent was 98% cellulose (Vance, *et al*, 1979) and Pearson (1982) has calculated that it constituted approximately 84% of the total organic carbon input into the loch over a one year period. Despite this, there has been no apparent long term increase in sediment cellulose levels (Stanley *et al*, 1978), although the effluent discharge has resulted in large decreases in the sediment redox potential of affected regions in the loch (Pearson and Stanley, 1979).

Sampling station E-70 was located in the deep basin of Loch Eil (water depth 49m), approximately 2 km from the effluent discharge point. As with many sea-loch basins the sediment was comprised of small particles such as fine sand, silt and clay. Due to the effluent pollution the sediment had a high

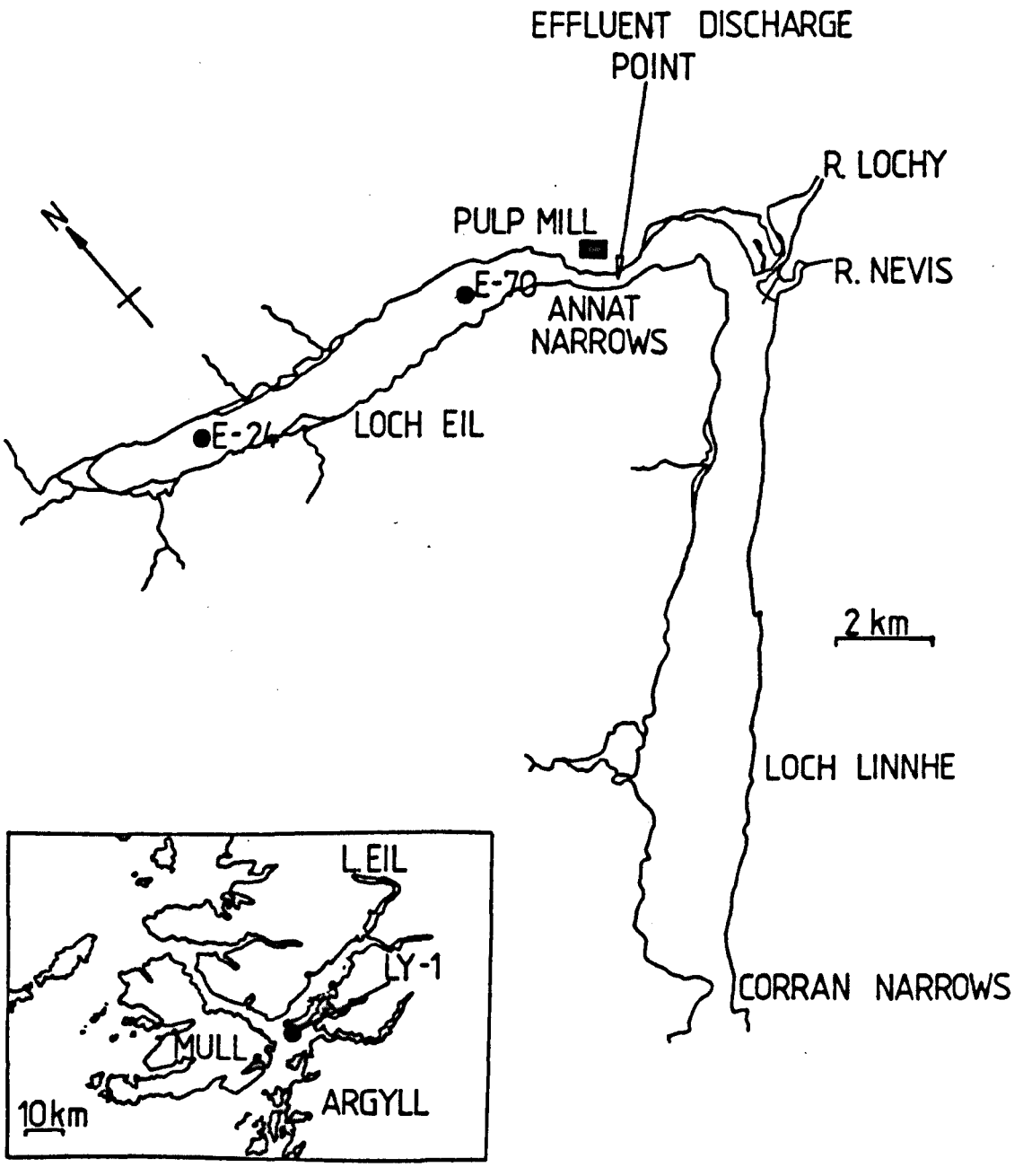


Fig.ii: Position of the Loch Eil sampling stations. Inset map of the west coast of Scotland showing the position of sampling station LY-1 in the Lynn of Lorne.

carbon content (5.9%) and as an area receiving a large input of organic carbon was characteristically highly reduced and rich in sulphide (table i). However, at most times the water overlying the sediment was oxygenated due to active water renewal above the sediment surface (Pearson and Stanley, 1977). Sampling station E-24 was located approximately 1.5 km from the head of the loch in a water depth of 34 m. The sediment was predominantly comprised of silt and, as the area was less influenced by the cellulose effluent, was correspondingly lower in organic carbon and less highly reduced (table i).

Sampling station LY-1 in the Lynn of Lorne (water depth 47 m) was used as a control station and was a comparable sediment to the two Loch Eil stations prior to effluent discharge (*i.e.* pre-1966). Being unaffected by the effluent, the sediment was low in carbon (2.5%) and oxidised.

LOCH CRERAN

Loch Creran is a sea loch approximately 15 km long and 2 km wide (figure iii) and forms part of the fjordic coastline on the west coast of Scotland of which Loch Eil is a part. The loch forms a double basin system with a shallow sill separating the main basin from the Firth of Lorne and one at Creagan enclosing the smaller upper basin. Considerable tidal flushing occurs in the loch with vertical mixing in the more turbulant regions of the main basin (Gage, 1972).

As with Loch Eil, the loch was subjected to an artificial input of organic carbon in the form of alginate waste from a seaweed factory situated at Barcaldine. However, the scale of organic loading was much less in this case (approximately

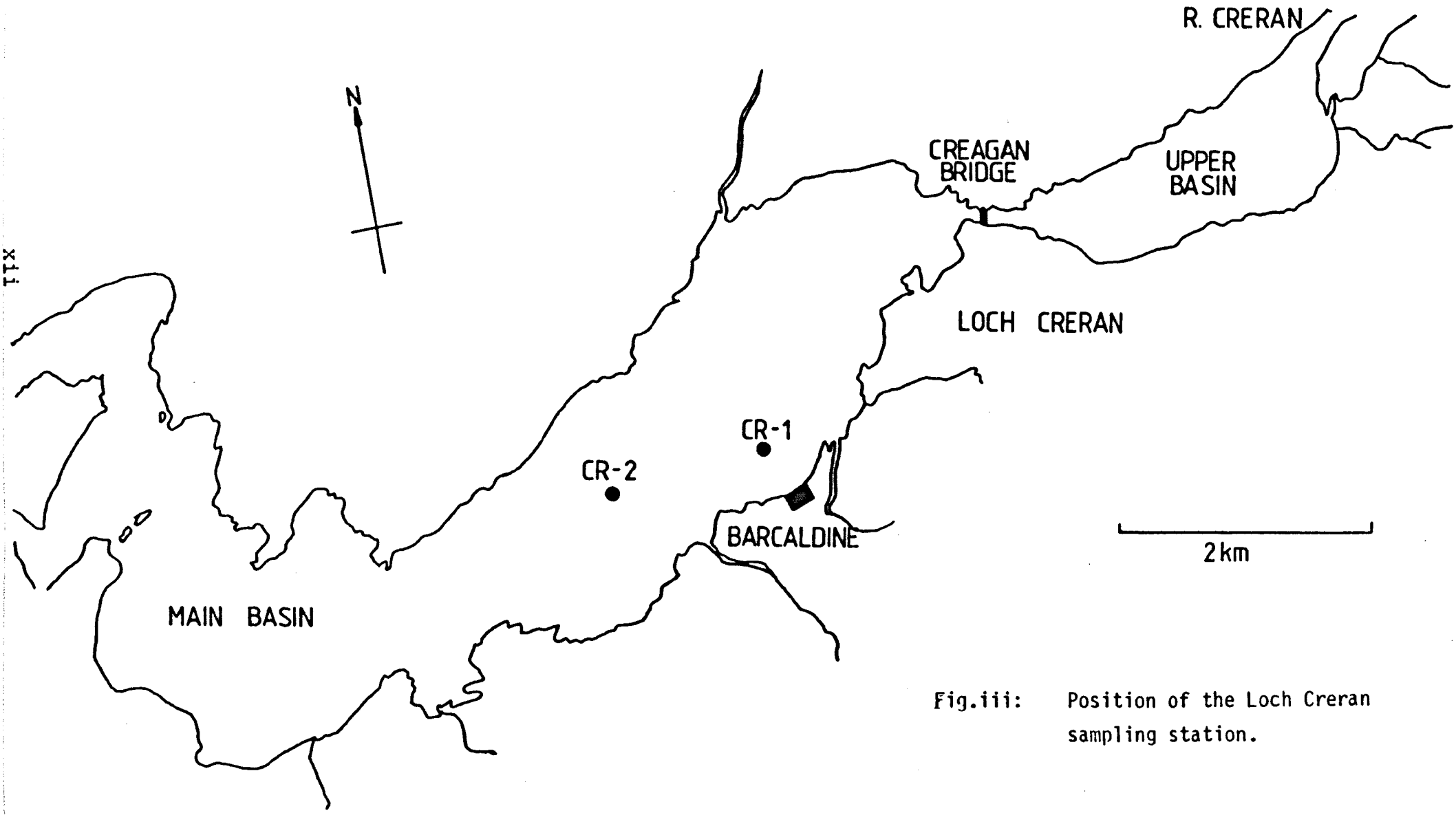


Fig.iii: Position of the Loch Creran sampling station.

800 tonnes organic carbon yr^{-1}) and accounted for some 27% of the organic carbon input per year - compared with 36% from river inflow, 27% from phytoplankton and 10% from macro-algae (S.M.B.A. annual report, 1979). The alginate waste tended to remain localized in a "dump" area and the extensive reduction of basin sediments that had arisen from effluent discharge in Loch Eil was not encountered in Loch Creran. Both sampling stations were located in the main basin of the loch where the sediments were similar in particle size to those found in the basin of Loch Eil and consisted mainly of silts. Station CR-1 was located in a water depth of 13 m as close to the alginate effluent dump as was navigable. The sediment was grossly polluted with a very high carbon content (20%) and was highly reduced. Sediment from station CR-2 in the centre of the basin was unaffected by the alginate waste and was more oxidised with an organic carbon content one tenth that of CR-1 (table i).

SULLOM VOE AND COLLA FIRTH

The positions of these two areas are shown in figure iv. Sullom Voe is the largest voe (fjord) on the Shetland mainland and has a major oil terminal situated on its eastern shoreline. The voe exits into Yell Sound and has a sill approximately 10 km from this opening which encloses a deep inner basin. Water circulation within this inner basin is restricted and during summer the formation of a thermocline often results in a stagnant body of water of low oxygen tension at the bottom of the basin (Stanley *et al*, 1978). Sampling station D-4 was situated in the inner basin (water depth 44 m) with sediment from this

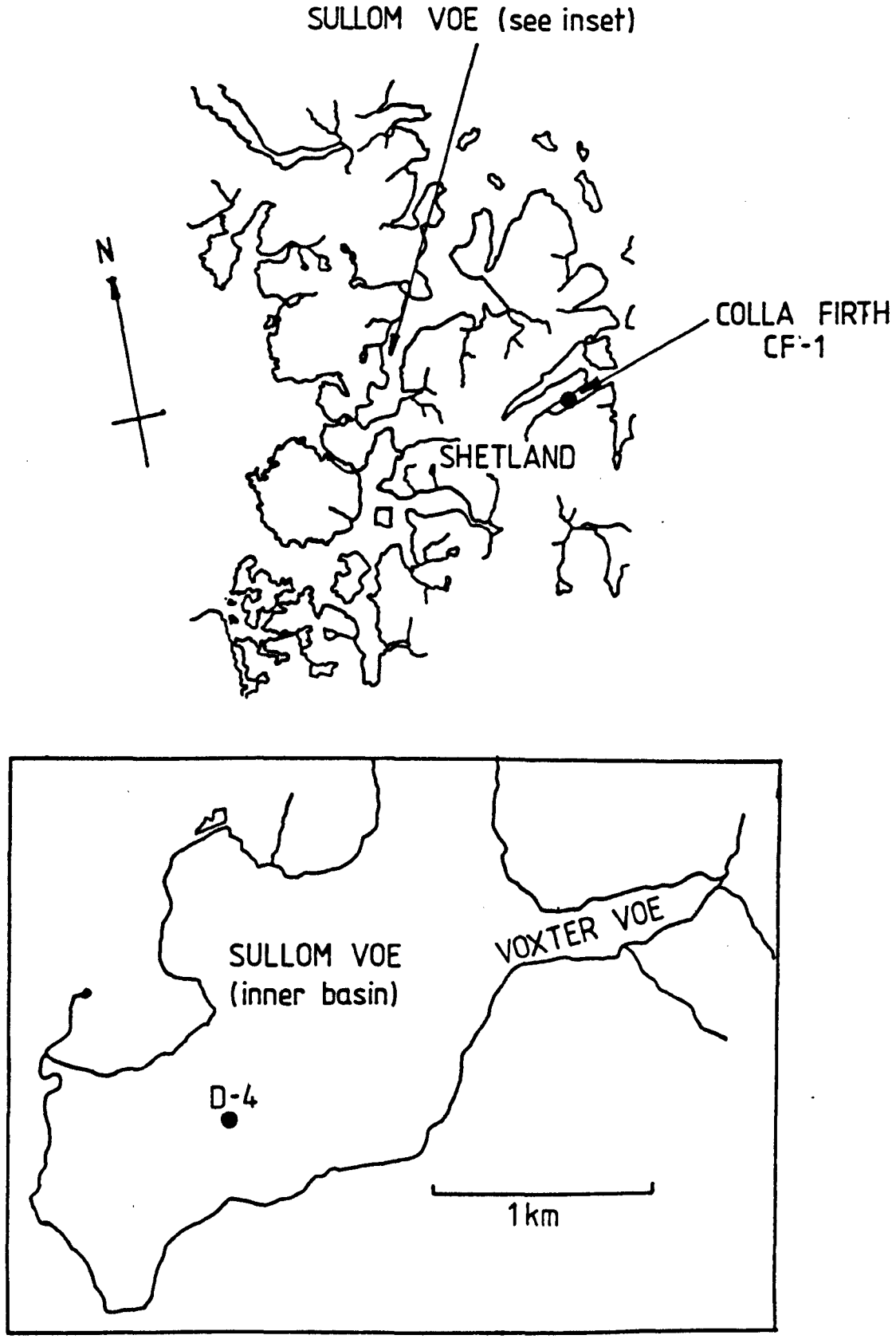


Fig.iv: Position of the Shetland mainland sampling stations.

station consisting of sand, silt and clay in similar proportions to those found at Station E-70 in the Loch Eil basin.

The sediment was high in carbon (9.6%) and sulphide, highly reduced and was thus an organically rich sediment similar to that found at E-70. However, the high organic deposition rates were due to natural influxes of detritus.

Colla Firth is a smaller voe on the east coast of Shetland and likewise received a naturally high input of organic carbon. Sampling Station CF-1 was located in 24 m of water and had silty sediments which were rich in carbon and highly reduced below the surface.

SECTION 1

DISSIMILATORY NITRATE REDUCTION IN MARINE SEDIMENTS

INTRODUCTION

Within the marine environment nitrogen is often a principle limiting nutrient for the production of biomass (Brezonik, 1972; Herbert, 1975). This is despite the fact that molecular nitrogen (N_2) constitutes approximately 80% of the atmosphere and is a reflection of its chemical inertness - the majority of living organisms requiring the element in a combined (fixed) form (Stanier *et al*, 1977). The predominant forms of combined nitrogen present in the marine environment range from the most oxidised form nitrate (NO_3^-), through nitrite (NO_2^-) to ammonia (NH_4^+), the most reduced. Combined nitrogen is also present in the form of organic nitrogen (org-N) as bacterial biomass, organic detritus, etc. The different forms of nitrogen in marine sediments are interrelated and cyclicly transformed in a series of biogeochemical reactions known collectively as the nitrogen cycle (fig.1.1). The majority of the steps in the cycle are performed by microorganisms although they constitute only a small fraction of the total sedimentary biomass (Rittenberg *et al*, 1955, Payne, 1973, Dunn *et al*, 1978). For example, Pearson (1982) estimated that in sediment from a sea-loch on the west coast of Scotland, bacteria accounted for only 0.03% of the total biomass. The importance of microbial processes in the nitrogen cycle is a reflection of their structure - the relatively large reactive surface of the microbial cell and their rapid growth rate responding rapidly to physical and chemical changes in their micro-environment (Jannasch, 1978). Whether such processes occur within a marine sediment are dependant on a variety of factors including types of bacteria present, presence of utilizable organic material, Eh, pH, temperature and pressure (ZoBell and Budge, 1965). One important step in the cycle which depends on such

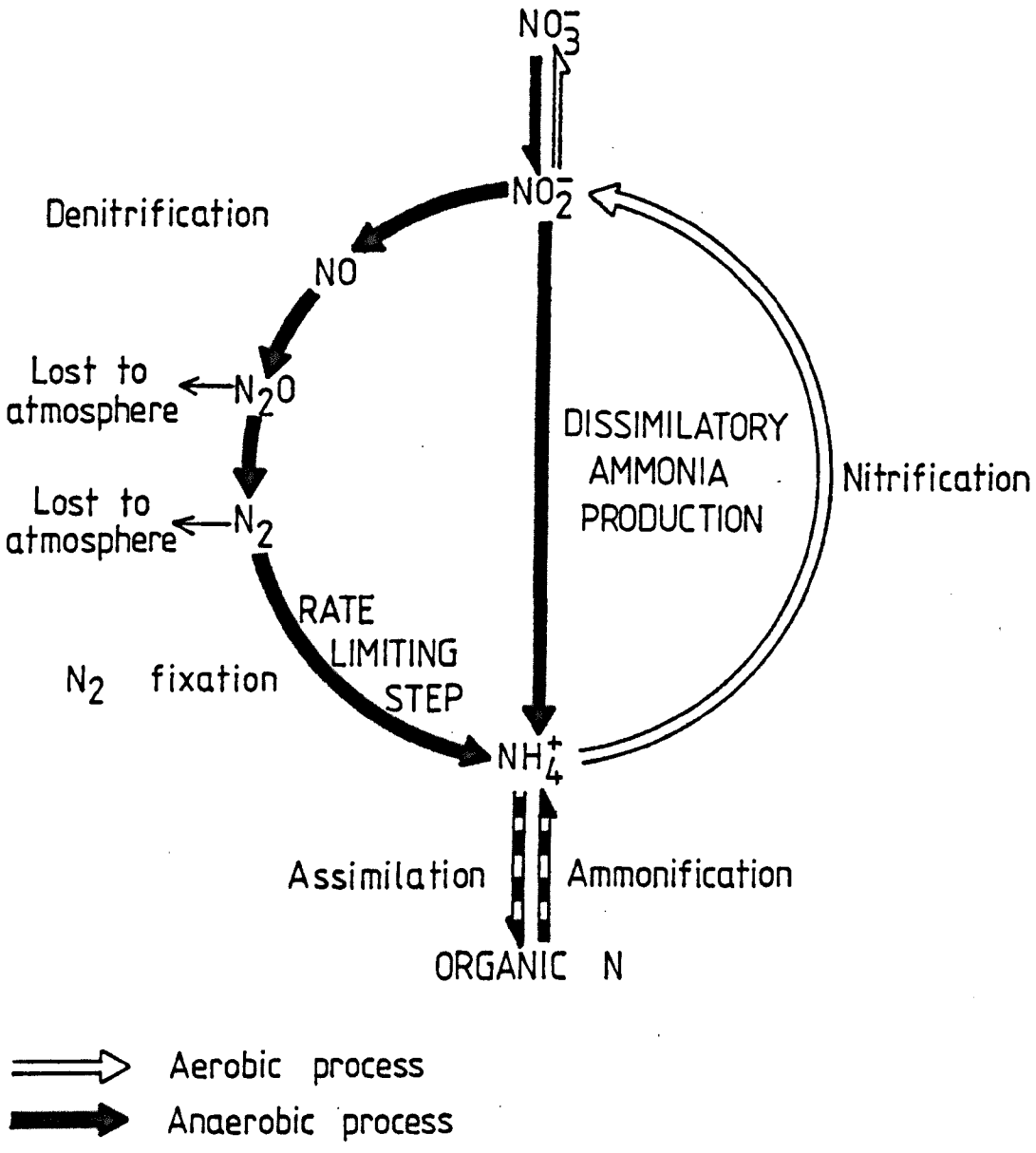


Figure 1.1: The biological nitrogen cycle.

factors (in this case anaerobiosis, a supply of NO_3^- as terminal electron acceptor and organic material as reducing power) is the dissimilatory reduction of NO_3^- to NO_2^-. This is a process whereby certain groups of sedimentary bacteria growing in anoxic environments utilise NO_3^- as an alternative, terminal inorganic electron acceptor to oxygen. The end product of nitrate respiration depends upon the type of bacterium and its growth conditions and will be discussed below.

The respiration of NO_3^- to NO_2^- is fairly widespread among bacteria growing under conditions of low O_2 tensions and has been reported to occur in some 40 genera of facultatively anaerobic and some strictly anaerobic organisms (Payne, 1973). ZoBell (1946, cited in ZoBell and Budge, 1965) has indicated that upto 50% of bacterial species in seawater are capable of reducing NO_3^- to NO_2^-. This process yields less energy than with O_2 as electron acceptor, although it is energetically superior to fermentative metabolism under anoxic conditions. Nitrate respiration involves a membrane bound nitrate reductase which accepts electrons from the existing electron transport chain, donating them to NO_3^- and whose synthesis is repressed by the presence of O_2 (Haddock and Jones, 1977). The reaction proceeds according to the equation:

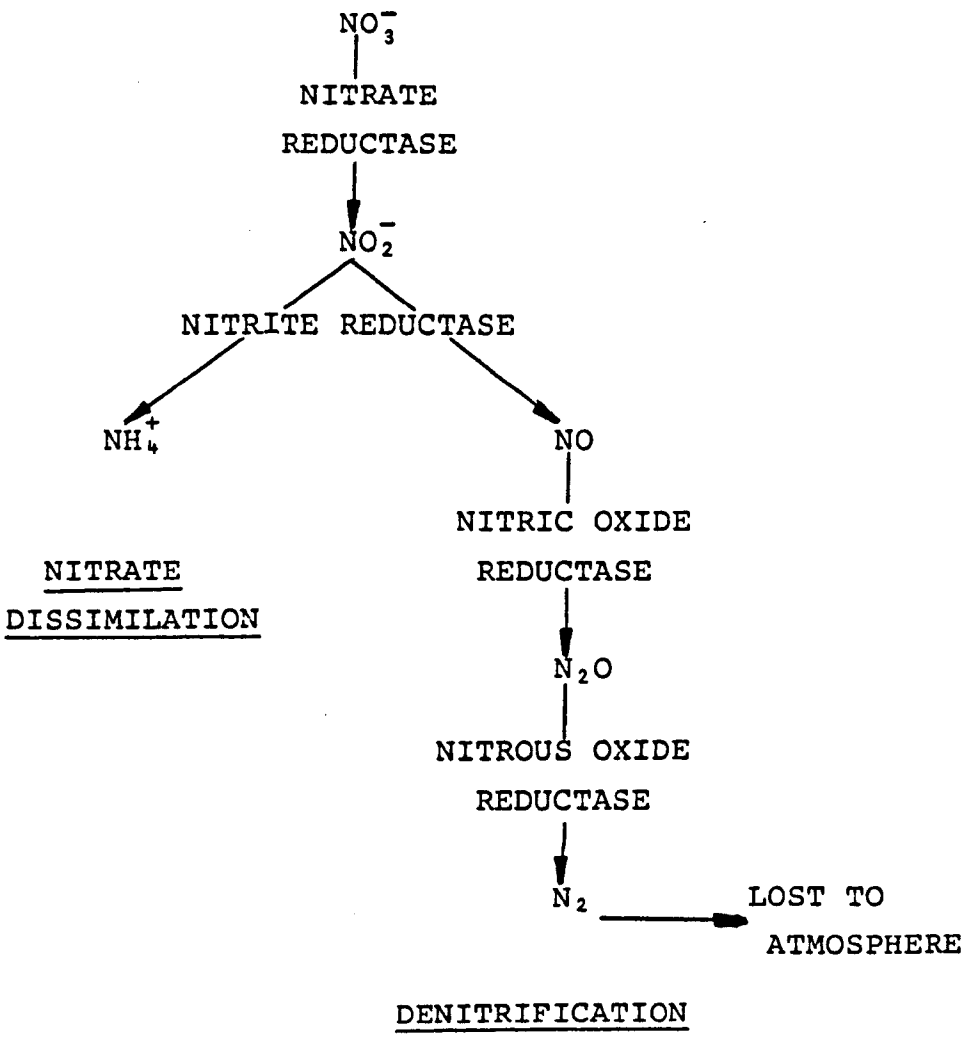
$$NO_3^- + H_2 \xrightarrow[\text{Reductase}]{\text{Nitrate}} NO_2^- + H_2O$$

$$\underline{\Delta G^{o1} = -163.2 \text{ kJ mol}^{-1}}$$

Several electron donors for nitrate reduction have been reported including NADH, hydrogen, formate, lactate, succinate and glycerol phosphate (Thauer *et al*, 1977). Linear hydrocarbons and aromatic compounds can be utilized by some pseudomonads (Taylor and Heeb, 1972; Traxler and Bernard, 1969), whilst

sulphur compounds (*e.g.* S^{2-}, SO_3^{2-}, $S_2O_3^{2-}$) are reported electron donors for *Thiobacillus denitrificans* (Adams *et al*, 1971).

Fewer bacteria (less than 5% in the marine environment, ZoBell and Budge, 1965) can reduce NO_2^- further to either gaseous nitrogen (denitrification) or to ammonia (nitrate dissimilation). The two processes are shown below:



NO_2^- reduction involves a soluble nitrite reductase and one possible function may be to remove toxic NO_2^- (Kemp and Atkinson, 1966). Denitrification involves nitrite, nitric oxide and nitrous oxide as electron acceptors in energy yielding reactions, with the subsequent gaseous products being lost to the atmosphere (Payne, 1973). Payne also reported that denitrification

occurred in some 15 genera of bacteria including *Achromobacter, Bacillus, Moraxella, Pseudomonas* and *Thiobacillus spp.* Recent studies on denitrification rates in marine sediments have involved the "acetylene blockage technique" in which the conversion of N_2O to N_2 is blocked by C_2H_2, enabling measurements of N_2O to yield rates of denitrification (*e.g.* Sørensen 1978a, Haines *et al*, 1981). Typical of the rates reported are those of Sørensen (1978a) for surface sediments from the Limfjorden, Denmark, which ranged from 0.10 µmol of N ml^{-1} sediment d^{-1} $NO_3^- \rightarrow N_2$ (N_2O) for 9-12 cm depth sediment segment to 0.87 µmol of N ml^{-1} sediment d^{-1} $NO_3^- \rightarrow N_2$ (N_2O) for the top 3 cm of sediment. Unless recaptured by nitrogen fixation (the rate limiting step of the sedimentary nitrogen cycle) the N_2 produced by such processes will result in a loss of combined nitrogen from the sediment.

Alternatively, nitrate can be dissimilated at low oxygen tensions to ammonia, preserving nitrogen within the sediment as a readily utilizable, combined nitrogen source (Dunn *et al*, 1980). At the time of writing the number of species capable of the dissimilatory reduction of NO_3^- to NH_4^+ is limited and only a handful have been described - *Klebsiella sp.* (Dunn, *et al*, 1978), *E.coli* (Cole, 1968), *Veillonella alcalscens* (Inderleid and Delwiche, 1973), *Vibrio sp.* (Herbert, 1982). Such bacteria use NO_2^- as an oxidising agent reducing it to NH_4^+. Although little useful energy is produced by this reaction, the process may act as an electron sink under conditions of nitrate limitation (Cole and Brown, 1980). Evidence for the importance of nitrate dissimilation in marine sediments has been accumulating recently (Cole and Brown, 1980). In the same study on Limfjorden sediments as mentioned above, Sørensen (1978a) reported rates

of NO_3^- dissimilation to NH_4^+ similar to those obtained for denitrification (0.12, 9-12 cm to 0.75, 0-3 cm µmol of N m^{-1} sediment d^{-1} $NO_3^- \rightarrow NH_4^+$) using the technique of incubating sediment samples with Na $^{15}NO_3$. In ^{15}N tracer incubations of anaerobic, coastal sediments from Mangoku-Ura, Simoda Bay and Tokyo Bay, Japan Koike and Hattori (1978a) recorded that between 20% and 67% of $^{15}NO_3^-$ added to samples was converted to ammonia or particulate organic nitrogen and concluded that dissimilatory reduction of NO_3^- was important in such coastal sediments. Ammonia has been shown to be the major end-product of nitrate reduction in KNO_3 supplemented sediments from the estuaries of the Rivers Leven and South Esk on the Scottish east coast and from a coastal sampling site in the North Sea (Herbert, 1982). Herbert also reported that fermentative bacteria (particularly *Aeromonas/Vibrio* and *Klebsiella spp.*) were the predominant nitrate reducers in the sediments, reducing NO_3^- to NO_2^- and NH_4^+. Oxidative, denitrifying bacteria capable of reducing nitrate to gaseous products were encountered only rarely. Similar observations have been recorded by Nedwell (1975) in a study on heterotrophic bacteria isolated from a tropical mangrove estuary (South Pacific, Fiji) and their action on NO_3^-. Nitrate was reduced to NO_2^- by 43% of the bacteria isolated, 30% reduced NO_2^- to NH_4^+ and only 2% denitrified NO_3^- to N_2 gas (determined by incubation in KNO_3 broth, containing an inverted Durham tube).

Within marine sediments nitrate reduction, either to NH_4^+ or to gaseous products, tends to maximaly occur between the aerobic zone and the zone of sulphate reduction. In most sediments this will be within the upper 5cm - nitrate reduction

activity being limited by the availability of NO_3^- which is usually at peak concentrations in this zone (Rittenberg *et al*, 1955; Sørensen 1978a,b; Sørensen *et al*, 1979). As a consequence, the rate of nitrate reduction decreases with depth down the sediment (due to NO_3^- depletion), although nitrate reduction can occur in deeper NO_3^- deficient sediments via the action of burrowing animals causing replenishment of NO_3^- levels- either by the transport of NO_3^- from surface waters directly or by the formation of aerobic zones and nitrification (Sørensen, 1978b). In some sediments nitrate reduction is inhibited by sulphate reduction, either by sulphide toxicity or by the decreasing redox potential (Sørensen, 1978b) and the two processes are often spatially separated (Sørensen *et al*, 1979). High rates of nitrate reduction have also been reported from continental shelf sediments at the sediment-water interface (Goering and Pamatmat, 1971). Although they are anaerobic processes, the reduction of NO_3^- to NH_4^+ or gaseous products can also occur in aerobic sediments within reduced microniches (50-200 μm diam.) as described by Jørgensen (1977a).

As previously mentioned, NH_4^+ is a readily utilizable combined nitrogen source and is the preferred nitrogen source for many marine microorganisms (Cole and Brown, 1980). In marine waters its concentration is usually low compared to that of NO_3^-, whilst the reverse is true in the interstitial waters of marine sediments. Dunn *et al*, (1978) showed that NH_4^+ levels in sediments from the Tay Estuary, N.E. Scotland were between 5 and 10 times greater than NO_3^- levels, whilst the reverse was true in the overlying water. Similar data has been recorded by Sørensen (1978a) with NH_4^+ levels in the top 3 cm of Limfjorden

sediments some 10 times higher than NO_3^- levels (1.15 *cf.* 0.09 μmol of N ml^{-1} sediment). Pearson and Stanley (1977) in work on fjords in Shetland and the north west coast of Scotland measured NH_4^+ levels in the interstial waters of the sediments some three times greater than NO_3^- levels. The source of this sedimentary NH_4^+ is generally thought to be from the degradation of particulate organic material via the process of ammonification which occurs in all marine sediments. However, appreciable amounts of NH_4^+ may be derived from the dissimilatory reduction of NO_3^- as described above. Sørensen (1978a) suggested that the process was quantitatively important in marine sediments and Koike and Hattori (1978a) concluded that in the anoxic sediments from Mangoku-Ura and Simoda Bay (Japan) ammonification was unlikely to form a major route for NH_4^+ production and that the source of sedimentary NH_4^+ was dissimilatory nitrate reduction.

The aim of the first part of this study was to gather information on the potential of a variety of marine sediments to perform dissimilatory reduction of NO_3^- to NH_4^+. The sediments ranged from polluted sea-loch to the N.E. Atlantic abyssal plain and covered a broad spectrum of organic carbon levels and redox states. The top 5 cm of the sediments only were examined as maximum rates of NO_3^- reduction generally occur in this region (see above). The study was conducted in two ways:

i. To determine whether dissimilatory nitrate reduction to NH_4^+ could be demonstrated in the sediments, by a KNO_3 supplementation and incubation technique.

ii. To determine the numbers and types of nitrate reducing bacteria present in the sediments, by anaerobic incubation on a nitrate agar.

MATERIALS AND METHODS

SAMPLE COLLECTION

Undisturbed sediment samples from the near shore sampling stations in Loch Linnhe (LY-1) and Loch Eil (E-24 and E-70) were obtained using a Craib corer (Craib, 1965) operated from R.V. "Calanus" (plate 1). Sediment cores of the upper 10-20 cm of the sediment were obtained in 24 cm long, 5.7 cm diameter transparent perspex core tubes, which were sealed with rubber bungs on removal from the corer. Undisturbed sediment samples from the N.E. Atlantic sampling stations (A-1, A-2 and A-3) were obtained using a replicate corer, designed by Dr. P. Barnett and Mr. J. Watson of the S.M.B.A. operated from R.R.S. "Challenger" (plate 2). Perspex core tubes of 60 cm length and 5.7 cm diameter enabled sediment cores of the upper 15-35 cm of the sediment to be obtained depending on the sampling station. Core tubes were again stoppered with rubber bungs. In the case of the N.E. Atlantic stations sediment cores were processed immediately, while samples from the near shore stations were stored in crushed ice prior to processing (usually within 24 hours).

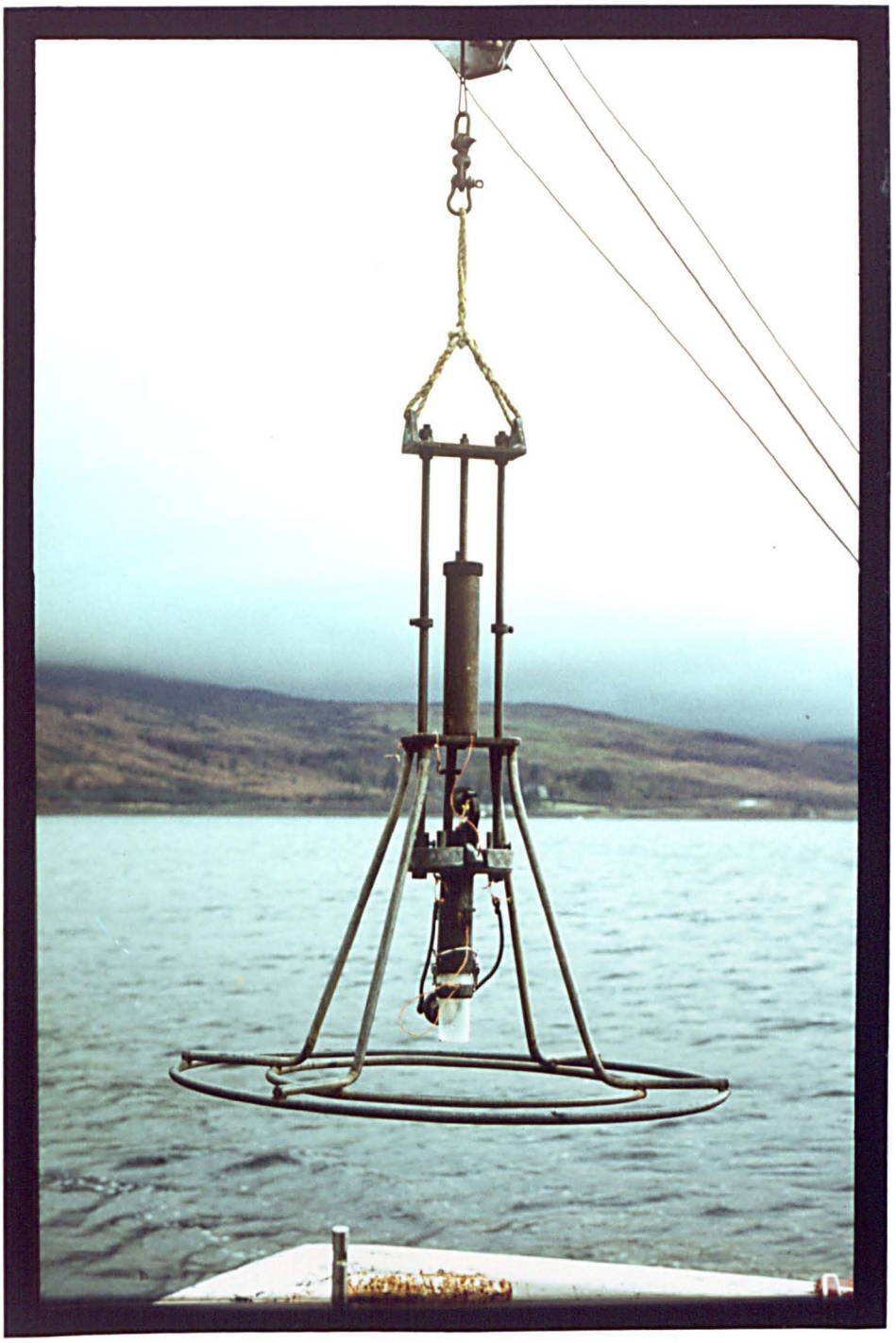


Plate 1: Craib corer (Craib, 1965) prior to sampling.

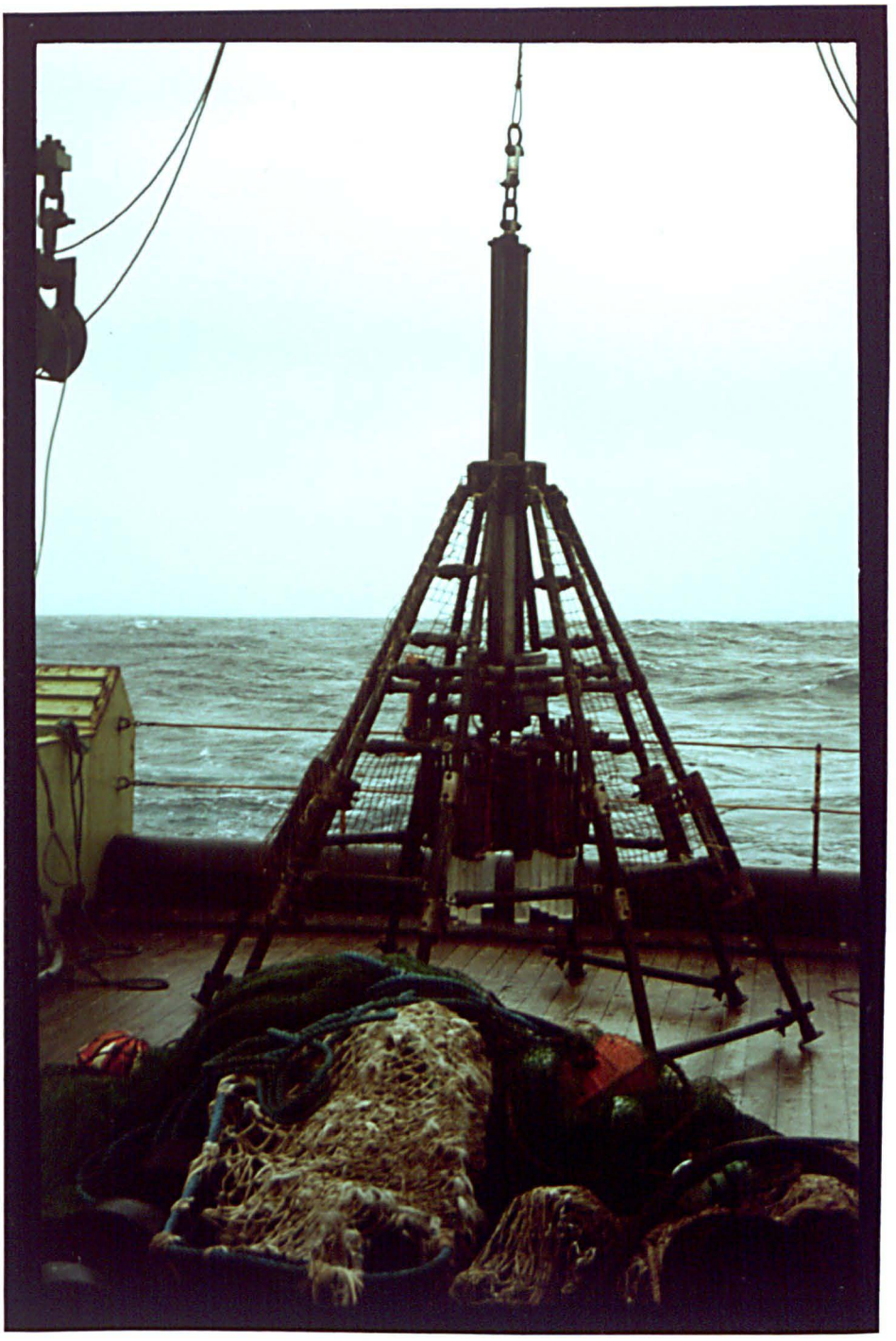


Plate 2: Barnett-Watson replicate corer prior to sampling.

NITRATE INCUBATIONS

The overlying water from undisturbed sediment samples was siphoned off through a silicone rubber tube attached to a 20 ml disposable syringe. The upper 5 cm of the sediment core was extruded with a plunger into a graduated tube of the same internal diameter as the core tube (5.7 cm), and placed into a sterile 250 ml beaker. The sediment was homogenised using a sterile spatula and 10 ml sub-samples placed into sterile universal bottles containing 0 mg, 2 mg or 10 mg KNO_3 (approximating to 0,2mM and 10mM nitrate in 10 ml sediment samples) by means of a truncated 10 ml disposable syringe. The bottles were vigorously shaken to mix the contents and dissolve the KNO_3, and duplicates of the three KNO_3 regimes immediately frozen and stored at -20°C. The remaining bottles were incubated in the dark at *in situ* temperature (4°C N.E. Atlantic samples, 10°C sea-loch samples) with duplicates of the three KNO_3 regimes being frozen after 24 hours and 5 days incubation. All bottles were stored at -20°C prior to analysis.

Samples were extracted from the sediment samples with 10 ml 2M KCl at room temperature for three hours. The samples were centrifuged for 20 minutes at 4,500 r.p.m. using a M.S.E. bench centrifuge and the resulting extracts stored at -20°C until the chemical analyses were made. Extracts were assayed for ammonia by a phenate-hypochlorite method (Fawcett and Scott, 1960). To 1.0 ml of sample were added: 1.0 ml H_2O, 2.0 ml sodium phenate (25 g phenol dissolved in 800 ml H_2O containing 78 ml 4N NaOH), 3.0 ml sodium nitroprusside (0.01% w/v) and 3.0 ml sodium hypochlorite ($^1/_{50}$ dilution of B.D.H. Chemicals sodium hypochlorite). The contents were mixed and

left at room temperature for 20 minutes. The absorbance of the resulting blue colour was read at 650 nm and the ammonia concentration of the sample calculated by means of a $(NH_4)_2SO_4$ standard curve and the relevant dilutions. Nitrite was assayed by the method of Montgomery and Dymock (1961) using Griess-Ilosvay reagents I and II (B.D.H. Chemicals). To 1.0 ml of sample were added: 1.0 ml Griess-Ilosvay reagent I (sulphanilic acid solution) and 1.0 ml Griess-Ilosvay reagent II (prepared from 8-aminonaphthalene-2-sulphonic acid). The contents were mixed and left for 10 minutes at room temperature. The absorbance of the resulting pink colour was read at 540 nm and the nitrite concentration of the sample calculated by means of a KNO_2 standard curve and the relevant dilutions.

ENUMERATION OF NITRATE REDUCING BACTERIA

10 ml of the homogenised sediment batch used for the nitrate incubations was added to 90 ml sterile 0.4M NaCl using a truncated 5 ml disposable syringe to give a 10^{-1} dilution of the sediment. From this 10^{-1} dilution further serial, ten-fold dilutions were made, down to 10^{-4}, with each dilution being well shaken by hand. 0.1 ml duplicates from each dilution were plated out on nitrate nutrient agar plates containing "Oxoid" nutrient agar (28 g $litre^{-1}$) (Oxoid Ltd., Basingstoke, Hampshire), 1.0% w/v KNO_3 and 0.4M NaCl. It had previously been shown (Abd-el-Malek *et al.* 1974, cited in Horsley, 1978) that a medium containing peptone and yeast extract gave maximum most probable number counts of denitrifiers from soils. "Oxoid" nutrient agar contains 5.0 g $litre^{-1}$ peptone and 2.0 g $litre^{-1}$ yeast extract and was found to be

a suitable base for a nitrate agar. Inoculated plates were incubated anaerobically under a H_2/CO_2 atmosphere derived from a GasPak (Becton, Dickinson, U.K. Ltd) in the presence of a palladium catalyst (Oxoid Ltd.) at *in situ* temperature for upto 3 weeks. After incubation the plates were counted and 50 colonies from each sediment were picked at random from a count plate for characterization. The validity of this method in representing the nitrate reducing bacterial population of the sediment having previously been shown (Horsley, 1978; Dunn *et al*, 1980),

CHARACTERIZATION OF THE NITRATE REDUCING BACTERIA

Characterization tests performed on the randomly selected isolates were as described by Cowan and Steel (1977) and consisted of Gram reaction, cell shape, oxidation or fermentation of glucose, catalase activity and oxidase (cytochrome C oxidase) activity. All tests were carried out at 15°C with cultures being incubated aerobically. Several isolates from the N.E. Atlantic sampling station A-2 were lost during tests at 15°C and were assumed to have been obligate psychrophiles.

The action of the isolates on nitrate was determined by incubation in a nitrate broth containing "Oxoid" nutrient broth no.2 (25 g $litre^{-1}$), 1.0% w/v KNO_3 and 0.4M NaCl at 15°C for 7 days. Gas production was tested for by an inverted Durham tube. Nitrite was detected by the addition of 1 ml of Griess-Ilosvay reagent I to the growth tube, followed by 1 ml of Griess-Ilosvay reagent II. A pink colour indicated the presence of nitrite and showed that nitrate had been reduced. Tubes which showed no pink colour within 5 minutes had powdered

zinc (up to 5 mg ml^{-1} of culture) added to them and were allowed to stand. A resulting pink colour indicated that nitrate was present in the medium (*i.e.* not reduced by the organism) whilst no pink colour indicated that nitrate was absent in the medium (*i.e.* reduced by the organism to nitrite, which was in turn reduced).

To investigate whether nitrite was being reduced to ammonia a peptone water tube test for ammonia production was used. Strains were inoculated into a medium comprising of "Oxoid" peptone water (0.1 g $litre^{-1}$), 0.01% w/v glucose and 0.4M NaCl containing either no KNO_3 or 0.001% w/v KNO_3. The low level of glucose was designed to raise the C:N ratio of the medium and so prevent the excretion of N as NH_4^+ during growth on a low C:N substrate (amino acids). The tubes were incubated at 15°C until visible growth was detectable. The presence of ammonia was detected by the previously described phenate-hypochlorite assay using 1 ml of culture and no water addition. Nitrite was detected with Griess-Ilosvay reagents I and II on the remainder of the culture as described above. In both cases the tubes were compared against their respective uninoculated controls (*i.e.* with and without KNO_3) as detectable ammonia levels were encountered in tubes containing 0.001% w/v KNO_3.

CHARACTERIZATION OF THE YEAST ISOLATES

In addition to the characterization tests performed on all the randomly selected isolates, several additional tests were performed on the yeast isolates obtained. Colony morphology was recorded after aerobic and anaerobic growth on nitrate nutrient agar plates at 15°C. A limited investigation into the physiology of the isolates used the nitrogen-limited medium of Evans (Evans *et al*, 1970) modified by lowering the

concentrations of all the components by 50% and by the addition of 0.01% w/v "Oxoid" yeast extract. The salt concentration was 0.4M NaCl, the nitrogen source was 15 mM KNO_3 and the carbon and energy source was 58mM sodium lactate:-

$NaH_2PO_4 . 2H_2O$	0.78 g
KCl	0.37 g
KNO_3	1.52 g
$Na_2SO_4 . 10H_2O$	0.14 g
Citric acid	0.21 g
$MgCl_2$	0.13 g
$CaCl_2$	0.001 g
Sodium lactate (70% w/w aq. solution)	6.7 ml
Yeast extract	0.1 g
Salts A (g $litre^{-1}$: $CaCl_2.2H_2O$ 4.0, $MnCl_2 . 4H_2O$ 4.0, $FeCl_3 . 6H_2O$ 2.8)	5 ml
Salts B (g $litre^{-1}$: $CoCl_2 . 6 H_2O$ 0.2, $(NH_4)_6 Mo_7O_{24} 4H_2O$, 0.2)	5 ml
Salts C (g $litre^{-1}$: $ZnSO_4 . 7H_2O$ 0.1, $SnCl_2.6H_2O$ 0.2, $NiCl_2 . 6H_2O$ 0.2, H_3BO_4 0.2, KI 0.1)	5 ml
NaCl	23.4 g
Distilled water	1 litre
pH 7.0	

The medium was solidified with 2% w/v "Oxoid" agar no.3 when used for plates. The effect of NaCl concentration on growth was investigated by inoculating plates of Evan's medium containing 0M, 0.2M and 0.4M NaCl. The effect of pH on growth was studied using plates of Evan's medium at pH 5.0, 6.0 and 7.0.

The presence or absence of growth at 4°C, 15°C, 30°C and 37°C was investigated using plates as described above. Glucose "petite" medium (Johnson and Brown, 1972) supplemented with 0.4M NaCl was used to detect growth and/or fermentation on glucose. An inverted Durham tube was used to detect any gas production. To determine whether the pigmentation of the strains was due to carotenoid pigments, colonies from plate grown cells were suspended in chloroform. Extraction of the pigment in the chloroform indicated that it was a carotenoid pigment (Lodder and Kreger-Van-Rij, 1967). Strains were examined for the production of capsule or slime material by the indian ink method described by Cowan and Steel (1977). Unless otherwise stated, all incubations were performed at 15°C for between 2 and 14 days.

MICROBIOLOGY OF THE NITRATE REDUCING FLORA FROM THE PULP MILL DISCHARGING INTO LOCH EIL

Samples were taken from the Wiggins Teape pulp and paper mill at Corpach, near Fort William by means of sterile universal bottles from the mill's paper machine, the input of the effluent holding tank (the effluent being released into Loch Eil according to the state of the tide) and the holding tank sediment (the heavier solids in the effluent settling out). The samples were diluted and plated out onto nitrate nutrient agar plates as described previously and incubated both aerobically and anaerobically (H_2/CO_2 atmosphere) at 15°C for up to 3 weeks. The pH of the samples was also recorded. After incubation the plates were counted and representative colonies from each sample were Gram stained and examined as described above.

R E S U L T S

NITRATE INCUBATIONS

The data presented in table 1.1 are the means of duplicate sediment samples. In all sediment samples with no nitrate supplementation low nitrite concentrations of around 0.01 µg N ml^{-1} sediment were encountered which remained constant or increased slightly on incubation. Maximum levels of nitrite production were measured in sediment samples from Loch Eil E-24 and N.E. Atlantic A-3 after 24 hours incubation with 10 mg KNO_3 supplementation (1.20 µg nitrate reduced ml^{-1} sediment day^{-1}). Lowest activities, with 10 mg KNO_3 supplementation after 24 hours incubation, were measured in sediment from Loch Linnhe LY-1 (0.14 µg nitrate reduced ml^{-1} sediment day^{-1}) and from the abyssal Atlantic stations (0.22 µg nitrate reduced ml^{-1} sediment day^{-1} A-2 and 0.06 µg nitrate reduced ml^{-1} sediment day^{-1} A-1). Although sediment from station E-70 in Loch Eil produced little nitrite after 24 hours incubation with 10 mg KNO_3 supplementation, it produced 2.62 µg of nitrite ml^{-1} sediment after 5 days incubation corresponding to a nitrate reduction rate over the 5 days of 0.52 µg nitrate reduced ml^{-1} sediment day^{-1}. However, in most samples the rate of nitrate reduction over 5 days was less than that over 24 hours and it appears that nitrate reduction in the sediment samples was at a maximum over the first 24 hours of incubation. Sediment samples from stations Loch Linnhe LY-1 and N.E. Atlantic A-1, A-2 and A-3 showed a production of nitrite after 24 hours at 2 mg KNO_3 supplementation equivalent to that with 10 mg KNO_3.

The ammonia levels recorded were much more variable and in many instances decreased on incubation. Sediment from N.E. Atlantic stations A-1 and A-2 showed decreasing ammonia

TABLE 1.1 INORGANIC NITROGEN ANALYSES OF SEDIMENT INCUBATIONS - MEANS OF DUPLICATE SAMPLES

Sampling Station	Nitrate Addition (10 ml sediment)	NO_2^- (µg N ml^{-1} Sediment)			NH_4^+ (µg N ml^{-1} Sediment)		
		Zero	24 hours	5 days	Zero	24 hours	5 days
Lynn of Lorne LY-1	0 mg KNO_3	0.001	0.01	0.001	1.66	3.77	1.01
	2 mg KNO_3	0.07	0.18	0.79	0.75	0.59	0.45
	10 mg KNO_3	0.07	0.21	0.82	0.31	0.29	0.18
Loch Eil E-24	0 mg KNO_3	0.01	0.02	0.01	2.06	1.16	1.18
	2 mg KNO_3	0.01	0.43	1.10	1.32	5.49	1.24
	10 mg KNO_3	0.36	1.51	2.94	1.31	1.49	1.08
Loch Eil E-70	0 mg KNO_3	0.01	0.01	0.03	2.01	0.73	2.84
	2 mg KNO_3	0.18	0.16	0.17	1.82	0.95	7.55
	10 mg KNO_3	0.29	0.35	2.91	1.53	2.70	5.00
N.E.Atlantic A-1	0 mg KNO_3	0.02	0.03	0.01	0.57	0.48	0.26
	2 mg KNO_3	0.01	0.08	0.16	0.57	0.53	0.31
	10 mg KNO_3	0.01	0.07	0.19	0.46	0.45	0.39
N.E.Atlantic A-2	0 mg KNO_3	0.01	0.01	0.01	0.38	0.22	0.22
	2 mg KNO_3	0.02	0.18	0.33	0.42	0.23	0.36
	10 mg KNO_3	0.02	0.24	0.33	0.45	0.24	0.19
N.E.Atlantic A-3	0 mg KNO_3	0.01	0.01	0.01	1.53	1.86	2.02
	2 mg KNO_3	0.02	1.23	1.55	1.39	2.13	2.52
	10 mg KNO_3	0.02	1.22	1.56	1.30	1.06	2.83

levels with and without KNO_3 supplementation, while sediment from Loch Linnhe LY-1 showed decreasing ammonia levels on incubation with KNO_3 supplementation. Net ammonia production, which in part was stimulated by KNO_3 addition, was measured in sediments from Loch Eil E-70 and N.E. Atlantic A-3 (net ammonia production with 10 mg KNO_3 supplementation of 2.64 and 1.04 $\mu gN\ ml^{-1}$ sediment after 5 days incubation respectively). Sediment from Loch Eil E-24 had increased ammonia levels on incubation with KNO_3 supplementation but these decreased on further incubation.

ENUMERATION OF NITRATE REDUCING BACTERIA

The results of a survey of nitrate reducing bacteria in samples from the same sediment batches as used for the nitrate incubation experiments are shown in table 1.2. Highest numbers of nitrate reducing bacteria were recovered from the inshore sampling stations with up to $1.6\text{-}2.0 \times 10^5$ bacteria ml^{-1} sediment being recovered from stations in Loch Eil. Fewer bacteria were recovered from the continental shelf station A-3 and the Goban Spur ($1.0\text{-}2.0 \times 10^3$ bacteria ml^{-1} sediment). Lowest numbers were recorded at the N.E. Atlantic station A-2, whilst attempts to recover nitrate reducers from station A-1 sediment failed on two occasions. The numbers of nitrate reducing bacteria remained fairly constant over a 5 to 7 month period in the Loch Linnhe LY-1 and N.E. Atlantic sediments whilst in Loch Eil the numbers recovered increased from winter to spring/summer.

CHARACTERIZATION OF THE NITRATE REDUCING BACTERIA

The results of the characterization tests performed on the

TABLE 1.2 COUNTS OF NITRATE REDUCING BACTERIA FROM LOCH LINNHE, LOCH EIL AND THE N.E. ATLANTIC - MEANS OF DUPLICATE SAMPLES

Sampling Station		Nitrate Reducing Bacteria (ml^{-1} Sediment)
Loch Linnhe LY-1	Nov.1978	2.0×10^4
	Jun.1979	2.0×10^4
Loch Eil E-24	Nov.1978	1.5×10^4
	Jun.1979	2.0×10^5
Loch Eil E-70	Nov.1978	3.2×10^4
	Mar.1979	1.6×10^5
	Jun.1979	4.0×10^4
N.E.Atlantic A-1	May 1979	-
	Oct.1979	-
N.E.Atlantic A-2	May 1979	1.0×10^2
	Oct.1979	1.0×10^2
N.E.Atlantic A-3	May 1979	1.5×10^3
	Oct.1979	1.9×10^3
Goban Spur	Oct.1979	2.2×10^3

- not detected

SYMBOLS USED IN TABLES 1.3 TO 1.8 AND EQUIVALENT DESCRIPTIVE TERMS:

GRAM REACTION	+	Gram positive
	-	Gram negative
SHAPE	R	Rod-shaped
	C	Coccus (sphere shaped)
	*S	Spores present
	*BY	Budding yeast

* Observed under x400 phase contrast microscopy.

O-F GLUCOSE	O	Oxidation of glucose
	F	Fermentation of glucose
	O/-	Little growth or oxidation of glucose
	O_W	Weak oxidation of glucose but growth
CATALASE AND	+	Reaction
OXIDASE	-	No reaction
	$+_W$	Weak reaction
ACTION ON	+	Reaction occurred
NITRATE	-	No reaction or end product undetected

TABLE 1.3 CHARACTERISTICS OF NITRATE REDUCING BACTERIA ISOLATED FROM LOCH LINNHE LY-1 (NOV.1978)

Isolate No	Gram Reac -tion	Shape	O-F Glucose	Cata- lase	Oxi- dase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	-	R	F	+	+	-	-	-	+
2	-	R	F	+	+	-	-	-	+
3	-	R	F	+	+	-	-	-	+
4	-	R	F	+	+	-	-	-	+
5	-	R	F	+	+	-	-	-	+
6	-	R	O	+	+	-	-	-	+
7	-	R	O	+	+	-	-	-	+
8	-	R	F	+	+	-	-	-	+
9	-	R	O	+	+	-	-	-	+
10	-	R	O	+	+	-	-	-	+
11	-	R	F	+	+	-	-	-	+
12	-	R	O	-	+ W	-	-	-	+
13	-	R	F	+	+	-	-	-	+
14	-	R	F	+	+	-	-	-	+
15	-	R	F	+	+	-	-	-	+
16	-	R	F	+	+	-	-	-	+
17	-	R	F	+	+	-	-	-	+
18	-	R	F	-	+	-	-	-	+
19	-	R	F	+	+	-	-	-	+
20	-	R	F	+	+	-	-	-	+
21	-	R	F	+	+	-	-	-	+
22	-	R	F	+	+	-	-	-	+
23	-	R	F	-	+	-	-	-	+
24	-	R	F	+	+	-	-	-	+
25	-	R	F	+	+	-	-	-	+

TABLE 1.3 LY-1 ISOLATES CONT.

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
26	+	R	F	+	+	-	-	-	+
27	-	R	O	-	+	+	-	-	-
28	-	R	F	+	+	-	-	-	+
29	-	R	F	+	+	-	-	-	+
30	-	R	F	+	+	-	-	-	+
31	-	R	O	+W	+	-	-	-	+
32	-	R	F	+	+	-	-	-	+
33	-	R	O	+W	+	-	-	-	+
34	-	R	O	+	+	-	-	-	+
35	-	R	F	+W	+	-	-	-	+
36	-	R	F	+W	+	-	-	-	+
37	-	R	F	+W	+	-	-	-	+
38	-	R	F	+	+	-	-	-	+
39	-	R	F	+	+W	-	-	-	+
40	-	R	F	+	+	-	-	-	+
41	-	R	F	+	+	-	-	-	+
42	-	R	F	+	+	-	-	-	+
43	-	R	F	-	+	-	-	-	+
44	-	R	O	-	+	-	-	-	+
45	-	R	F	+	+	-	-	-	+
46	-	R	F	+	+	-	-	-	+
47	-	R	F	+	+	-	-	-	+
48	-	R	F	+	+	-	-	-	+
49	-	R	F	+	+W	-	-	-	+
50	-	R	O	-	+	+	-	-	-

TABLE 1.4 CHARACTERISTICS OF NITRATE REDUCING BACTERIA ISOLATED FROM LOCH EIL E-24 (NOV.1978)

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	-	R	O	+	+	+	-	-	-
2	-	R	F	+	+	-	-	-	+
3	-	R	F	+	+	-	-	-	+
4	-	R	F	+	+	-	-	-	+
5	-	R	O	+	+	-	-	-	+
6	-	R	F	+	+	-	-	-	+
7	-	R	F	+	+	-	-	-	+
8	-	R	F	+	+	-	-	-	+
9	-	R	F	+	+	-	-	-	+
10	-	R	F	+	+	-	-	-	+
11	-	R	F	+	+	-	-	-	+
12	-	R	F	+w	+	-	-	-	+
13	-	R	F	+	+	-	-	-	+
14	-	R	F	+	+	-	-	-	+
15	-	R	F	+	+	-	-	-	+
16	-	R	F	+	+	-	-	-	+
17	-	R	O	+	+	-	-	-	+
18	-	R	F	+w	+	-	-	-	+
19	-	R	F	+	+	-	-	-	+
20	-	R	F	+	+	-	-	-	+
21	-	R	F	+	+	-	-	-	+
22	-	R	F	+w	+	-	-	-	+
23	-	R	F	+	+	-	-	-	+
24	-	R	F	+	+	-	-	-	+
25	-	R	F	+w	+	-	-	-	+

TABLE 1.4 E-24 ISOLATES CONT.

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
26	-	R	F	$\overset{+}{w}$	+	+	-	-	-
27	-	R	F	+	+	-	-	-	+
28	-	R	F	+	+	-	-	-	+
29	-	R	O	-	+	-	-	-	+
30	-	R	F	+	+	-	-	-	+
31	-	R	F	-	+	-	-	-	+
32	-	R	F	$\overset{+}{w}$	+	-	-	-	+
33	-	R	F	-	+	-	-	-	+
34	-	R	F	-	+	-	-	-	+
35	-	R	F	+	+	-	-	-	+
36	-	R	O	+	+	-	-	-	+
37	-	R	F	-	+	-	-	-	+
38	-	R	F	+	+	-	-	-	+
39	-	R	F	+	+	-	-	-	+
40	-	R	F	-	+	-	-	-	+
41	-	R	F	-	+	-	-	-	+
42	-	R	F	+	+	-	-	-	+
43	-	R	F	-	+	-	-	-	+
44	-	R	F	+	+	-	-	-	+
45	-	R	F	$\overset{+}{w}$	+	-	-	-	+
46	-	R	F	+	+	-	-	-	+
47	-	R	F	-	+	-	-	-	+
48	-	R	F	+	+	-	-	-	+
49	-	R	F	-	+	-	-	-	+
50	-	R	O	+	+	-	-	-	+

TABLE 1.5 CHARACTERISTICS OF NITRATE REDUCING FLORA ISOLATED FROM LOCH EIL E-70 (NOV.1973)

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	+	RS	O	+	+	-	-	-	+
2	-	R	F	+	+	-	-	-	+
3	-	R	O|-	+	+	-	-	-	+
4	+	R	O|-	+	+	-	-	-	+
5	-	R	O	-	+	-	-	-	+
6	-	R	F	+	+	-	-	-	+
7	+	C	O	+	+/W	+	-	-	-
8	-	R	O/W	+	+	-	-	-	+
9	-	R	F	+	+	-	-	-	+
10	-	R	F	+	+	-	-	-	+
11	+	R	F/W	+	+	-	-	-	+
12	-	BY	-	+	+/W	-	+	-	-
13	-	R	O|-	+	+	-	-	-	+
14	+	RS	-	+	+	+	-	-	-
15	-	R	O|-	+	+	-	-	-	+
16	-	R	O	+	+	-	-	-	+
17	-	R	F	+	+	-	-	-	+
18	-	R	O|-	+	+	-	-	-	+
19	-	R	O|-	+	+	-	-	-	+
20	+	BY	-	+	+/W	+	-	-	-
21	-	R	O	+	+	-	-	-	+
22	+	R	F	+	+	-	-	-	+
23	+	RS	F	+	+	-	-	-	+
24	-	R	O|-	+	+	-	-	-	+
25	-	R	F	+	+	-	-	-	+

TABLE 1.5 E-70 ISOLATES CONT.

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
26	-	R	F	+	+	-	-	-	+
27	-	R	O|-	+	+	-	-	-	+
28	-	R	-	+	+	-	-	-	+
29	+	R	F	+	+	-	-	-	+
30	-	R	F	+	+	-	-	-	+
31	-	R	F	+	+w	-	-	-	+
32	-	R	-	+	+	-	-	-	+
33	-	R	F	+	+	-	-	-	+
34	-	R	O	+	+	-	-	-	+
35	+	RS	O|-	+	+	-	-	-	+
36	-	R	F	+	+	-	-	-	+
37	+	RS	F	+	+	-	-	-	+
38	-	R	O|-	+	+	-	-	-	+
39	-	R	F	+	-	-	-	-	+
40	-	R	F	+	+	-	-	-	+
41	-	R	O	+	+	-	-	-	+
42	-	R	F	+	+	-	-	-	+
43	-	R	F	+	+	-	-	-	+
44	-	R	O|-	+	+	-	-	-	+
45	-	R	-	+	+	-	-	-	+
46	-	R	-	+w	+	-	-	-	+
47	-	R	F	+	+	-	-	-	+
48	+	BY	O|-	+	+	-	+	-	-
49	+	RS	F	+	+	-	-	-	+
50	-	R	O|-	+	+	+	-	-	-

TABLE 1.6 CHARACTERISTICS OF NITRATE REDUCING BACTERIA ISOLATED FROM N.E. ATLANTIC A-2 (MAY 1979)

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	-	R	F	+	+	-	-	-	+
2	-	R	F	+	$\overset{+}{w}$	-	-	-	+
3	-	R	F	+	$\overset{+}{w}$	-	-	-	+
4	-	R	F	+	+	-	-	-	+
5	-	R	F	+	+	-	-	-	+

TABLE 1.7 CHARACTERISTICS OF NITRATE REDUCING BACTERIA ISOLATED FROM N.E. ATLANTIC A-3 (MAY 1979)

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	-	R	F	+	+	-	-	-	+
2	-	R	F	+	+	-	-	-	+
3	-	R	F	+	+	-	-	-	+
4	-	R	F	+	+	-	-	-	+
5	-	R	F	+	+	-	-	-	+
6	-	R	F	$\overset{+}{w}$	+	-	-	-	+
7	-	R	F	+	+	-	-	-	+
8	-	R	F	+	+	-	-	-	+
9	-	R	F	+	+	-	-	-	+
10	-	R	F	+	+	-	-	-	+
11	-	R	-	+	+	-	-	-	+
12	-	R	F	$\overset{+}{w}$	+	-	-	-	+
13	-	R	F	+	+	-	-	-	+
14	-	R	F	+	+	-	-	-	+
15	-	R	F	+	+	-	-	-	+
16	-	R	F	+	+	-	-	-	+
17	-	R	F	+	+	-	-	-	+
18	-	R	F	+	+	-	-	-	+
19	-	R	F	$\overset{+}{w}$	+	-	-	-	+

TABLE 1.8 CHARACTERISTICS OF NITRATE REDUCING BACTERIA ISOLATED FROM N.E. ATLANTIC GOBAN SPUR (OCT.1979)

Isolate no.	Gram reaction	Shape	O-F Glucose	Catalase	Oxidase	Action on Nitrate			
						None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
1	-	R	F	+	+	-	+	-	-
2	-	R	F	+	+	-	-	-	+
3	-	R	F	+	+	-	-	-	+
4	-	R	F	+	+	-	-	-	+
5	-	R	F	+	+	-	-	-	+
6	-	R	F	+	+	-	-	-	+
7	-	R	F	+	+	-	-	-	+
8	-	R	F	+	+	-	-	-	+
9	-	R	F	+	+	-	-	-	+
10	-	R	F	+	+	-	-	-	+
11	-	R	F	+	+	-	-	-	+
12	-	R	F	+	+	-	-	-	+
13	-	R	F	+	+	-	-	-	+
14	-	R	F	+	+	-	-	-	+
15	-	R	F	+	+	-	-	-	+
16	-	R	F	+	+	-	-	-	+
17	-	R	F	+	+	-	-	-	+
18	-	R	F	+	+	-	-	-	+
19	-	R	F	+	+	-	-	-	+
20	-	R	F	+	+	-	-	-	+
21	-	R	F	+	+	-	-	-	+
22	-	R	F	+	+	-	-	-	+

randomly selected isolates are presented in tables 1.3 to 1.8. The majority of the isolates were Gram negative, fermentative rods together with appreciable numbers of Gram negative oxidative rods in samples from the inshore sediments. In most instances growth in nitrate broth resulted in the reduction of nitrate to nitrite and the reduction of this nitrite in turn. Results from the peptone water tube test indicated that this nitrite was reduced to ammonia. The Gram negative rods isolated were characterized to genus level using the identification scheme of Lee, *et al*, (1979) in which the oxidase test was used to distinguish between the enterobacteria and the pseudomonads and *Aeromanas/Vibrio* organisms (table 1.9). The latter two groups were in turn distinguished by their oxidation or fermentation of glucose in the Hugh and Leifson (O-F) test.

TABLE 1.9 CHARACTERS DISTINGUISHING THE ENTEROBACTERIA FROM THE PSEUDOMONADS AND *AEROMONAS/VIBRIO spp*.

(from Lee, *et al*, 1979)

	Aeromonas/Vibrio	Pseudo-monads	Entero-bacteria
Oxidase	+	+	-
Glucose Metabolism			
O-F Medium	F	O, O/-	F

Gram positive, non-acid fast, aerobic, spore-forming rods were assigned to the genus *Bacillus* (Cowan and Steel, 1977). Non-sporing Gram positive rods were all recorded in one group as were Gram positive cocci. Budding yeasts were identified

TABLE 1.10 SUMMARY OF BACTERIAL COUNTS, GROUPS AND ACTION ON NITRATE OF NITRATE REDUCING BACTERIA FROM LOCH LINNHE LOCH EIL AND N.E. ATLANTIC SEDIMENTS

Sampling Station	Nitrate Reducing Bacteria (ml^{-1} sediment)	Bacterial Identification (% Total)						Action on Nitrate (% Total)			
		A/V	PLO	Bacillus	Gram+ rods	Gram+ cocci	Budding yeasts	None	$NO_3^- \rightarrow NO_2^-$	$NO_3^- \rightarrow N_2$	$NO_3^- \rightarrow NH_4^+$
Loch Linnhe LY-1	2.0×10^4	76	22	2	-	-	-	4	-	-	96
Loch Eil E-24	$1.5 \times 10^4 - 2.0 \times 10^5$	88	12	-	-	-	-	4	-	-	96
Loch Eil E-70	$3.2 \times 10^4 - 1.6 \times 10^5$	32	40	12	8	2	6	8	4	-	88
N.E.Atlantic A-1	-	-	-	-	-	-	-	-	-	-	-
N.E.Atlantic A-2	1.0×10^2	100	-	-	-	-	-	-	-	-	100
N.E.Atlantic A-3	$1.5\text{-}1.9 \times 10^3$	95	5	-	-	-	-	-	-	-	100
N.E.Atlantic Goban Spur	2.2×10^3	100	-	-	-	-	-	-	5	-	95

A/V *Aeromonas/Vibrio*

PLO pseudomonads

by their size, structure and mode of reproduction using phase-contrast microscopy. The results of these identification procedures on the isolates are shown in table (1.10) as percentage total of the nitrate reducing population of each sediment sample and show the preponderance of members of the *Aeromonas/Vibrio* group in the nitrate reducing flora. Significant numbers of pseudomonads were also recovered from the inshore sediment samples. Sediment from Loch Eil E-70 contained the most varied nitrate-reducing flora, including a high percentage of Gram positive organisms including *Bacillus spp.* and substantial numbers of budding yeasts. The isolates' action on nitrate recorded as percentage total of the nitrate-reducing population show that 92 to 100% of the bacteria recovered were able to reduce nitrate and 88 to 100% to reduce nitrite to ammonia. Reduction of nitrite to gaseous products was not encountered.

CHARACTERIZATION OF THE YEAST ISOLATES.

The results of the characterisation tests performed on the yeasts isolated from Loch Eil E-70 are presented in table 1.11. All 22 isolates were similar in colony morphology when grown aerobically or anaerobically on nitrate nutrient agar plates, being circular and entire or undulate, 1 to 4 mm in diameter, flat, shiny and conspicuously tan/yellow or orange/yellow in colouration. All reproduced by budding and all could assimilate KNO_3 as sole nitrogen source. All but one of the isolates could grow at salt concentrations of 0M, 0.2M and 0.4M NaCl with several isolates showing an increased lag period at 0M NaCl and others growing better at 0.2M NaCl than at

0.4M NaCl. All of the isolates could grow at 15°C and 30°C, most at 37°C and none showed growth at 4°C. All the isolates could grow at pH 6.0 and pH 7.0 and anaerobically. None could grow or ferment on glucose and no isolate possessed a capsule or slime, or a carotenoid pigment.

SYMBOLS USED IN TABLE 1.11 AND EQUIVALENT DESCRIPTIVE TERMS:

COLONY MORPHOLOGY	CE	Circular and entire
	U	Undulate
	F	Flat
	T	Tan
	T/Y	Tan/yellow
	O/Y	Orange/yellow
	S	Shiny colony surface
OTHER TESTS	+	Assimilation, growth
	-	No growth, no fermentation, absent
	•	Growth after long lag phase
	o	Better growth at 0.2M NaCl than at 0.4M NaCl

TABLE 1.11 CHARACTERISTICS OF LOCH EIL E-70 YEAST ISOLATES

Isolate	Colony morphology					Mode of reproduction	NO_3^- Assm^n	NaCl growth			Anaer-obic growth	Growth at (^oC)				Glucose ferm^n	Growth at		Capsule or slime	Caro-tenoid pigment
	Shape	Diam (mm)	Eleva-tion	Colour	Shiny /dull			0M	0.2M	0.4M		4^o	15^o	30^o	37^o		pH6	pH7		
A	U	2	F	T/Y	S	Budding	+	+	+	+	+	-	+	+	-	-	-	+	-	-
B	U	4	F	O/Y	S	Budding	+	-	+	+	+	-	+	+	+	-	+	+	-	-
C	U	3	F	O/Y	S	Budding	+	+	^{o}+	+	+	-	+	+	+	-	+	+	-	-
D	CE	1-5	F	T/Y	S	Budding	+	+	+	+	+	-	+	+	-	-	+	+	-	-
E	CE	1-5	F	T/Y	S	Budding	+	+	^{o}+	+	+	-	+	+	-	-	+	+	-	-
F	U	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
G	U	2	F	O/Y	S	Budding	+	•+	+	+	+	-	+	+	-	-	+	+	-	-
H	U	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
I	CE	1.5	F	T/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
J	CE	1.5	F	T/Y	S	Budding	+	+	^{o}+	+	+	-	+	+	-	-	+	+	-	-
K	U	3	F	O/Y	S	Budding	+	•+	+	+	+	-	+	+	+	-	+	+	-	-
L	CE	2.5	F	O/Y	S	Budding	+	•+	+	+	+	-	+	+	+	-	+	+	-	-
M	CE	1.5	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	-	-	+	+	-	-
N	CE	1	F	T/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
O	U	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
P	CE	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
Q	CE	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
R	U	2	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
S	CE	2	F	T	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
T	CE	1	F	T/Y	S	Budding	+	+	+	+	+	-	+	+	+	-	+	+	-	-
U	CE	2	F	O/Y	S	Budding	+	•+	+	+	+	-	+	+	-	-	+	+	-	-
V	CE	1.5	F	O/Y	S	Budding	+	+	+	+	+	-	+	+	-	-	+	+	-	-

MICROBIOLOGY OF THE NITRATE REDUCING FLORA FROM THE PULP MILL DISCHARGING INTO LOCH EIL.

In an attempt to determine the origin of the Loch Eil E-70 yeast isolates, samples from the pulp and paper mill discharging close to station E-70 were screened for nitrate reducing bacteria as before. The results of both anaerobic and aerobic incubations are shown in table 1.12. Although budding yeasts were isolated from aerobically incubated samples from the paper machine, they did not possess the characteristic coloration of the previous isolates and were not recorded on the anaerobically incubated plates. Gram positive spore-forming rods were recorded on aerobic nitrate plates of samples from the effluent holding tank input (very similar to the discharged effluent) and were presumably *Bacillus spp.*, whilst Gram positive cocci were the predominant colonies on the anaerobic plates. Both these types were recorded from Loch Eil E-70 samples and from no other station.

No yeasts were recovered from the holding tank and only Gram+ cocci were found on the aerobic plates. The low counts of nitrate reducing bacteria recorded from the anaerobically incubated nitrate plates (zero to 1.0×10^2) were in contrast to the number of nitrate reducers found in sediment from Loch Eil E-70 (table 1.2).

TABLE 1.12 MICROBIOLOGY OF NITRATE REDUCING FLORA FROM THE PULP MILL DISCHARGING INTO LOCH EIL

Sample	pH	Aerobic Count (Bacteria ml^{-1})	Representative Types	Anaerobic Count (Bacteria ml^{-1})	Predominant Bacterial Type
Paper Machine	6.0	6.0×10^2	Non-pigmented budding yeasts	1.0×10^2	Gram negative rods
Input of effluent holding tank (*ie.* discharge)	6.1	1.0×10^2	Gram positive spore-forming rods	1.0×10^2	Gram positive cocci
Sediment in effluent holding tank	3.0	1.0×10^2	Gram positive cocci	-	-

D I S C U S S I O N

NITRATE INCUBATIONS

The observation that the concentration of ammonia in the interstitial water of marine sediments is generally higher than that of nitrate is well documented. Rittenberg and co-workers (1955) have demonstrated that NH_4^+ was the dominant form of inorganic nitrogen in sediments from the Santa Barbara, Santa Monica and Catalina Basins off the southern Californian coast (average water depth 1,650m). Ammonia levels ranged from double those of nitrate in the surface 10 cm of Catalina Bay sediments (310μM NH_4^+; 138μM NO_3^-), to greater than 500-fold in samples from the Santa Barbara Basin (560μM NH_4^+; NO_3^- undetected). Haines *et al*, (1981) in a study on the surface 5 cm of Alaskan continental shelf sediments recorded ammonia concentrations which were greater than nitrate by from three times in samples from the Beaufort Sea (water depth 19m; 102μM NH_4^+, 33μM NO_3^-) to forty times in Upper Bering Sea samples (water depth 35m; 720μM NH_4^+, 17μM NO_3^-). Within fjordic sediments Sørensen (1978a) has recorded levels of ammonia up to 175-fold greater than those of nitrate in the surface 6 cm of sediment. In sediment samples from the Tay Estuary in N.E. Scotland, Dunn and co-workers (1978) found that ammonia was five times more common in the surface 5 cm of sediment than nitrate. Similar results have been reported by Kaplan *et al*, (1979) for sediment samples from the Great Sippewissett salt marsh in New England (50μM NH_4^+, 10μM NO_3^-).

Inorganic nitrogen analyses performed on Loch Eil sediments at the time of sampling showed a similar trend to that described above (see table 4.1, appendix). At station E-24 nitrate was the predominant form of inorganic nitrogen down to a sediment

depth of 4 cm but below this point ammonia was more abundant, with a concentration at 10 cm depth some twenty times that of nitrate. Ammonia was the dominant form of inorganic nitrogen at all depths investigated in sediment from the polluted inner basin sampling station E-70 and was more than two thousand-fold the concentration of nitrate at 10 cm sediment depth.

Sedimentary NH_4^+ is usually considered to originate from bacterial decomposition of organic nitrogen and this process of ammonification occurs in many marine sediments (*e.g.* Rittenberg, *et al*,1955; Dunn *et al*, 1980). As has already been mentioned, NH_4^+ can also be formed by the dissimilatory reduction of NO_3^- to NO_2^- and then to NH_4^+, and this process has been demonstrated in several marine sediments (Koike and Hattori,1978a; Sørensen 1978a; Dunn *et al*, 1980; Herbert, 1982). The data obtained in this study (table 1.1), would tend to suggest that this latter reaction is a source of ammonia in the diverse marine sediments examined.

The low levels of NO_2^- encountered (with no KNO_3 supplementation) of around 0.4μM were in agreement with published values for other marine sediments (Rittenberg *et al*, 1955; Grundmanis and Murray, 1977; Dunn *et al*, 1980) and suggest that NO_2^- may not be the final end product of nitrate respiration in marine sediments. The negligible change in NO_2^- levels on the incubation of unamended sediments has been observed by Herbert (1982) in samples from the Rivers Don and Leven and the North Sea. All the sediments produced NO_2^- on supplementation with nitrate which indicated the potential of the sediments to perform dissimilatory nitrate reduction. As this stimulation occurred in both organically rich (*e.g.* E-70, 6.2%C) and poor

(*e.g.* A-1, 0.3%C) sediments the data would suggest that the process was rate limited by electron acceptor availability. This has been shown to be the case in Alaskan shelf sediments, with organic carbon amendment only stimulating nitrate reduction when combined with nitrate supplementation (Haines *et al*, 1981). Studies on coastal marine sediments by Sørensen *et al*, (1979) have demonstrated that the availability of NO_3^- was limiting for dissimilatory nitrate reduction.

The lowest rates (after 24 hours) of nitrate reduction with KNO_3 supplementation were recorded in the low carbon sediments of the N.E. Atlantic (A-1 and A-2) and the Lynn of Lorne (LY-1). A 24 hour incubation period was chosen for comparative purposes as in the majority of samples nitrate reduction occurred at a maximum over this period. As the counts of nitrate reducing bacteria recovered from LY-1 sediment were of the same order as those from the organically rich stations E-24 and E-70 in Loch Eil (table 1.2), it would appear that when NO_3^- is in excess, nitrate reduction is limited by electron donor (*i.e.* organic matter) availability. This may also be the case in the two Atlantic sediments although fewer potential nitrate reducing bacteria have been isolated from A-2 and none from A-1. The effects of rapid decompression on samples from these two deep sea stations (pressure changes of approximately 490 atm. and 300 atm. for A-1 and A-2 respectively) may also have had an effect on the nitrate reducing capability of the sediment samples and indeed on the viable cell counts (see below). The highest rates of NO_3^- reduction (after 24 hours incubation) on supplementation with nitrate occurred in sediment from stations E-24 in Loch Eil and the continental shelf

station A-3 and were presumably a reflection of the greater availability of organic carbon. The slower rate of nitrate reduction in E-70 sediment would not appear to be due to lack of organic material and may have been due to sulphide toxicity. Sørensen (1978b) reported this as a possible explanation for the spatial separation of nitrate and sulphate reduction in coastal marine sediment from Kysing fjord, Denmark and at the time of sampling, sulphide levels of upto 380μM were recorded in the surface 5 cm of E-70 sediment (F.Drake, unpublished data). In most of the sediments the nitrate reduction rate over 24 hours with 2 mg KNO_3 supplementation (approx.2mM) was the same as that with 10 mg (approx.10mM) addition and would suggest that nitrate reduction was rate limited by a NO_3^- concentration <2mM. The nitrate reduction activity measured by this method over 5 days for the Loch Eil and Lynn of Lorne sediments was of the same order as that measured by Herbert (1982) using an essentially similar technique in sediments from the east coast of Scotland.

As can be seen from table 1.1, there was an appreciable variation in NH_4^+ levels on incubation both with and without KNO_3 supplementation. The decrease in NH_4^+ levels on incubation in sediment from sampling stations A-1, A-2 and LY-1 presumably reflected the assimilation of NH_4^+ by sediment organisms and it was unclear as to whether dissimilatory nitrate reduction to ammonia had occurred. However, the increase in NO_2^- in these sediments after 5 days incubation with KNO_3 supplementation indicated that dissimilatory reduction of $NO_3^- \rightarrow NO_2^-$ was possible and the isolation from these sediments of bacteria capable of reducing NO_3^- through to NH_4^+ (see below)

provided indirect evidence for this process occurring within A-2, LY-1 and possibly A-1, sediments. Nitrate stimulated net NH_4^+ production was recorded in sediment samples from E-70 in Loch Eil, A-3 on the British continental shelf and to some extent E-24 in Loch Eil and showed that dissimilatory reduction of NO_3^- to NH_4^+ could be a potential source of the high NH_4^+ levels found in the interstitial waters of these marine sediments. Sørensen (1978a) has indicated that the process may be quantitatively important in marine sediments and net NH_4^+ production stimulated by nitrate addition has been recorded in a variety of estuarine and coastal sediments (Dunn *et al*, 1980; Herbert, 1982). Koike and Hattori (1978a) have demonstrated that over 50% of $^{15}NO_3^-$ was reduced to ammonia in organically rich sediments such as those found at E-70 in Loch Eil, whilst in organically poor sediment nitrate reduction proceeded in the main to gaseous products (denitrification). This may have been the fate of reduced nitrate in the low organic carbon sediments of A-1, A-2 and A-3 and the technique employed in this study would not have detected denitrification. However, for reasons mentioned above, the products of NO_3^- reduction were assumed to be NH_4^+.

It is worth pointing out here that although this study has demonstrated the potential of nitrate reduction to NH_4^+ as a source of NH_4^+ in Loch Eil sediments, reference to the low levels of NO_3^- in the overlying water of the sediments and to the very high levels of NH_4^+ in E-70 sediment and in the lower reaches of E-24 sediment (table 4.1, appendix), would indicate that some other process was supplying a large proportion of the NH_4^+ found in the interstitial waters of E-70

and E-24 sediments. This was presumably due to ammonification, although the decrease of NO_3^- with depth did indicate that NO_3^- reduction was occurring and the observed bioturbation in Loch Eil sediments, carrying surface water down to deeper NO_3^- deficient layers, may have resulted in higher rates of nitrate reduction than was assumed at first glance. NO_3^- may also be regenerated by nitrification, a process whereby NH_4^+ is oxidised to NO_3^- under aerobic conditions. Faunal burrows formed by polychaete worms are common in Loch Eil sediments (Pearson, 1975) and result in oxidised patches below the redox transition zone where nitrification can take place (Sørensen 1978a,b; Grundmanis and Murray, 1977). This may have been the cause of the elevated NO_3^- levels at around 30 cm depth in E-24 and E-70 sediments.

The sediment samples in this study were incubated in closed containers under an initially aerobic environment and it was assumed that nitrate reduction occurred in anaerobic microenvironments as has been reported by Sørensen (1978a), Knowles (1979) and Dunn *et al* (1980). In work on lake sediment, Knowles (1979) has recorded that the reduction of added NO_3^- under initially aerobic conditions proceeded at a rate similar to that under anaerobic (He) conditions. Hence, it was possible that in the sediments studied both denitrification and nitrification occurred simultaneously within microniches or below the aerobic-anaerobic interface. ^{15}N studies by Koike and Hattori (1978b) have demonstrated this in marine sediments from Odawa Bay, Japan.

NITRATE REDUCING FLORA

The use of KNO_3 supplemented yeast extract–peptone containing media has been reported in several studies on the enumeration of nitrate reducing bacteria from aquatic environments (*e.g.* Horsley, 1978; Blake, 1980). In this study the sediment samples were incubated on plates of such a nitrate medium under anaerobic conditions to yield a count of bacteria capable of growing anaerobically at the expense of NO_3^-. Highest counts of nitrate reducing bacteria were recorded from sediments obtained from sampling stations at LY-1 in the Lynn of Lorne and the two Loch Eil stations, E-24 and E-70. Although there are large differences in organic carbon content and Eh in sediments from the two areas (table i), the variation in numbers of nitrate reducers was small (counts of 10^4-10^5 nitrate reducers ml^{-1} sediment). This observation and the fact that the counts obtained were much lower than those from freshwaters (10^9-10^{10} nitrate reducers g^{-1} sediment, recorded by Jones and Simon, 1981) suggested that nitrate reduction was relatively unimportant as a means of anaerobic oxidation of organic material within such marine sediments due to electron acceptor (NO_3^-) limitation (Sørensen *et al*, 1979; Blake *et al*, 1982). When corrected to g^{-1} dry wt. of sediment the values obtained were between ten and a hundred-fold lower than those obtained from Tay Estuary sediments by similar methods (Dunn *et al*, 1980).

The lowest counts of nitrate reducing bacteria were recovered from the N.E. Atlantic samples and ranged from 10^2-10^3 nitrate reducing bacteria ml^{-1} sediment. The highest

counts were obtained from the shallower, continental shelf stations on the Malin Shelf and Goban Spur and when corrected to g^{-1} dry weight sediment were some ten times greater than counts obtained from similar sediments in the North Sea (Blake, 1980). The lower counts from station A-2 and the fact that attempts to isolate nitrate reducers from A-1 sediment failed, was a reflection of the much lower occurrence of bacteria in deep sea sediments, probably caused by the very low organic carbon levels encountered in such sediments (Gage, 1978). Published counts of nitrate reducing bacteria from deep sea sediments are rare, although Morita and ZoBell (1955) reported that an "appreciable percentage" of bacteria isolated from pelagic Pacific sediments (depth 1700-5000m) could reduce nitrate. The counts of nitrate reducers isolated from A-2 sediment (depth 2,880m) in this study were within their range of counts for the surface 10 cm of sediment (average aerobic count 10^2 g^{-1} wet sediment; anaerobic count $0.5x10^2$ g^{-1} wet sediment). One of the inherent problems in attempting to enumerate and isolate nitrate reducing, or any other type of, bacteria from deep sea sediments such as A-2 and A-1, is the rapid decompression that cells undergo on recovery of sediment samples from the ocean depths. It was unclear as to what reduction in recovery this caused and indeed whether the failure to recover nitrate reducing bacteria from A-1 sediment was due to loss of bacterial viability by decompression or to the absence of such cells initially. The pressure on bacteria in A-1 surface sediments is around 490 atm. assuming a change of 1 atmosphere pressure for each 10 m depth of sea water. ZoBell and Budge (1965) have shown that although

the processes were retarded, bacteria could still reduce nitrate and reproduce at pressures in excess of 500 atm. Furthermore, they concluded that bacteria could reduce nitrate at pressures greater than those at which reproduction occured and that pressure should not prevent nitrate reduction at any depth in the ocean. These observations, together with the production of NO_2^- on supplementation with KNO_3 by A-1 sediments (see above) and the recovery of upto 10^4 bacteria g^{-1} dry wt. sediment from A-1 (Blake, 1980), suggest that the failure to recover any nitrate reducing bacteria from station A-1 was due to rapid pressure change, and that nitrate reduction may in fact be occurring at low rates in the abyssal plain of the N.E. Atlantic.

The method of examining randomly selected bacteria from plates to determine the characteristics of the nitrate reducing flora has been employed in several studies on nitrate reduction in aquatic environments and appears to give a good approximation of the identity of the organisms involved (Nedwell, 1975; Horsley, 1978; Dunn *et al*, 1980; Herbert, 1982). As can be seen from tables 1.3 to 1.8, summarized in table 1.10, *Aeromonas/Vibrio* organisms were the dominant bacterial types in all the sediments examined, except E-70 where they made up 32% of the nitrate reducers recovered. Oxidative organisms such as pseudomonads were rarely recovered, except again at E-70 which had the most varied nitrate reducing flora of any of the sediments examined. This preponderance of fermentative bacteria suggested that the sediments examined had a similar nitrate reducing flora to that encountered in the Tay Estuary and North Sea (Dunn *et al*, 1980) and in

estuaries on the east coast of Scotland (Herbert, 1982). The majority of the organisms isolated could reduce NO_3^- through to NH_4^+, whilst oxidative organisms capable of reducing NO_3^- through to gaseous products (denitrification) were never encountered. This suggested that within the marine sediments examined, fermentative bacteria were the dominant nitrate reducers, retaining a readily assimilable source of inorganic nitrogen within the sedimentary ecosystem as NH_4^+. This process of course has a distinct ecological advantage compared with denitrification, where nitrogen is exported from the sediment as gaseous products (N_2O, N_2) and subsequently lost to the atmosphere. Furthermore, the dissimilatory reduction of NO_3^- to NH_4^+ is proposed by Cole and Brown (1980) as a "short circuit" to the rate-limiting step of nitrogen fixation in the nitrogen cycle (see fig.1.1). The *Aeromonas/Vibrio* organisms isolated would appear well adapted to existence within the sediment environment, being capable of anaerobic fermentation, or respiration when NO_3^- was available either from the interstial water, faunal burrows or ammonification (Sørensen, 1978a).

The direct plating method used in this study yielded information of the nitrate respiring flora but gave no indication of which organisms were most active in the process. However, comparative studies with chemostat enrichments have shown that fermentative bacteria are numerically predominant and the most active in estuarine sediments (Herbert, 1982).

As indicated in table 1.10 sediment from station E-70 in Loch Eil had the most diverse nitrate reducing flora of the sediments examined including a substantial proportion of

budding yeasts. This was initially surprising as E-70 sediment was rich in sulphide and the enumeration regime was designed to select for nitrate respiring bacteria. However, yeasts have been isolated from H_2S zones in the Black sea and N.W. Pacific and many marine yeasts are capable of reducing NO_3^- to NO_2^- (Kriss, 1963). Yeast isolates 12 and 48 from E-70 (table 1.5) were observed to be capable of reducing $NO_3^- \rightarrow NO_2^-$ in NO_3^- nutrient broth and could presumably use this compound as an alternative electron acceptor to oxygen under anoxic conditions. All of the isolates could assimilate NO_3^- as a nitrogen source for growth. The limited investigation into the physiology of the yeast isolates indicated that they could all grow in fresh or salt water and could grow anaerobically. Their inability to grow at 4°C, coupled with the observation that most of the strains could grow at 37°C, suggested that the majority of the isolates could be classified as in-shore forms of yeast as opposed to off-shore, marine forms (van Uden and Fell, 1968). It was suspected that the yeasts were dormant in E-70 sediment and originated in the effluent discharge from the pulp and paper mill at Annat Point.

However, the examination of samples from the paper making process and the effluent holding tank yielded budding yeasts only from the paper machine, (table 1.12) which were not the same types as those isolated from station E-70. Neither were yeasts recorded in samples from the holding tank, where it was thought that the low pH would tend to favour yeast development. It must be concluded, therefore, that the origin of the yeasts in E-70 sediment was unlikely to be

the pulp effluent outflow. Whether the yeasts were dormant forms derived from terrestrial run-off or whether they were active and if so what processes within the sediment were they associated with, are questions which are poorly understood and which would benefit from further detailed research. Yeasts have been reported to be capable of growth on the hydrolysis products of cellulose breakdown (Imshenetsky, 1967) and this may be a reason for their occurrence in Loch Eil E-70 sediment.

SECTION 2

DISSIMILATORY SULPHATE REDUCTION IN MARINE SEDIMENTS

INTRODUCTION

Sulphur occurs abundantly on this planet and as with nitrogen is an essential component of the biosphere. The cytoplasm generally contains between 0.4 and 1% dry weight sulphur (Postgate, 1979) which is present in proteins, amino acids, vitamins and nucleic acids. Although sulphur is present in some marine algae (*e.g. Gigartina spp.*) at levels of up to 3% dry weight (Goldhaber and Kaplan, 1974), the sulphur content of cells is usually low when compared with the other elements involved in biosynthesis and sulphur is generally not a growth-limiting nutrient in the natural environment. Even the sulphate reducing bacterium *Desulfovibrio vulgaris* contains only 1.3% S compared with 46.5% C; 12.5% N and 7.1% H (Postgate, 1979) and such an elemental composition is typical of many bacteria.

As with nitrogen, sulphur can exist in a number of oxidation states from the most oxidised form sulphate (SO_4^{2-}, oxidation state +6) to the most reduced form sulphide (S^{2-}, -2), with intermediary forms such as elemental sulphur (S^O), sulphite (SO_3^{2-}) and thiosulphate ($S_2O_3^{2-}$). Sulphur is mainly available to the biosphere in its most oxidised state in the form of soluble sulphates. In freshwaters sulphate is present at a concentration of 3 to 300µM (Campbell, 1977), although sulphide (upto 18 µM S^{2-}) can accumulate during the summer months in the hypolimnion zone (1m above the sediment surface) of eutrophic lakes (Jones, J; 1982). Sulphate is also present in most soils at a concentration of 10 to 125 $mgkg^{-1}$ (Campbell, 1977) and oxidised forms of sulphur (SO_4^{2-} and SO_2) can also comprise a minor component of the atmosphere at concentrations of <1 to 20 ng l^{-1} depending on the degree of pollution *via* fossil fuel combustion (Kellog *et al*, 1972). However, by far the largest

reservoir of sulphur available to the biosphere is found in the marine environment where seawater at a standard salinity of 35‰ contains 28.9mM sulphate (Dyrssen and Wedburg, 1974).

Sulphate enters the sea at a rate of approximately 3.7×10^{11} kg yr^{-1} as river run-off *via* terrestrial erosion (Sieburth, 1979). The abundance of sulphate in seawater however, tends to remain at a constant level, as shown by its concentration with respect to the conservative element chlorine. Chlorine is not used biologically and the resulting sulphate : chlorine ratio for seawater is generally constant at a value of 0.14 (Wilson, 1975), indicating that the influx of sulphate is removed in the marine environment. This can occur either by precipitation with Ca^{2+} ions to form $CaSO_4$ (Sieburth, 1979) or by the biological reduction of sulphate to sulphide under anaerobic conditions by a small, specialised group of bacteria which utilize sulphate as a terminal electron acceptor for the anaerobic oxidation of organic matter (Goldhaber and Kaplan, 1974). These bacteria are collectively known as the sulphate reducing bacteria (Postgate, 1979) and their activities in the marine environment results in sulphate removal with a concomitant lowering of the sulphate : chlorine ratio in their locality (Vosjan, 1974).

The reduction of sulphate to sulphide is part of a series of biologically mediated oxidation-reduction transformations of sulphur termed the biological sulphur cycle (fig.2.1), which is in many respects analogous to the better known nitrogen cycle (fig.1.1). Due to the oxic conditions that usually exist in the marine water column and to the anaerobic nature of the sulphate reducers it is usually only within the sediment

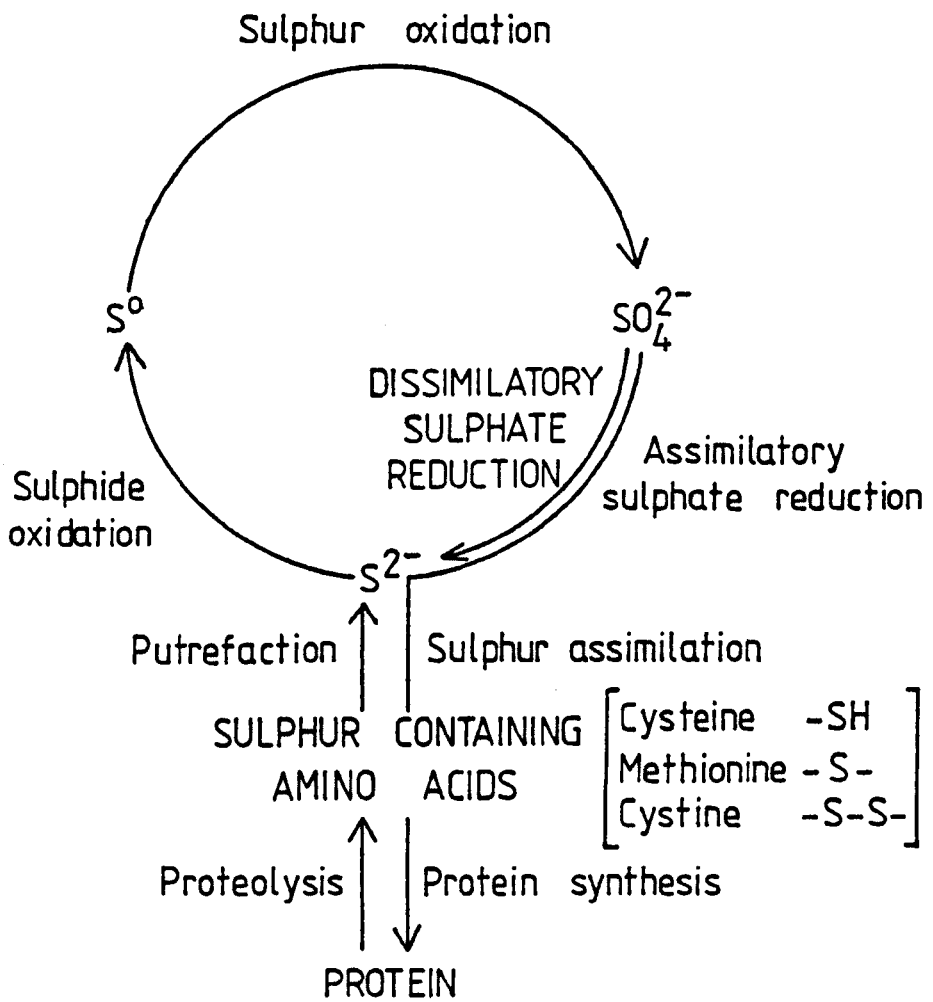


Figure 2.1: The Biological Sulphur Cycle

(after Postgate, 1979)

environment that the full cycle is found. The production of sulphide by dissimilatory sulphate reduction results in sediment redox potentials falling to as low as -200mV (Jørgensen and Fenchel, 1974) which can have a profound effect on the fauna and chemistry of the sediment (Gray, 1981) and on any subsequent diagenetic processes (Goldhaber and Kaplan, 1974; Jørgensen, 1978c). Although much of this sulphide is trapped within the sediment as metal sulphides, its diffusion into oxic and photic layers of the sediment can result in its oxidation back to sulphate by chemoautotrophic and photoautotrophic sulphur bacteria. This often results in the formation of sulfureta, either on a micro- or macro-environment scale in which consecutive electron transfer reactions convert sulphate to sulphide and back again (see fig.2.1 and Postgate, 1979). The presence of such a sulphide-oxidising organism (*Beggiatoa*) in Loch Eil inner basin sediments has been reported (Stanley *et al*, 1978) and the characteristic white fibrous mat was noticed on the surface of E-70 cores on several occasions. *Beggiatoa spp.* are typical gradient organisms (Jørgensen and Fenchel, 1974) indicating that free sulphide was reaching the E-70 sediment surface and that at this sampling station at least the full sulphur cycle as shown in fig.2.1 was occurring.

As can be seen from fig.2.1 sulphate can be reduced during sulphur assimilation by plants and microorganisms to the level of sulphydryl (HS^-) groups which react with the amino acid serine to form cysteine ($CH_2SH\ CHNH_2COOH$). Methionine and cystine, two more sulphur-containing amino acids, can be subsequently formed from cysteine and used in the biosynthesis of proteins, etc. As a rule organisms only assimilate enough

sulphur for their immediate biosynthetic needs (as indicated by the low levels of sulphur in the cytoplasm) and do not excrete reduced forms of sulphur into the environment (Stanier *et al*, 1977). Hence, on the decomposition and mineralization of sulphur-containing organic matter the release of sulphides into the sediment is low when compared with that originating from dissimilatory sulphate reduction (Goldhaber and Kaplan, 1974). Less than 3% of the total sulphide production measured in a shallow fjordic sediment (Limfjorden, Denmark) which received an organic detrital input of 170 g org-C $m^{-2}yr^{-1}$ originated from putrefaction (Jørgensen, 1977b). Data from the Black Sea by Deuser (1970) has indicated that only 5% of the sulphide measured originated from putrefaction with sulphate reduction accounting for between 94% and 97% of the total. Hence, the bacterial use of sulphate as an oxidant for the anaerobic mineralization of organic matter is the greatest source of sulphide in the marine environment.

Although the dissimilatory reduction of sulphate by the sulphate reducing bacteria requires reduced conditions with a redox potential of about 0 to -100mV (Alico and Liegey, 1966; Postgate, 1979), the process can occur within the water column in the marine environment. Sea water is normally well oxygenated by physical mixing and photosynthesis but bodies of water can become anoxic as a result of hydrographic conditions (*e.g.* thermoclines or haloclines) which prevent the exchange of water and high oxygen consumption in the sediment or overlying water (see Deuser, 1975). Such a situation occurred at sampling station D-4 in the inner basin of Sullom Voe (Shetland Islands) and has been attributed to the formation of a thermocline during

the summer months (Stanley *et al*, 1978). Sorokin (1962) reported low rates of sulphate reduction in the bottom waters and the surface of the H_2S zone in samples from the Black Sea and Ivanov (1968, 1978) has reported similar low rates of activity in meromictic lakes in the Soviet Union, together with counts of 0.1 to 10 sulphate reducing bacteria ml^{-1} recovered from the benthic water. One well documented area where anoxic bodies of water occur is the Baltic Sea where the hydrography often results in the formation of stable thermo-haloclines which prevent water exchange (see Schneider, 1977). The lack of oxygen supply can be further exacerbated by organically rich terrestrial run-off within estuarine and fjordic regions. Counts of sulphate reducing bacteria of 10^2 to 10^3 ml^{-1} have been reported by Bansemir and Rheinheimer (1970, cited by Schneider, 1977) in deep holes in Schlei fjord in the western Baltic - a region where water exchange was restricted. Later work by the same authors (1974, cited by Schneider, 1977) demonstrated low numbers of sulphate reducing bacteria (ca. 20 ml^{-1}) in water samples from Kiel fjord in the Baltic, with higher numbers recovered from a deep hole in the fjord. However, Schneider concluded that only negligible sulphate reduction was occurring in these areas due to oxygen still being present. Low numbers of sulphate reducing bacteria have also been recovered from the water column in Hiroshima Bay, Japan (Kimata *et al*, 1955a) and from the North Sea (Hardy, 1981).

Biogenic sulphide production has been found in the deep water of stagnant basins off the Southern Californian, Greenland and Canadian coasts and in the Cariaco Trench off Venezuela (Fonselius, 1976). However, sulphate reduction is of only

local importance in the water column, although its contribution to sulphate reduction activity in the geologic past may have been greater due to a lower atmospheric oxygen tension (Goldhaber and Kaplan, 1974). As a consequence sulphate reducing activity in the water column at the sampling stations investigated in this study was not measured, despite the anoxic conditions encountered in the inner basin waters of Sullom Voe.

It is within marine sediments that the anoxic reduced environment essential for dissimilatory sulphate reduction is found, in which the process is most active and on which this section concentrates. In the majority of marine sediments reducing conditions are maintained below a thin, upper oxidised zone by the action of heterotrophic microorganisms degrading macromolecules to lower molecular weight forms. The structure and extent of such reduced areas has already been discussed and it is here that sulphate reduction is most active. The process has been detected in the oxidised layer of a fjordic sediment by means of $^{35}SO_4^{2-}$ incubation, although this has been attributed to reduced microniches formed from faecal pellets within the overall oxidised environment (Jørgensen, 1977a) - reducing conditions within the particles being maintained by the rapid O_2 consumption of the heterotrophic organisms and by the production of reducing compounds such as H_2S.

Within the reduced zone of marine sediments dissimilatory sulphate reduction is often the major means of anaerobic mineralization and evidence for its importance has accumulated over the past eight years. Jørgensen and Fenchel (1974) in experiments on a model system based on a sandy estuarine sediment demonstrated that sulphate reduction accounted for over half of the organic detritus mineralization. A similar conclusion

was reached by Jørgensen (1977b) in a definitive series of experiments over a two year period on shallow coastal sediment from the Limfjorden (Denmark), with sulphate reduction mineralizing 53% of the organic material in the sediment. The use of sulphate as a terminal electron acceptor oxidised 1,800 g org-C m^{-2} yr^{-1} in a New England salt marsh, whilst the use of O_2 or NO_3^- as electron acceptors was of much less importance (Howarth and Teal, 1979). Data reported by Sórensen *et al*, (1979), for two Danish estuaries indicated that sulphate reduction oxidised 30.2 mmols C m^{-2} d^{-1} compared with 110 mmols Cm^{-2} d^{-1} for aerobic respiration with dissimilatory nitrate reduction being of little significance. This importance of dissimilatory sulphate reduction in the degradation of organic matter in marine sediments is a reflection of the abundance of the electron acceptor SO_4^{2-} (28.9 mmol l^{-1} at a salinity of 35‰) compared with O_2 (225.2 µmol l^{-1} at 20°C and S = 35‰ Kester, 1975) or NO_3^- (ca. 0 to 100 µmol l^{-1}, on average).

The rate of sulphate reduction in marine sediments can be measured by radiotracer techniques, calculated from mathematical models or estimated from chemical and bacteriological field data. These different methods are compared by Jørgensen (1978a,b,c). The most widely used method is that involving the incubation of sediment samples with the radiotracer $^{35}SO_4^{2-}$ which can be introduced into the sediment in true tracer amounts. The labelled sulphide produced can be measured with a high degree of sensitivity allowing low rates of sulphate reduction to be measured over a short time period (Jørgensen, 1978a). Since its inception by Ivanov in 1959 (cited in Sorokin, 1962) the method has been used in several variations which differ

mainly in the way in which $^{35}SO_4^{2-}$ is introduced into the sample (*e.g.* Sorokin, 1962; Ivanov, 1968; Jørgensen and Fenchel, 1974) and such a variation was employed in this study.

As sulphate is usually abundant in surface marine sediments the maximum rates of sulphate reduction are generally found in areas of high organic content (*i.e.* electron donor concentration). The highest published values of sulphate reduction rates commonly come from studies on intertidal sediments, which have a high productivity and large detrital organic content (Nedwell, 1982). Rates of 6,000 n mol SO_4^{2-} ml^{-1} d^{-1} have been measured in the surface 20 cm of peat from a U.S. salt marsh during summer (Howarth and Teal, 1979). However, in general the activity of such intertidal sediments is between 10^2 and 10^3 nmol SO_4^{2-} ml^{-1} d^{-1}. Intermediary sulphate reduction rates have been reported in the literature from coastal sediment samples taken from areas such as fjords, bays in the Baltic and Black Sea and on the continental shelf. For example, Jørgensen (1977b) measured sulphate reduction rates from 25 to 200 n mol SO_4^{2-} ml^{-1} d^{-1} in surface sediments from the Limfjorden in northern Denmark and these rates are typical of coastal sediments. The lowest rates of sedimentary sulphate reduction reported in the literature have been measured in deep sea and abyssal sediments which receive low inputs of refractory organic material (Stanley *et al*, 1978). In addition the low temperatures, high Eh and high pressure commonly found in such regions result in very low activities of the order of 0 up to 10 n mol SO_4^{2-} ml^{-1} d^{-1} in some reduced sediments.

Sulphate reducing activity also tends to show a decrease with increasing depth down the sediment from a maximum near

the top of the anaerobic zone where presumably the concentration of electron donors and acceptor is greatest (Nedwell, 1982). Nedwell and Abram (1978) have demonstrated that sulphate reduction rates in two English saltmarsh sediments decreased rapidly over the surface 20 cm with the maximum activity being recorded in the top 10 cm of sediment. Similar trends have been reported by Jørgensen (1977b) and Aller and Yingst (1980) in coastal sediments and most of the sulphate reducing activity in marine sediments appears to occur over a depth of 0-10 cm. Sulphate reduction can occur at much greater depths in marine sediments. Data plotted by Goldhaber and Kaplan (1974) from interstitial sulphate levels in porewater from a deep water sediment off the north western coast of North America demonstrated that sulphate reduction was occurring at sediment depths in excess of 300 m over time periods of several millions of years. However, sulphate reduction at this depth is of more interest from a geochemical viewpoint as in coastal sediments sulphate reduction below 170 cm sediment depth contributed only 2.6% of the total activity (Jørgensen, 1977b).

Little has been said until now on the biological catalysts of sedimentary sulphate reduction - the sulphate reducing bacteria. Quantification of their numbers and activity in marine sediments has been infrequently reported (Oremland and Silverman, 1979) and this is partly a reflection of their anaerobic nature and recalcitrance as research organisms (Postgate, 1979). Data gleaned from the literature suggests that the highest numbers of sulphate reducing bacteria are recovered from intertidal and coastal sediments (areas of typically high organic deposition) with correspondingly lower counts from low carbon, oxidised, deep-sea sediments. This decrease in numbers with increased

water depth also occurs with heterotrophic bacteria and is presumably due to the availability of organic substrates. For example, Schröder and van Es (1980) estimated that there were 10^7 to 10^8 aerobic heterotrophs ml^{-1} in surface sediment from the Ems-Dollard Estuary (Wadden Sea), whilst Blake (1980), using essentially similar methods, recovered only 10^4 aerobes ml^{-1} from an abyssal N.E. Atlantic sediment.

In general the numbers of sulphate reducing bacteria are greatest in the surface layers of intertidal, inshore and coastal sediments and as a rule are not as affected by water depth and location as sulphate reduction rate. The rate is not thought to be limited by bacterial numbers but by environmental variables such as electron donor availability, temperature, Eh, pressure, etc. (Nedwell and Abram, 1978). The numbers of sulphate reducers in the surface layers of intertidal sediments are of the order of 10 to 10^5 ml^{-1}. Sandkvist (1968) has recovered from $8x10^2$ to $2x10^4$ bacteria ml^{-1} from tidal flats in the Dutch Wadden Sea, whilst Ivanov (1968) has estimated that there were $8x10^2$ to $4x10^4$ sulphate reducers ml^{-1} in littoral sediment from Krasnovodsk Bay (U.S.S.R.). In coastal and inshore sediments the numbers of sulphate reducing bacteria appear to similarly range from $10-10^5$ ml^{-1}, Ivanov (1978) has reported counts of 12.5 and 50 ml^{-1} in the surface 5 cm of sediment from the Pacific (water depth 140 m) and Indian (43 m) Oceans respectively. Kimata *et al*, (1955a), recovered from 10^2 to 10^5 sulphate reducers ml^{-1} from Hiroshima Bay sediments, whilst Jørgensen (1977b) estimated that the population of sulphate reducing bacteria in the upper 10 cm of a Danish fjordic sediment was of the order of $4.3x10^4$ to $9.3x10^4$ ml^{-1}.

Counts of sulphate reducing bacteria from deep sea sampling stations are as expected much lower than those from intertidal, inshore or coastal sediments and tend to lie within a range from <10 to 10^3 ml^{-1}. For example, Ivanov (1978) has reported counts of sulphate reducers in the surface 5 cm of deep sea sediment from 8 ml^{-1} (water depth 3,260m, Gulf of California) to 50 ml^{-1} (1,820m, Indian Ocean). Higher numbers of bacteria have been recovered from a depth of 2,000m in the Black Sea by Sorokin (1962) who reported counts from $8x10^2$ to $5x10^3$ sulphate reducers ml^{-1}.

Although little published data is available on the numbers of sulphate reducing bacteria at different depths in marine sediments, the discernable trend is for numbers to decrease with increasing depth (Nedwell, 1982). This is again thought to be a reflection on the decreasing abundance of available organic substrates (Aller and Yingst, 1980). Nedwell and Abram (1978) have shown a decrease in the numbers of sulphate reducing bacteria from 10^4 to 10^2 ml^{-1} over a 20 cm depth in saltmarsh creek sediments and from 10^5 to 10^4 ml^{-1} in saltmarsh pan sediments. Counts of sulphate reducing bacteria reported by Sorokin (1962) in a contrasting sediment from the Black Sea (water depth 2,000m) showed a decrease from $3x10^3$ ml^{-1} at the sediment surface to 8 ml^{-1} at a depth of 10 cm.

The rate of decrease in bacterial numbers is usually at a maximum in the surface layers of marine sediments where the measured sulphate reducing activity tends to show a similar rapid decrease. However, there is often no significant correlation between the two values (Jørgensen, 1977b; Nedwell and Abram, 1978, 1979). One explanation for this occurrence is

that the standard enumeration procedures employed are capable of underestimating the natural populations of sulphate reducing bacteria by 10^2- to 10^3-fold or more (Jørgensen, 1977b). The media commonly used in such studies, however, give good recoveries of sulphate reducers in pure culture (Pankhurst, 1971) although as will be seen, such media would not recover novel types of sulphate reducing bacteria. Such a lack of correlation between bacterial numbers and activity has often been reported in ecological studies and demonstrates that bacterial activity is dependent more on environmental factors as described above.

Most sulphate reducing bacteria are capable of fixing nitrogen (Postgate, 1979) and are thought to play a role in the supply of fixed nitrogen to nitrogen deficient sediments (Herbert, 1975). The process is conveniently measured by the acetylene reduction assay (Stewart *et al*, 1967) in which sediment samples are incubated in the dark (to discount any photosynthetic bacterial activity) in the presence of acetylene. As the nitrogen fixing enzyme nitrogenase is also capable of catalysing the reduction of acetylene to ethylene (see Masterson and Murphy, 1980), the production of ethylene can be sensitively measured by gas chromatography to yield an indirect measure of the sedimentary heterotrophic nitrogen fixation rate. These rates are usually reported in terms of n mol ethylene produced g dry wt sediment^{-1} per unit time. Although factors do exist to convert this rate into nitrogen fixation rate (see Hardy *et al*, 1973), the theoretical ratios such as 3 moles C_2H_2: 1 mole N_2 reduced are rarely obtained in sediment studies (Masterson and Murphy, 1980) and it is usual to quote and discuss nitrogen fixing activity in terms of acetylene reduction rate.

The published rates for heterotrophic acetylene reduction in marine sediments are generally low and are of the order of 10^{-2} to 10 n mol C_2H_4 prod. g dry wt^{-1} hr^{-1} (Blake *et al*, 1982). The evidence for sulphate reducing bacteria contributing to these rates is somewhat lacking. Herbert (1975) working on surface Tay Estuary sediments with acetylene reduction rates of 0.05 to 0.14 n mol C_2H_4 prod. g dry wt^{-1} hr^{-1} concluded that sulphate reducers were probably the predominant members of the heterotrophic nitrogen fixing microflora. This was due to their numerical predominance over other N-fixing bacteria (up to 50%) and to the salt requirement of their nitrogenase enzymes. Similar conclusions have been reported by Blake and Leftley (1977) using comparable methods on the surface 5 cm of sediment from two Scottish west coast sea lochs which had an acetylene reducing activity of 0.06-4.10 n mol C_2H_4 prod. g dry wt^{-1} hr^{-1}. An interesting observation by the authors was that the measured activity was stimulated by the addition of lactate (a known electron donor for sulphate reduction), although only after a possibly significant lag period of 24 to 30 hours. However, there was no correlation between the counts of sulphate reducing bacteria and activity, with a later paper (Blake *et al*, 1982) suggesting that the sulphide formed by the sulphate reducers could be exerting an inhibitory effect on acetylene reduction. Sediment supplementation experiments with $BaCl_2$ (Dicker and Smith, 1980b) and molybdate (Nedwell and Abdul Aziz, 1980) have been reported to inhibit acetylene reduction in salt marsh sediments and heterotrophic nitrogen fixation has been attributed to the sulphate reducing bacteria as these compounds are known inhibitors of dissimilatory

sulphate reduction. In an attempt to shed new light on this subject the acetylene reducing activity of selected inshore sediments was compared with the sulphate reduction (*i.e.* sulphate respiration) rate in an effort to determine the relationship between the activities of the sulphate reducing bacteria and sedimentary nitrogen fixation.

As already mentioned, the sulphate reducing bacteria are a specialized group of prokoryotes which conserve energy by the dissimilatory reduction of sulphate, which involves a turnover of sulphur some 10 to 100 times greater than that in other sedimentary bacteria (Postgate, 1965). This unique form of anaerobic respiration, coupled with the abundance of sulphate in the biosphere, enables them to outcompete many other bacteria for the available organic material within the anaerobic layers of marine sediments. The group were discovered by Beijernick in 1895 (cited in Postgate, 1979) in samples from a freshwater ditch in Holland. He described a strain called *Spirillum desulfuricans* which was to become the type species of the main genus of sulphate reducing bacteria (*Desulfovibrio desulfuricans*, see below). The organisms were first isolated in pure culture by van Delden (1903, cited in Sieburth, 1979) in sediment and water samples from the North Sea and this appears to have been the first description of marine sulphate reducing bacteria. Baars (1930, cited in Roy and Trudinger, 1970) in his doctoral thesis undertook the first extensive study of the metabolism and physiology of the sulphate reducing bacteria and together with other Dutch microbiologists such as Kluyver and van Delden, established the theory and methods essential for the study of the activities of this group

(Postgate, 1979). Baars' medium was in fact quoted in a tabulation of media for sulphate reducers by Pankhurst (1971) and survives today in a modified form as one of the most widely used liquid media for sulphate reducing bacterial studies, namely medium B of Postgate (1979) and was used in this study. These media use lactate as the electron donor and enumeration, isolation and pure culture research on sulphate reducing bacteria over the past half century has predominantly used this compound as the growth substrate (Postgate, 1965; Pankhurst, 1971; LeGall and Postgate, 1973; Postgate, 1979). However, since the completion of a major portion of the work described in this chapter reports have been published of previously undetected genera of sulphate reducing bacteria (Widdel, 1980; Widdel and Pfennig, 1981) isolated on novel media containing compounds such as acetate, benzoate, butyrate, palmitate or propionate as electron donors (see Pfennig *et al*, 1981). The presence and activities of such bacteria have also been demonstrated in intertidal, estuarine sediments using essentially similar methods (Laanbroek and Pfennig, 1981). As the majority of data in this chapter were obtained prior to these reports becoming available, *via* the use of lactate based media, the remainder of this introduction is concerned primarily with sulphate reducing bacteria isolated on lactate as the electron donor. However, the significance of these newly described genera and a brief series of experiments on their activities in marine sediments and in pure culture is described in the final section of this thesis.

The lactate oxidising sulphate reducing bacteria (with the exception of one strain) have been classified into two

genera: *Desulfovibrio* and *Desulfotomaculum* (Campbell and Postgate, 1965; Postgate and Campbell, 1966). Members of the genus *Desulfotomaculum* are anaerobic, Gram negative rods which are motile by the action of peritrichous flagella. They are distinguished from *Desulfovibrio spp.* primarily by terminal or subterminal sporulation which is detected by microscopy or heat resistance. The genus contains mesophillic (optimum temperature 30-37^{o}C) and thermophillic (55^{o}C) species of which five have been reported in Postgate's 1979 monograph: *Dm. acetoxidans* (until recently the only isolated sulphate reducer that could utilise acetate or butyrate as electron donor); *Dm. antarticum* (a strain isolated from Antarctic soil); *Dm. nigrificans* (formerly classified as *Clostridium nigrificans*, optimum growth temperature 55^{o}C); *Dm. orientis* and *Dm. ruminis*. In contrast to the larger genus *Desulfovibrio*, these organisms lack the pigment desulfoviridin and have lower amounts of guanine and cytosine in their DNA (37 to 46% GC). Members of the genus *Desulfotomaculum* are generally isolated from soils, freshwaters, rumen contents, geothermal regions and have been implicated as food spoilage organisms. Although isolated strains have been shown to adapt to saline conditions (Postgate, 1979) there are no reports of naturally occurring marine forms (Ochynski and Postgate, 1963). ZoBell and Rittenberg (1948) failed in attempts to recover *Desulfotomaculum spp* from marine sediments and it would appear that the majority of marine sulphate reducing bacteria belong to the genus *Desulfovibrio* (Goldhaber and Kaplan, 1974).

Desulfovibrio spp. are anaerobic, Gram negative vibrios (some sigmoid or spirilloid forms) which do not form endospores and are motile by means of polar flagella (two species have

lophotrichous flagellation). All of the strains except for one (Norway 4) possess desulfoviridin and have higher DNA base ratios than the previous genus (46 to 61% GC). The genus consists of seven species: *D.africanus* (isolated from African waters), *D.baculatus*, *D.desulfuricans* (the type species described in 1895, see above), *D.gigas* (dimensions approx. 2x those of other species), *D.salexigens* (chloride ion required for growth), *D.thermophilus* (a thermophillic species) and *D.vulgaris*. The habitats for these organisms include soil and freshwaters; however it is in sea water and predominantly in marine sediments where the bulk of *Desulfovibrio spp.* occur and this is a reflection of their salt tolerance (freshwaters to saturation) and utilisation of sulphate as electron acceptor in energy conservation (see Postgate, 1979).

The use of sulphate as an inorganic terminal electron acceptor, proceeds according to the equation:

$$SO_4^{2-} + 4H_2O + H^+ \rightarrow HS^- + 4H_2O \qquad \text{(Thauer } et\ al\text{, 1977)}$$

Prior to its use as an electron acceptor, sulphate must first be activated to a high-energy organic sulphur compound adenosine phosphosulphate (APS) in an energy consuming enzymic reaction (Peck, 1962). APS is reduced to sulphite (SO_3^{2-}) which is in turn reduced to sulphide by the action of several sulphite reductases (sometimes known as bisulphite reductases as SO_3^{2-} is usually present as HSO_3^- at cellular pH; see Peck, 1962; Postgate, 1979; Jones C,W., 1982). The major reductases are genera-specific with the diagnostic pigment desulfoviridin (probably the dissimilatory sulphite reductase; Postgate, 1979) being present in all *Desulfovibrio spp.* except Norway 4

(desulforubidin) and pigment P_{582} in *Desulfotomaculum spp.* In addition to sulphate several other sulphur compounds can act as electron acceptors for the anaerobic respiration of *Desulfovibrio spp.* These tend to be intermediaries involved in the reduction of sulphate to sulphide and include sulphite (SO_3^{2-}), metabisulphite ($S_2O_5^{2-}$), dithionite ($S_2O_4^{2-}$) and thiosulphate ($S_2O_3^{2-}$). Hata (1960) in an extensive survey of potential electron acceptors for a marine sulphate reducing bacterium reported similar results. However, these compounds are generally found at low concentrations from 0 to 0.5mM in marine sediments (Novitsky *et al*, 1981) and due to the abundance of sulphate in the marine environment it is unlikely that they are used as terminal electron acceptors, although they may play a role in Eh poising (Boulègue and Michard, 1979).

The range of electron donors that can be used by *Desulfovibrio spp.* as sources of reducing power for sulphate reduction tends to be narrow and is confined mainly to simple C_3- and C_4- substituted fatty acids, glycerol and primary alcohols (Postgate, 1979). Reports of growth substrates are scattered and often contradictory and reflect the use of impure cultures in the past which gave a false wide range of potential electron donors. These historical anomalies are discussed at length in Postgate's monograph (1979) and highlight the difficulties in cultivating these strictly anaerobic organisms. Members of the genus *Desulfovibrio* are also capable of mixotrophic growth in which an inorganic or non-assimilable organic molecule provides the reducing power for sulphate reduction, whilst a different compound acts as a source of cell carbon. This type of growth involves the use of mixotrophic or "incomplete" substrates which,unlike "complete" substrates,

provide energy only and not energy and cell carbon. Many of the electron donors used by *Desulfovibrio spp.* are borderline between the two and this has further complicated the picture of their nutrition.

Complete substrates utilized by the genus are usually incompletely oxidised to acetate and CO_2 and include lactate, pyruvate, glycerol, fumarate, malate, succinate, and in some strains glucose and amino acids (Smith and Klug, 1981). Compounds such as ethanol, propanol and butanol are oxidised to the homologous fatty acids; whilst methanol, formate, oxalate and oxamate, if utilized, are metabolized to CO_2. However, growth yields on these alcohols, C_1-compounds and C_2- derivatives are low and it is thought that many of these substrates are on the borderline of being mixotrophic. Two established mixotrophic substrates are isobutanol (see Mechalas and Rittenberg, 1960) and molecular hydrogen.

Sulphate reducing bacteria are capable of scavenging H_2 (a product of anaerobic fermentations, see below) from the sediment porewater and using it as an electron donor for dissimilatory sulphate reduction by means of the enzyme hydrogenase. This enzyme is present in most sulphate reducers (Postgate, 1965) and is located peripherally in some species (Bell *et al*, 1974) which will facilitate H_2 uptake from the environment. There is now firm evidence that it is a competition for a common electron donor (H_2) which is the cause of the observed spatial separation between sulphate reducing and methanogenic bacteria in marine sediments (see Abram and Nedwell, 1978a,b). As the electron acceptor sulphate is only rarely limiting for sulphate reducing bacteria in marine sediments, sulphate reduction is

generally the predominant pathway of anaerobic decomposition. However, under conditions of low sulphate concentration this situation can be reversed (Mountfort *et al*, 1980). This can occur deep in marine sediments or within freshwater sediments, where methanogenesis is capable of mineralizing 25% of the available carbon (Jones and Simon, 1981).

The growth of *Desulfovibrio spp.* on carbohydrates is a contentious issue at the present moment, although there have been reports of glucose, lactose, sucrose, fructose and maltose supporting low levels of growth in *D.desulfuricans* (MacPherson and Miller, 1963). However, the low growth obtained on such substrates may have been due to media impurities. A wide ranging assessment of possible electron donors for sulphate reducing bacteria from a thermodynamic aspect has been published by Wake and co-workers in Australia (1977). This study agreed with the previously reported growth substrates for sulphate reducing bacteria and speculated that co-metabolism of unavailable compounds such as hydrocarbons by other sedimentary bacteria could enable them to be used as electron donors for sulphate reduction. As sulphate reducing bacteria in marine sediments are dependent on heterotrophic bacteria for the degradation of complex macromolecules to lower molecular weight forms (Goldhaber and Kaplan, 1974), this process is potentially of great importance to the activity of sulphate reducers *in situ*.

Work by Kimata *et al* (1955e) on sulphate reducing bacteria isolated from Hiroshima Bay has demonstrated that in pure culture only alanine, formate, glycerol, lactate and pyruvate supported sulphate reduction, whilst crude enrichments of sulphate reducers and heterotrophic bacteria utilized over 30 compounds

including cellobiose, glucose, starch, alginic acid and a range of amino acids. A later laboratory study by Tezuka (1966) has demonstrated a syntrophy between a glucose metabolizing fermentative bacterium which supplied lactate to a sulphate reducer.

The electron donors for dissimilatory sulphate reduction in marine sediments are generated and replenished by two main pathways. Sediment invertebrates can release a range of low molecular weight material into the sediment by secretion through the epidermis and excretion (Fry, 1982). However, little detail is known on this contribution to electron donor levels and it is thought that this contribution is often small, with many organisms being in fact net utilizers of such low molecular weight compounds (Miller *et al*, 1979).

The most important source of growth substrates for sedimentary sulphate reduction is the bacterial degradation of organic detritus such as plant material, macrofaunal faecal pellets and carcases. The dependance of the activity of sulphate reducing bacteria on sedimentary heterotrophic bacteria has been reported by several workers (Sorokin, 1962; Vacelet, 1971; Nedwell and Floodgate, 1972). In many marine sediments, especially those inshore ones which are in proximity to land (*e.g.* sea-lochs), the main detrital input into the sediment will be plant polysaccharides such as cellulose. Loch Eil which was polluted by effluent from a pulp and paper mill received particularly high levels of cellulose as the effluent accounted for 84% of the total carbon input (Pearson, 1982) and was 98% cellulose (Vance *et al*, 1979).

Within the sedimentary environment cellulose is depolymerised

by cellulolytic bacteria to yield glucose and the disaccharide, cellobiose (Doelle, 1981). The process can occur either aerobically (*Cellvibrio spp.*, *Cytophaga spp.*, *Sporocytophaga spp.*) or anaerobically by *Bacillus spp.* and clostridia (Rodina, 1972). The mono- and disaccharides formed from polysaccharide oxidation are further degraded within the by now anoxic sediment by bacterial fermentations which are capable of utilizing most carbohydrates (Stanier *et al*, 1977). The carbohydrates are metabolized *via* glucose, with the products of this metabolism replacing O_2 as the terminal hydrogen acceptor in a series of balanced oxidation-reduction reactions which end with the reoxidation of the reduced pyridine nucleotide ($NADH_2$) and the formation of fermentation products. These end-products are often characteristic of the bacterial groups involved, although environmental conditions are also important in determining the end-products formed.

Lactate is a common end-product of microbial fermentation and is produced by many bacteria, with the lactic acid bacteria yielding it as their major end product. Anaerobic clostridia can produce ethanol, succinate, acetate, hydrogen and CO_2 by carbohydrate fermentation depending on the species involved. Likewise, enteric bacteria can yield lactate, succinate, formate, ethanol, acetate, hydrogen and CO_2 as their principal fermentation end-products depending on the strain of bacterium involved in the fermentation (see Mandelstam and McQuillen, 1973; Doelle, 1981).

Many of the above organisms can dispose of "excess" electrons in the form of molecular hydrogen. Under the conditions of low H_2 tension that exist in many marine sediments the

fermenting heterotrophic bacterium can reoxidise its $NADH_2$ by H_2 transfer to a H_2-scavenging sulphate reducer as already mentioned. As $NADH_2$ oxidation occurs in the terminal stages of the fermentation pathway, the end-products are shifted from reduced compounds such as lactate and succinate to yield more acetate and H_2.

Data on the electron donors used by members of the genus *Desulfovibrio in situ* is lacking at the time of writing. As already mentioned H_2 has been shown to be a natural electron donor and accounted for up to 10% of the sulphate reducing activity in a shallow, anaerobic coastal sediment (Sørensen *et al*, 1981). It is thought that pyruvate is not excreted into the sediment by heterotrophic bacteria and is therefore unlikely to be a naturally available electron donor (Nedwell, 1982). Lactate has been shown to stimulate sulphate reducing activity in marine sediments (*e.g.* Sorokin, 1962). The technique of stimulating sedimentary sulphate reduction by carbon source amendment however suffers from the problem that it is difficult to assess whether any measured stimulation has occurred *via* a compound derived from the added substrate or by the substrate *per se* (*e.g.* see Blake and Leftley's paper, 1977). One method to assess the ability of sedimentary sulphate reducing bacteria to utilise various electron donors is by pure culture work on isolated strains (Blake *et al*, 1982).

The few reports published on naturally occurring marine sulphate reducing bacteria tend to be limited to substrates of diagnostic importance. For instance, a survey of 41 sulphate reducers from various marine environments by Trüper *et al*, (1969) only investigated the ability of the strains to grow on

malate and formate in the presence of sulphate, and choline and pyruvate in its absence. Growth rate and sulphate reduction studies on lactate for a salt-tolerant *Desulfovibrio sp.* isolated from San Francisco Bay have been reported in the literature (Leban *et al*, 1960), as have respiration and fermentation data for a *D.desulfuricans* strain from the Dutch Wadden Sea growing on lactate or pyruvate (Vosjan, 1975). However, in each of these papers the range of growth substrates given is very small. A wider range of electron donors for sulphate reduction and other physiological data has been reported for a marine sulphate reducer from Hiroshima Bay in Japan by Kimata *et al* (1955b,c,d,e). However, the strain used was not identified and must be considered atypical as it was incapable of growth at 15.5°C (the temperature of the overlying water) and could not utilize ammonia (NH_4^+) as a sole inorganic nitrogen source - the preferred nitrogen source of the majority of sulphate reducing bacteria (Postgate, 1979).

The aims of the second section of this study were to investigate the activities of the sulphate reducing bacteria in a range of marine sediments from low carbon, aerobic abyssal to polluted, reduced fjordic. In addition the range and preferance of electron donors for growth by naturally occurring sulphate reducing bacteria were determined.

The study was conducted in four ways:

i. Sedimentary sulphate reduction rate was measured by a $^{35}SO_4^{2-}$ radiotracer technique,with sulphate reducing bacteria being enumerated in a lactate based medium by a most probable number method and heterotrophic nitrogen fixation by the acetylene reduction assay. The values obtained from the various sediments

were compared and at several sampling stations profiles of these measurements with sediment depth were constructed and compared.

ii. The electron donors utilized by crude enrichments of sulphate reducing bacteria from the surface sediment of two sea-lochs and three N.E. Atlantic stations were determined.

iii. Lactate oxidising organisms from the area of highest sulphate reducing activity (Loch Eil E-70 surface sediment) were isolated, identified and their electron donor range determined.

iv. A representative of the strains isolated in iii. was studied to determine its nutritional characteristics and its rate of growth and sulphate reduction on various electron donors.

MATERIALS AND METHODS

SAMPLE COLLECTION

Undisturbed sediment samples from the three N.E.Atlantic sampling stations (A-1, A-2 and A-3) were collected using the S.M.B.A. replicate corer operated from R.R.S. "Challenger" as described previously in section one. Sediment samples from sampling station LY-1 in the Lynn of Lorne were obtained by means of a Craib corer operated from R.V. "Calanus", also as described previously. Samples of sediment from the near shore sampling stations in Loch Creran, Loch Eil, the inner basin of Sullom Voe and Colla Firth were collected using a Sholkovitz gravity corer operated from R.V. "Calanus". (Plate 3). The use of 100 cm length by 10 cm diameter acrylic plastic core tubes enabled sediment cores of the upper 60 to 80 cm of the sediment to be obtained, depending on the sampling station. On removal from the gravity corer the core tubes were sealed with rubber bungs. Sediment samples from the N.E.Atlantic, Sullom Voe and Colla Firth were processed immediately onboard ship, whilst samples from the near shore stations were stored in crushed ice until landfall at the laboratory - usually an interval of less than ten hours.

SEDIMENT HANDLING

As sulphate reduction and heterotrophic nitrogen fixation are essentially anaerobic processes the exposure of anaerobic sediment samples to the atmosphere was kept to a minimum. Sediment manipulations therefore, whenever possible, were performed under an anaerobic atmosphere within a nitrogen filled glove bag (Plate 4). The glove bag was of a design essentially

Plate 3. Sholkovitz gravity corer on recovery.

Plate 4. Nitrogen filled glove bag.

similar to that of Leftley and Vance (1979) and consisted of a PVC gnotobiotic canopy (Vickers Medical Engineering, Basingstoke, Hants) with rubber gloves attached to the frosted PVC sleeves *via* a metal collar and "O"-ring. The open end of the canopy (on the right hand side of plate 4) was used for cleaning and for the introduction of large pieces of equipment into the glove bag, and during use was sealed by means of a wooden clamp and by folding the canopy over itself several times. To facilitate the transfer of materials between the atmosphere and the glove bag, an acrylic airlock was fitted into the canopy (Plate 5), with inner and outer doors sealed by means of rubber "O"-rings and two metal clamps each. Prior to use the glove bag was evacuated by means of an electric vacuum pump and filled with oxygen-free nitrogen passed through a "Supelco" carrier gas purifier (Supelco, Inc.) to remove any residual oxygen to levels <1 ppm (up to 10 ppm residual oxygen being present in "oxygen-free" nitrogen). This process was repeated three times to ensure anaerobiosis within the glove bag. (All tubing for the gas lines was butyl rubber which is impermeable to oxygen). The air-lock was likewise twice evacuated to a vacuum of 15 mm Hg and filled with oxygen-free nitrogen by means of the valve system shown in plate 5 when in use.

On landfall at the laboratory the overlying water from sediment samples was siphoned off as described previously and the sediments rapidly sectioned at 5 cm intervals using a graduated tube of the same internal diameter as the core tube. The sectioned sediments were placed in the airlock for two evacuation-flushing cycles and then transferred to the glove bag for homogenization by gentle stirring. All further

Plate 5. Side-view of glove bag showing airlock and gas/vacuum supply.

sediment manipulations (*e.g.* sediment dilutions, inoculations, etc.) were performed within the glove bag-bottles and reaction flasks only being removed when tighty sealed. It was assumed that during the brief period of sectioning there would be enough buffer capacity, with respect to sediment sulphide, to protect anaerobic bacteria from any exposure to the atmosphere (Leftley and Vance, 1979).

Sediment samples from Sullom Voe and Colla Firth could not be handled in the glove bag as space precluded its use onboard R.V. "Calanus". The cores were therefore sectioned at 5 cm intervals as above but placed into nitrogen filled polythene bags in which they were homogenized by gentle squeezing of the bags under a stream of nitrogen. Further sediment manipulations were performed using the Hungate technique (Hungate, 1969) in which the sediment is handled under a stream of oxygen-free gas (such as carbon dioxide, hydrogen or in this case nitrogen) which displaces the air from the culture bottle or reaction flask. Again sulphide was assumed to provide some protection against any effects of oxygen. As the N.E.Atlantic sediments were initially aerobic they were sectioned at 5 cm intervals and homogenized as described in section one. However, all future manipulations were carried out under a nitrogen gas stream to preserve the reduced conditions of culture media and reaction flasks.

ENUMERATION OF SULPHATE REDUCING BACTERIA

10 ml of homogenized sediment was added to 90 ml sterile diluent by means of a sterile, truncated 5 ml syringe to give

a 10^{-1} dilution. The diluent contained 0.4 M NaCl, 0.1 g $litre^{-1}$ ascorbic acid and sodium thioglycollate as reducing agents and 0.0002% w/v resazurin as a redox indicator. The diluent was distributed as 90 ml lots into medical flat bottles and sterilised by autoclaving for 20 minutes at 103.4 kPa. As soon as the autoclave pressure fell to atmospheric the bottles were tightly sealed to ensure reduced conditions within the diluent. Any oxidised bottles, indicated by a pink coloration from the resazurin, were discarded. Further ten-fold dilutions down to 10^{-6} were made from this 10^{-1} dilution by means of a 1-5 ml Finnpipette (A. and J. Beveridge, Edinburgh) fitted with sterile polypropylene tips, with each dilution being well shaken by hand. 1 ml aliquots from each dilution were inoculated, by means of a 200-1000 µl Finnpipette fitted with sterile tips, into quintiplicate universal bottles containing Postgate's medium B (Postgate, 1979):-

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$CaSO_4$	1.0 g
$MgSO_4.7H_2O$	2.0 g
Sodium lactate (70% w/w aqueous solution)	3.6 ml
NaCl	23.4 g
Yeast extract	1.0 g
Ascorbic acid	0.1 g
Sodium thioglycollate	0.1 g
$FeSO_4.7H_2O$	0.5 g
Resazurin (0.1% w/v aqueous solution)	2 ml
Tap water	1 litre

The pH of the medium was adjusted to 7.5 and the medium distributed into universal bottles, with autoclaving and sealing as described for the diluent. After sealing tightly the inoculated bottles were incubated for upto four weeks in the dark at 30°C - it having previously been noted that there appeared to be no psychrophillic sulphate reducing bacteria in the sediments examined, the only effect of incubation at 4°C being very slow growth. Growth was indicated by blackening of the medium due to the production of ferrous sulphide *via* sulphide production. Black bottles were counted as positives and the most probable number of sulphate reducers in the sediment sample estimated from the statistical tables of Alexander (1965).

ESTIMATION OF SULPHATE REDUCTION RATES

The method used for the estimation of sulphate reduction rate in the sediments examined was in essence that of Vosjan (1974). Special reaction flasks were used which consisted of "Pyrex" 50 ml conical flasks (Corning Ltd., Stone, Staffordshire) modified by the replacement of the neck by a "Sovirel" SVL 22 screw thread (V.A. Howe and Co. Ltd., London). The flasks were sealed by means of a "Sovirel" screw cap and a "Teflon"-silicone rubber disc (Pierce and Warriner U.K. Ltd., Chester, Cheshire) - the "Teflon" preventing adsorption of sulphide to the sealing disc, whilst the silicone rubber had good resealing capabilities after puncture. 5 ml aliquots of homogenized sediment were added by means of a truncated 5 ml syringe to triplicate flasks containing 10 ml deoxygenated 0.4 M NaCl and 0.01% w/v sodium thioglycollate to retain a negative redox potential. The

flasks were tightly sealed ensuring that the "Teflon" side of the sealing disc was lowermost. Triplicate flasks from each sample were held at 80°C for 20 minutes as controls. Carrier free $^{35}SO_4^{2-}$ (Amersham International, Amersham, Buckinghamshire) was then injected into each flask in amounts of between 1 to 20 µCi depending on the suspected activity of the sediments - more label being added to aerobic NE.Atlantic sediments than to anoxic near shore sediments. The contents of the flasks were then mixed using a vortex mixer prior to incubation for 24 hours in an orbital shaker incubator at *in situ* temperature (4°C N.E.Atlantic samples, 10°C sea-loch samples) in the dark. The incubations were terminated by the injection of 5 ml 0.75 M cadmium acetate into the flasks which fixed any sulphides present as cadmium sulphide. The sediment slurries were then vortex mixed and stored at -20°C until analysed.

Although the estimation of sediment sulphate reduction rates by such a slurry method has been widely used over the past two decades (*e.g.* Sorokin, 1962; Vosjan, 1974; Oremland and Taylor, 1979; Winfrey *et al*, 1981), the method does have two disadvantages when compared to the other widely used method - the ^{35}S-SO_4^{2-} core-injection method of Jørgensen (1978a). The sediment dilution can decrease the measured sulphate reduction rate, whilst in surface sediments the slurry method can result in an overestimation of the true level of activity (although the core injection method may give a slight under-estimation - Jørgensen, 1978a). However, the slurry method of estimating sulphate reducing activity in marine sediments required fewer replicates than the core-injection method because

of a smaller statistical variation due to bacterial heterogenecity (Jørgensen, 1978a). In addition, the method was less affected by bioturbation which occurs in the sediments studied (Stanley *et al*, 1978) and was the only method capable of estimating sulphate reducing activity in the very fluid surface sediment found at sampling station CR-1 in Loch Creran.

For the estimation of $^{35}S^{2-}$ the reaction flasks whilst still frozen, were attached *via* their screw-threads to a custom-built distillation apparatus (plate 6). The design was basically that of Jørgensen and Fenchel (1974) with the S^{2-} from the reaction flask being flushed by an oxygen-free gas through a pair of sulphide traps. The apparatus was constructed from "Sovirel" glassware with any joints being made by means of PTFE gaskets and "Sovirel" sealing rings. Tubing throughout was 6 mm diameter borosilicate tubing with butyl rubber tubing being used for the nitrogen supply. The contents of the flasks were mixed using a heater-magnetic stirrer.

The frozen sediment slurries were thawed under a stream of oxygen-free nitrogen carrier gas further purified to an oxygen content of <1 ppm O_2 by passage through a "Nilox" gas scrubber (Jencons Ltd., Hemel Hempstead). The trapped sulphide was liberated by injection of 10 ml deoxygenated 6 M HCl through side arms in the apparatus with heating at 80°C for 1 hour and continuous stirring. This lowering of the slurry pH to below 1 and heating has been shown to liberate both the free, uncombined and the acid volatile sulphide (Nedwell and Floodgate, 1972; Jørgensen and Fenchel, 1974). Liberated sulphide was flushed from the reaction flasks by the N_2 carrier gas and trapped in 10 ml 1.5 M NaOH with a second trap of 20 ml 0.2 M

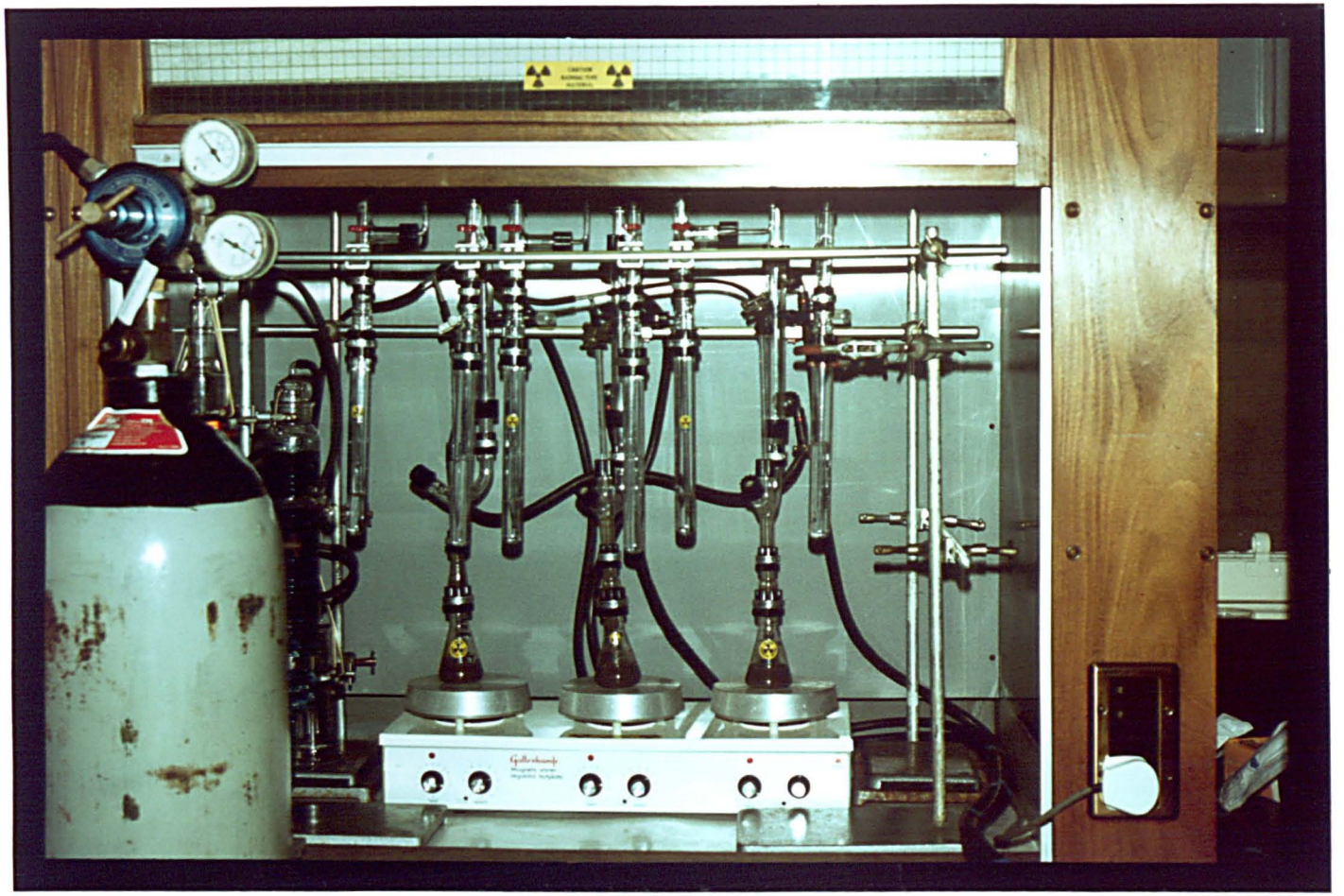


Plate 6. ^{35}S-Sulphide distillation apparatus.

cadmium acetate to detect any carry over of sulphide. No carry over was ever detected and distillations of known Na_2S standard solutions, together with iodine-thiosulphate titrations (Mosey and Jago, 1977) of any sulphide trapped, indicated that the trapping efficiency of the apparatus was 95%, which compared favourably with efficiencies obtained by other workers (*e.g.* Jørgensen and Fenchel, 1974; Jones and Simon, 1981). The radioactivity of the sulphide trapped was measured by counting 1.0 ml samples from the NaOH traps in 10 ml "Dimilume-30" (Packard Instrument Ltd., Caversham, Berks) in a Packard Tri-Carb liquid scintillation spectrometer. Quench corrections were performed using the internal standard method (Horrocks, 1974) and a [^{35}S] dioctyl sulphide standard (Amersham International, Amersham, Bucks.), which gave a counting efficiency of 84%. Although no recounting of the sample was possible using this system, the internal standard method did have the advantages of being rapid, unaffected by different types of quenchers and measured the counting efficiency in the actual samples (Horrocks, 1974). In addition, the method is widely used for quench correction and is the most accurate method of quenching determination (Peng, 1977). Background corrections were also performed.

Although the addition of alkali to many scintillation cocktails can result in chemiluminescence (Horrocks, 1974; Peng, 1977; Gibbs *et al*, 1978) a survey of propriatory cocktails showed that "Dimilume-30" when used as directed (Anon, 1976 Packard liquid scintillation counting application notes no.2/76) inhibited chemiluminescence to background levels in under one hour. Chemiluminescence was detected for by a

decrease in a second repeat count of a sample after an appropriate interval (Gibbs *et al*, 1978). The use of lead acetate papers in the scintillation vial caps indicated that no sulphide was released from the scintillation cocktail during counting.

The use of 1.5M NaOH as the sulphide trapping agent has been used in other ecological studies (*e.g.* Langstone Harbour study, 1975) and eliminated the time-consuming filtration step of many previous methods (*e.g.* Sorokin, 1962; Ivanov, 1968; Nedwell and Abram, 1978). In addition, the trapping of $^{35}S^{2-}$ in NaOH, coupled with the use of "Dimilume-30", enabled a homogeneous radioactive sample to be counted. This is one of the most ideal conditions for radioactive measurement (Peng, 1977; Gibbs *et al*, 1978) and eliminated the problems which can arise when a heterogeneous system is counted - *e.g.* ZnS counted as an emulsion in a gelling scintillation liquid (*e.g.* Jørgensen, 1978a) or CdS counted as a precipitate on a solid support such as a membrane filter (*e.g.* Ivanov, 1968). These considerations are further discussed by Peng (1977) and Gibbs *et al* (1978).

The rate of sulphate reduction in the sediment sample was calculated from the following equation derived from that of Sorokin (1962):

$$\text{Rate} = \frac{[SO_4^{2-}] \,.\, {}^{35}S^{2-} \,.\, 1.06}{{}^{35}SO_4^{2-} \,.\, t} \quad \text{n mol } SO_4^{2-} \text{ ml}^{-1} \text{d}^{-1}$$

where $[SO_4^{2-}]$ was the sulphate concentration of the sediment in n mol ml^{-1}, $^{35}S^{2-}$ was the radioactivity of the sulphide in µCi corrected for trapping efficiency, dilution, background

and quenching, 1.06 was a correction factor for the microbial fractionation of the ^{32}S and ^{35}S isotopes, $^{35}SO_4^{2-}$ was the radioactivity of the sulphate in μCi and t was the period of incubation in days. Porewater sulphate concentrations in the cores examined were determined by co-workers in the S.M.B.A. geochemical cycling group by either $BaSO_4$ precipitation or by complexometric titration as detailed in the appendix (tables 4.2 to 4.6).

ESTIMATION OF HETEROTROPHIC NITROGEN FIXATION (ACETYLENE REDUCTION) RATES.

The method employed in this study was similar to that of Blake and Leftley (1977). 5 ml aliquots of homogenized sediment were added to duplicate sterile universal bottles by means of a truncated 5 ml syringe and sealed with sterile "Suba-seals" (W. Freeman and Co.Ltd., Barnsley, Yorks). Control bottles were prepared by either injecting 0.1 ml of 1% w/v $HgCl_2$ into duplicate bottles from each sediment section, vortex mixing and heating at 80°C for 20 minutes or by the absence of acetylene. The surface area of the sediment was increased by coating the sides of the bottle by vortex mixing with the bottle held almost horizontally. The bottles were then evacuated to 740 mmHg and observed for leaks before being filled with oxygen free argon. This process was repeated three times before the bottles were finally gassed out with argon for approximately 60 volume changes. The internal pressure of each bottle was then equalised to atmospheric before being made hyperbaric by the injection of 3 ml argon. This was done,

as several samples would be removed from each bottle. 1 ml of acetylene was then injected into each bottle, the bottle agitated and 0.6 ml of gas removed to give the zero time sample. The bottles were incubated in the dark at *in situ* temperature (10°C) and samples removed at time intervals for analysis. Sampling syringes were flushed with argon prior to use and samples stored prior to analysis by sticking the syringe into an inverted rubber bung. No detectable loss of sample was encountered over several hours storage in this way. The gas samples were analysed by gas chromatography using a Pye Unicam 204 gas chromatograph. Separation of gases was carried out on a 900 mm long by 2 mm diameter glass column packed with Porapak T held at a temperature of 90°C. Porapak T gave a better separation of acetylene and ethylene than Porapak R which has been used in several published studies (*e.g.* Macgregor, *et al*, 1973; Werner *et al*, 1974; Knowles *et al*, 1974; Marsho *et al*, 1975). Although Porapak T showed "tailing" for acetylene compared with a sharp peak with Porapak R the high amplifier gain necessary for this assay produced noticeable "tailing" with "R" and so favoured the use of "T". Nitrogen was used as a carrier gas at a flow rate of 40 ml min^{-1} with a hydrogen flow rate of 40 ml min^{-1} and an air flow rate of 550 ml min^{-1}. The injector heater was at 100°C and the detector heater at 125°C. The sample volume was 0.5 ml with the separated gases being measured by a hydrogen flame ionization detector. Approximate retention times using this system were ethylene 18 seconds and acetylene 30 seconds. Acetylene and ethylene peaks were integrated on a programmable computing integrator (Supergrator-2, Columbia

Scientific Industries) calibrated against known standards by the method of Postgate (1972). At the end of the sampling period each bottle was weighed with wet sediment, dry sediment (105°C, 24 hours), empty and filled with water to yield the volume of the gas phase in each bottle. The total amount of ethylene in the bottles at each time interval could therefore be calculated (assuming most of the ethylene was in the gas phase) and a time course plotted of ethylene production. The gradient of the linear part of the graph yielded the acetylene reduction rate expressed as n mol C_2H_4 produced g dry wt.$^{-1}$ hr^{-1}.

ENRICHMENTS OF SULPHATE REDUCING BACTERIA ON DIFFERENT ELECTRON DONORS

2 ml aliquots of homogenized 0-5 cm sediment were inoculated using a truncated 5 ml syringe into Postgate's medium B modified by the replacement of sodium lactate by sodium acetate, sodium benzoate, butanol, sodium butyrate, ethanol, sodium formate glucose, glycerol, sodium lactate,propanol, sodium propionate, sodium pyruvate, sodium succinate or valeric acid from sterile 10% w/v stock solutions to give a final concentration of 0.1% w/v. Basal medium only blanks were inoculated, all the bottles completely filled and sealed tightly. The use of hydrogen as an electron donor was investigated by incubating inoculated basal medium bottles under a H_2/CO_2 atmosphere generated from a GasPak in the presence of a palladium catalyst within an anaerobic jar. All samples were incubated at 30°C for upto four weeks with growth being indicated by blackening of the medium as compared with a basal medium blank. Positive enrichments were subcultured at least three times before being

recorded as growth of sulphate reducing bacteria occurring on that electron donor.

ISOLATION OF SULPHATE REDUCING BACTERIA

Isolates of sulphate reducing bacteria were obtained from Loch Eil E-70 0-5 cm lactate enrichments by means of an anaerobic roll-tube method (Hungate, 1969). 25 ml "Astell" roll tube bottles (Astell Hearson, Catford, London) containing 4 ml aliquots of anaerobic media under an atmosphere of oxygen-free nitrogen (further purified by passage over copper turnings heated to 350°C) were prepared using standard anaerobic handling techniques (see Hungate, 1969). The medium employed was medium E of Postgate (1979):

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	1.0 g
$CaCl_2 . 6H_2O$	1.0 g
$MgCl_2 . 6H_2O$	2.0 g
Sodium lactate (70% w/w aqueous solution)	3.6 ml
NaCl	23.4 g
Yeast extract	1.0 g
Ascorbic acid	0.1 g
Sodium thioglycollate	0.1 g
$FeSO_4 . 7H_2O$	0.5 g
Agar (Oxoid No.1)	7.5 g
Resazurin (0.1% w/v aqueous solution)	2 ml
Tap water (distilled for growth studies)	1 litre

The pH of the medium was adjusted to pH 7.6 using NaOH.

The bottles were autoclaved with their butyl rubber stoppers clamped down for 15 minutes at 103.4kPa and then held at 45°C. 0.5 ml of enrichment was then injected into one bottle, the contents mixed and 0.5 ml from this bottle inoculated into a second bottle, usually upto a maximum of nine bottles. After inoculation the bottles were placed on an "Astell" water cooled, roll tube spinner so as to coat the inside surface of each bottle with a thin layer of agar. The bottles were incubated at 30°C until discrete black colonies were visible in the agar layer. Well separated colonies were picked from the agar by means of sterile Pasteur pipettes with the capillary bent at right angles and mouth suction. The manipulations were performed under a dissection microscope with the picked colonies being rapidly introduced into universal bottles of Postgate's medium B. Cultures showing growth after incubation at 30°C were tested for purity using the methods of Postgate (1979) with tryptone-soya agar (Oxoid Ltd.). Six pure cultures were selected for future work and maintained by fortnightly subculture in medium B, with the cultures being plated out onto tryptone-soya agar supplemented with 0.4M NaCl on each handling to detect any aerobic contaminants.

IDENTIFICATION OF SULPHATE REDUCING BACTERIA ISOLATES

The isolates were identified using the test characters and working classification scheme of Postgate and Campbell (1966) and Postgate (1979). Morphology was compared using young cultures (2 to 3 days) in medium B at 30°C and phase-contrast microscopy of wet preparations. Sporulation was detected for by the ability of cells to grow after exposure

to 80°C for 5 minutes, the presence of desulfoviridin by a red fluorescence of centrifuged alkaline (2M NaOH) culture under ultraviolet light (365 nm) and the utilization of substrates was as described overleaf. Sulphate-free growth on choline or pyruvate was tested for in medium D of Postgate (1979) modified by the addition of ascorbate and thioglycollate as reducing agents:

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
$CaCl_2.2H_2O$	0.1 g
$MgCl_2.6H_2O$	1.6 g
Yeast extract	1.0 g
$FeSO_4.7H_2O$	0.004 g
Choline chloride/sodium pyruvate	1.0 g
NaCl	23.4 g
Ascorbic acid	0.1 g
Sodium thioglycollate	0.1 g
Resazurin (0.1% w/v aqueous solution)	2 ml
Distilled water	1 litre

The pH was 7.5. An increase in turbidity compared with a choline/pyruvate-free inoculated blank which was sustained after three subcultures was recorded as positive. Salt requirement was tested for using concentrations of 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% w/v NaCl in medium B (strains with a salt requirement >1% w/v being recorded as positive). Whilst thermophily was tested for by incubation of cultures in medium B at 60°C, with any positives being subcultured at least once.

GROWTH OF ISOLATES ON DIFFERENT ELECTRON DONORS

1 ml aliquots of the isolates were inoculated into medium B modified by the use of distilled water instead of tap water, the replacement of sodium lactate by sodium acetate, sodium benzoate, butanol, sodium butyrate, ethanol, sodium formate, glucose, sodium glutamate, glycerol, sodium lactate, malic acid, propanol, sodium propionate, sodium pyruvate, sodium succinate or valeric acid at final concentrations of 50mM and by the reduction of the yeast extract content to 0.001% w/v. 10 ml of a general trace elements solution (Ware and Postgate, 1971) were included which contained (in 1 litre distilled water): H_3BO_3 232 mg, $CoSO_4.7H_2O$ 95.6 mg, $CuSO_4.5H_2O$ 8.0 mg, $MnSO_4.4H_2O$ 8.0 mg, $Na_2MoO_4.2H_2O$ 30.0 mg, $ZnSO_4.7H_2O$ 174.0 mg and the final pH of the medium was 7.6. Basal medium only blanks were inoculated as before and growth on hydrogen was again investigated in gas jars under a H_2/CO_2 GasPak atmosphere. Bottles were incubated at 30°C for a period of up to four weeks with growth being determined by comparing the amount of blackening and turbidity in test bottles with that in inoculated blanks. Electron donors which supported growth over at least three subcultures were recorded as positives.

PRELIMINARY GROWTH STUDIES

As the rest of this work required the measurement of growth by direct counting or turbidometric means, it was desirable that a medium low in iron (Fe^{2+}) was used to avoid any interference from precipitated FeS. However, all six strains of sulphate reducing bacteria isolated from station E-70 in Loch Eil showed

only poor or no growth on the most widely used medium in such studies, namely medium C of Postgate (Postgate, 1979; see also Pankhurst, 1971). Experiments with a known growth medium (B) together with medium C (supplemented with 0.01% w/v ascorbate and thioglycollate as reducing agents) showed that this failure to grow was due to media composition and not to any biophysical factors such as failure of anaerobic technique. Therefore a series of preliminary growth experiments were performed in an attempt to determine the reason for the poor growth of the isolated strains on a recommended and widely used medium and if necessary, to develop a low Fe^{2+} medium suitable for growth studies. Isolate 19 was selected as a representative strain of the sulphate reducing flora encountered in E-70 surface sediment (see discussion) with Postgate's medium C as the reference low Fe^{2+} growth medium. The isolation medium (medium E of Postgate) was used in liquid form as a base for these preliminary growth studies and supported good growth of all six strains.

i. EFFECT OF REDUCING AGENTS ON GROWTH

Medium E was prepared with 30mM sodium lactate and sodium sulphate, with either 0.01% w/v ascorbic acid, 0.01% w/v sodium thioglycollate, both together or no reducing agents. 15 ml duplicates were dispensed into Pankhurst anaerobic tubes (Astell Hearson, London) prepared as described by Pankhurst (1971). The tubes were autoclaved at 103.4 kPa for 20 minutes and cooled under oxygen free nitrogen purified by passage over copper turnings heated to 350°C. The tubes were stoppered with sterile suba-seals and 0.5 ml aliquots of saturated aqueous

pyrogallol and an alkali solution (10% w/v NaOH + 15% w/v K_2CO_3) added to the side arms of the tubes prior to sealing (Postgate, 1972). The tubes were gassed out with nitrogen and left hyperbaric to allow for any absorption of oxygen by the alkaline pyrogallol. After a period of 12 hours to allow any traces of oxygen to be removed from the tubes, a filter sterilized solution of washed $Na_2S.9H_2O$ was injected into the duplicate tubes containing no reducing agents to give a final concentration of 5mM sulphide. The tubes were inoculated with 1.0 ml of a 48 hour culture of strain 19 by injection, the contents mixed and samples taken for cell counts by means of 1 ml syringes flushed with sterile nitrogen. Direct counts were performed using phase contrast microscopy and an improved "Neubauer" counting chamber. The precautions recommended by Norris and Powell (1961) were used to improve precision in conjunction with the statistical procedure of Cassell (1965) to improve the significance of the counts (both cited in Mallette, 1969). The tubes were incubated at 30°C until no further increase in growth was observed. At the end of growth direct cell counts were again performed and the final pH of the cultures recorded.

ii. Fe^{2+} REQUIREMENT

Medium E was prepared as above but with $FeSO_4.7H_2O$ concentrations from zero to 6.0mM Fe^{2+}. 15ml duplicates were dispensed into Pankhurst tubes and treated as described above. The inocula were 0.3 ml aliquots of a 48 hour culture of strain 19 and incubation was at 30°C. Initial and final cell counts were performed as before.

iii. EFFECT OF CITRATE ON GROWTH

Medium E was prepared in the usual manner but with 25 μM Fe^{2+} and with/without 1mM sodium citrate. 15 ml duplicates in Pankhurst tubes were treated as before and inoculated with 0.5 ml of 48 hour culture of strain 19. The tubes were incubated and counted as before.

iv. SULPHATE REQUIREMENT

Sodium sulphate concentrations from zero to 22mM were set up in medium E prepared with 30mM lactate, 25 μM $FeSO_4.7H_2O$ and 1mM citrate. Pankhurst tubes containing 15 ml of medium were prepared as above and duplicates inoculated with 0.5 ml of a 48 hour sulphate-limited culture of 19. Incubation and counting were as above.

v. MODIFIED MEDIUM E

The above experiments resulted in a modified version of Postgate's medium E which was low in Fe^{2+} and produced no FeS during the growth of sulphate reducing bacteria. The medium was as formulated previously with the following modifications - Na_2SO_4 concentration was 20mM, $FeSO_4.7H_2O$ was present at a lowered concentration of 25μM, 1mM sodium citrate was present as a chelating agent and the electron donor concentration (usually sodium lactate) was 30mM. The derivation of these medium concentrations is examined in the results and discussion sections.

MIXOTROPHIC GROWTH OF STRAIN 19

Modified medium E was prepared as above but with yeast

extract concentrations of 0, 0.01, 0.05, 0.1 and 0.2% w/v and no sodium lactate. 10 ml aliquots of media were distributed into Pankhurst tubes, autoclaved and cooled under nitrogen in the usual manner. After cooling butanol, ethanol, lactate or propanol were added to sets of tubes, from sterile pH 7.6 0.3M stock solutions to give a final concentration of 30mM. A set of tubes were left containing basal medium only. All the tubes were sealed and made anaerobic as described above. Duplicate tubes were inoculated with 0.5 ml of a 48 hour culture of 19 and incubated at 30°C until no further increase in turbidity was observed. Initial and final counts were determined by direct counting as before.

SOURCES OF CELL CARBON ONLY FOR THE GROWTH OF STRAIN 19 UNDER A H_2/CO_2 ATMOSPHERE

15 ml aliquots of modified medium E containing no yeast extract or lactate were autoclaved in Pankhurst tubes and cooled under nitrogen as described previously. From sterile pH 7.6 stock solutions tubes were set up which contained sodium acetate, sodium benzoate, sodium butyrate, glucose, sodium lactate, sodium propionate or valeric acid at concentrations of 0, 2.5, 5.0, 7.5 and 10mM, and yeast extract at concentrations of 0, 0.5, 1.0, 2.0 and 3.0% w/v. The tubes were sealed and reduced as previously described and inoculated with 0.3 ml of a 48 hour culture of 19. Incubation was at 30°C with initial and final counts as above. In addition the final sulphide concentration in the tubes was determined by injecting 2.5 ml of 0.2M cadmium acetate into each tube to fix any sulphide present (after the removal of culture by syringe for direct counting). The

sulphide was assayed using an iodine-thiosulphate titration method similar to that of Mosey and Jago (1977). The following were added sequentially to a 250 ml conical flask: 100 ml distilled H_2O, 1.5 g KI, 25.0 ml 3.3μM KIO_3 and 10 ml 10% v/v H_2SO_4. 10 ml of the culture were then added with the pipette tip below the surface of the liquid to prevent loss of H_2S and the mixture back titrated with 0.02M $Na_2S_2O_3.5H_2O$ using 1% w/v starch solution as an end-point indicator. The concentration of sulphide in the culture was calculated from the volume of thiosulphate with correction being made for the dilution caused by the cadmium acetate solution.

EFFECT OF Mg^{2+} CONCENTRATION ON THE GROWTH OF STRAIN 19

Modified medium E was prepared with $MgCl_2.6H_2O$ concentrations of 0, 0.25, 2.5, 5.0 and 10.0mM Mg^{2+}. 10 ml aliquots were dispensed into 20 ml reductase tubes, autoclaved at 103.4 kPa for 10 minutes until reduced (resazurin redox indicator colourless) and cooled under a stream of oxygen free nitrogen further purified by passage over heated copper turnings as before. The tubes were then sealed with "Astell" butyl rubber roll tube stoppers and autoclaved with the tops clamped down for 15 minutes at 103.4 kPa. The tubes were inoculated by injection of 0.5 ml of a 48 hour culture of strain 19 (1.5 x 10^8 bacteria ml^{-1}) and incubated at 30°C. Growth was followed by the increase in absorbance at 580nm measured by the insertion of tubes into a modified Cecil Instruments CE 404 colorimeter (Cecil Instruments, Cambridge) with an Ilford Bright Spectrum series filter 626. The tubes were read against

an uninoculated medium blank. No interference of turbitidy measurements by precipitated iron sulphide occurred with modified medium E. The measurement of growth in sulphate reducing bacteria by the increase in turbidity at 580 nm has been shown to be comparable with other growth determination methods, such as direct gravimetric estimation or ^{35}S-incorporation (Badziong *et al*, 1978), under such conditions of no iron sulphide precipitation. At the end of logarithmic growth the final population in each tube was estimated by direct counting in the usual manner. The maximum specific growth rate (μm) for each Mg^{2+} concentration was calculated from the steepest portion of a semilogarithmic plot of $\log_{10}$ absorbance against time using the formula:

$$\mu = 2.303 \frac{(\log_{10}X - \log_{10}Xo)}{(t - to)} \quad hr^{-1} \quad \text{(Koch, 1981)}$$

where $\log_{10}X$ and $\log_{10}Xo$ were the values of $\log_{10}$ absorbance at times t and to (hr).

EFFECT OF Mg^{2+} CONCENTRATION ON THE GROWTH OF *DESULFOVIBRIO DESULFURICANS* STRAINS IN POSTGATE'S MEDIUM C.

Postgate's medium C was prepared with $MgSO_4.7H_2O$ omitted and $MgCl_2.6H_2O$ concentrations of 0, 0.25, 2.5, 5.0 and 10.0 mM Mg^{2+}; 25 μM $FeSO_4.7H_2O$ and 0.01% w/v sodium thioglycollate and ascorbic acid as reducing agents:

KH_2PO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.84 g
$CaCl_2.6H_2O$	0.06 g

$MgCl_2.6H_2O$	see above
Sodium lactate (70% w/v aqueous solution)	3.5 ml.
Yeast extract	1.0.g
$FeSO_4.7H_2O$	0.007 g
Sodium citrate.$2H_2O$	0.3 g
Ascorbic acid	0.1 g
Sodium thioglycollate	0.1 g
NaCl	23.4 g
Resazurin (0.1% w/v aqueous solution)	2 ml
Distilled H_2O	1 litre

The pH of the medium was 7.6. 10 ml lots were dispersed into reductase tubes and treated as in the previous experiment. The tubes were inoculated with 0.5 ml aliquots of 48 hour cultures of three marine *D.desulfuricans* strains: Loch Eil strain 19 (see discussion), Norway 4 (National Collection of Industrial Bacteria 8310) and Hossegor (NCIB 8400). The two NCIB strains were obtained from the NCIB, Torry Research Station, MAFF, P.O.Box 31, 135 Abbey Road, Aberdeen as freeze-dried cultures and maintained on either medium B or C. The inoculated tubes were incubated at 30°C with growth being again followed by turbidimetry. At the end of logarithmic growth the total amount of sulphide present in each tube was determined in the usual manner whilst total growth of the strains was calculated by means of calibration curves of absorbance at 580 nm against dry cell weight (see below), using the formula:

$$\text{Total Growth} = x_{max} - x_o \qquad \text{(Monod, 1949)}$$

where x_o and x_{max} were the initial and maximum cell densities respectively. The maximum specific growth rate μm was determined as before.

CALIBRATION OF ABSORBANCE MEASUREMENTS

The turbidometric estimations of cell density were calibrated against dry cell weight using a method similar to that used by Khosrovi and Miller (1975). 500 ml cultures of strains Loch Eil 19, Norway 4 and Hossegor were grown up in medium C (30 mM lactate, 20 mM sulphate, 2.0 g litre^{-1} $MgCl_2$ $6H_2O$) and harvested during logarithmic growth (24 hours). 240 ml of each culture were centrifuged at 25,000 xg for 30 minutes at 15^{o}C and then combined into a pre-weighed tube with distilled water before further centrifugation at 25,000 xg for 20 minutes at 15^{o}C . The supernatant was decanted and the cells washed with 20 ml of distilled water prior to centrifugation at 30,000 xg for 30 minutes at 0^{o}C. The supernatant was again decanted and the cells dried *in vacuo* at 50^{o}C over NaOH until a constant weight was recorded. The remainder of the culture was used to prepare 1/10 to 8/10 dilutions in basal medium and the absorbance of these dilutions at 580 nm read against a medium blank. A calibration curve of absorbance against dry cell weight (mg litre^{-1}) was plotted for each of the three strains and yielded values of ΔA_{580} 0.1 corresponding to cell densities of 28.5 mg cells (Loch Eil 19), 34.8 mg cells (Norway 4) and 31.4 mg cells (Hossegor) dry weight litre^{-1}.

EFFECT OF Mg^{2+} CONCENTRATION ON THE SULPHATE-FREE GROWTH OF STRAIN 19 ON PYRUVATE

Medium C was prepared as described previously but with $MgCl_2.6H_2O$ concentrations of 0, 0.25, 2.5, 5.0 and 10.0 mM Mg^{2+}; no Na_2SO_4 or resazurin and 30 mM sodium pyruvate replacing

sodium lactate. The medium was distributed as 30 ml lots in universal bottles and sterilized as in previous experiments. The bottles were inoculated with 2 ml of a 48 hour culture of strain 19 and after being fully filled with media and sealed, were incubated at 30°C for six days. Growth was determined by the increase in absorbance at 580 nm when read against a medium blank and compared to an inoculated bottle at zero time. The readings obtained were converted into mg dry cells litre^{-1} using the aforementioned calibration curve.

EFFECT OF Mg^{2+} AS CHLORIDE OR SULPHATE, AND OF Mn^{2+} ON THE GROWTH OF STRAIN 19

Reductase tubes were set up as in previous experiments and contained 10 ml medium C with $MgCl_2.6H_2O$; $MgSO_4.7H_2O$ or $MnSO_4.4H_2O$ at concentrations of 0, 0.25 or 10.0 mM M^{2+}. The tubes were inoculated with 0.5 ml of a 48 hour culture of strain 19 and incubated at 30°C. Growth was followed by the increase in absorbance at 580 nm,with the total growth at the stationary phase being calculated by means of the calibration curve as detailed above.

GROWTH OF STRAIN 19 IN POSTGATE'S MEDIUM C IN THE PRESENCE OR ABSENCE OF CITRATE, AND WITH Mg^{2+} OR Ca^{2+} SUPPLEMENTATION.

Postgate's medium C was prepared as detailed by Postgate (1979) with 0.2 mM $MgSO_4.7H_2O$ as the Mg^{2+} source and with 1 mM citrate as listed, no citrate, 1 mM citrate + 10 mM $MgCl_2.6H_2O$ or 1 mM citrate + 10 mM $CaCl_2.6H_2O$. The medium was autoclaved as 30 ml lots in universal bottles in the usual manner. The bottles were inoculated with 1 ml aliquots of a 48 hour culture

of strain 19 and after filling and sealing were incubated at 30°C. The bottles were subcultured twice before the growth of cultures after 48 hours incubation was determined as described above.

GROWTH AND SULPHATE REDUCTION OF STRAIN 19 ON LACTATE, MALATE OR PYRUVATE

Medium C was prepared with the same composition as that in the absorbance calibration experiment but with 30 mM sodium lactate, malic acid or sodium pyruvate as the electron donor. 10 ml aliquots were dispensed into reductase tubes, reduced and sterilized as in previous experiments. Sets of ten tubes containing each substrate were inoculated with 1 ml of a 48 hour culture of strain 19 grown in the same medium and mixed. The absorbance of all the tubes at 580 nm was recorded and 2.5 ml of 0.2M cadmium acetate injected into one tube of each substrate to fix any sulphide present. These tubes represented the sulphide present at zero time which was determined using the previously detailed iodine-thiosulphate titration. The remaining tubes were incubated at 30°C with the absorbance of the tubes and the amount of sulphide present in sacrificed tubes from each substrate being recorded at noted time intervals (usually 3 hours) until the stationary phase of growth was reached. Growth was followed by a plot of the arithmetic mean of absorbance converted to dry cell weight against elapsed time in hours. The maximum specific growth rate μm for each substrate was calculated in the usual manner, whilst the maximum rate of sulphide production (sulphate reduction) was calculated from the gradient of the steepest portion of the

curve obtained by a plot of sulphide concentration (mmol l^{-1}) against time (hour). Sulphate is reduced quantitatively to sulphide during dissimilatory sulphate reduction by *Desulfovibrio spp.* (Leban *et al*, 1966; Wake, *et al*, 1977; Postgate, 1979) and so sulphide production is commonly used as a convenient means of examining sulphate reduction rate.

EFFECT OF NaCl CONCENTRATION ON THE GROWTH OF STRAIN 19.

Medium C was prepared as in the previous experiment with 30 mM lactate and NaCl concentrations of 0, 1.0, 3.0, 5.0 or 7.0% w/v. 10 ml lots of each media were dispensed into reductase tubes and treated in the usual manner. The tubes were inoculated with 0.5 ml of a 48 hour culture of 19, with growth on incubation at 30°C being measured turbidometrically as in previous experiments. The maximum specific growth rate for each NaCl regime was calculated as described above, as was the total growth at the end of logarithmic growth.

GROWTH OF STRAIN 19 ON DIFFERENT NITROGEN SOURCES

Medium B was prepared with NH_4Cl, sodium lactate and yeast extract omitted, with distilled water replacing tap water and with 10 ml $litre^{-1}$ of the general trace elements solution of Ware and Postgate (1971). To one portion of media, sodium lactate was added to a final concentration of 30 mM as electron donor and nitrogen sources of 20 mM NH_4Cl, $NaNO_3$, $NaNO_2$ or 1% w/v amino acid mixture ("Oxoid" casein acid hydrolysate) set up. Another set of bottles were prepared without lactate but with either 1% w/v amino acid mixture + 20 mM NH_4Cl or

1% w/v amino acid mixture alone. The bottles were autoclaved as in previous experiments and inoculated with 1 ml of a 48 hour culture of strain 19, filled with media and sealed. Incubation was at 30°C with growth being determined by an increase in blackening/turbidity when compared with an inoculated basal medium only blank. A nitrogen source sustaining growth over at least three subcultures was recorded as positive.

USE OF NO_3^- AS A POTENTIAL ELECTRON ACCEPTOR FOR STRAIN 19

To investigate the ability of strain 19 to utilise nitrate as an alternative terminal electron acceptor to sulphate, medium B was prepared with the following modifications: $CaSO_4$ was replaced by 1.0 g litre^{-1} $CaCl_2.6H_2O$, $MgSO_4.7H_2O$ was replaced by 1.6 g litre^{-1} $MgCl_2.6H_2O$, NH_4Cl and yeast extract were omitted and distilled water and trace elements were again used. The following regimes were then set up: 20 mM Na_2SO_4 as electron acceptor +20 mM NH_4Cl or $NaNO_3$ as nitrogen source, 20 mM $NaNO_3$ as electron acceptor and nitrogen source or with 20 mM NH_4Cl as nitrogen source; basal medium only. These media were autoclaved as 30 ml lots in universal bottles and inoculated with 2.5 ml aliquots of an 8 day culture of strain 19 grown on NO_3^- as sole nitrogen source. Growth was again determined by an increase in turbidity of test bottles when read against an inoculated basal medium blank. Bottles scored positive supported growth over at least three subcultures.

1% w/v amino acid mixture alone. The bottles were autoclaved as in previous experiments and inoculated with 1 ml of a 48 hour culture of strain 19, filled with media and sealed. Incubation was at 30°C with growth being determined by an increase in blackening/turbidity when compared with an inoculated basal medium only blank. A nitrogen source sustaining growth over at least three subcultures was recorded as positive.

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R E S U L T S

N.E. ATLANTIC

Sulphate reduction rates for the three N.E. Atlantic sampling stations examined are shown in table 2.1. No sulphate reduction was detected in A-1 surface sediment and attempted enrichments for sulphate reducing bacteria (table 2.4) failed on three separate occasions. A low sulphate reduction rate was measured in sediment from the Rockall Trough and sulphate reducing bacteria were shown to be present. The rate of sulphate reduction in A-3 surface sediment from the continental shelf was some ten times greater than that at A-2 at a value of 1.0 n mol SO_4^{2-} $ml^{-1}d^{-1}$ and again sulphate reducers were shown to be present.

LYNN OF LORNE

Counts of sulphate reducing bacteria in LY-1 sediment (fig. 2.2) were at a maximum in the lower section of the core examined (10-15 cm) and decreased to around 10^2 ml^{-1} sediment in the surface 10 cm. A similar situation was encountered with sulphate reduction rate, with the lowest rate measured in the surface 5 cm of sediment (0.1 n mol $SO_4^{2-}ml^{-1}d^{-1}$) which increased some ten fold to a value of 1.1 n mol $SO_4^{2-}ml^{-1}d^{-1}$ at a depth of 10-15 cm. Acetylene reduction rates recorded were low and constant at a value of approximately 0.1 n mol C_2H_4 prod.g dry $wt^{-1}hr^{-1}$ over the top 15 cm of sediment.

LOCH EIL

Data obtained for the two Loch Eil sampling stations are presented in table 2.2 and figure 2.3. Maximum sulphate

TABLE 2.1 PRESENCE OF SULPHATE REDUCING BACTERIA AND SULPHATE REDUCTION RATES IN THE SURFACE 5 cm OF N.E. ATLANTIC SEDIMENTS.

Sampling Station and Depth	Sulphate Reduction Rate (nmol SO_4^{2-} $ml^{-1}d^{-1}$)	Sulphate Reducing Bacteria
DEEP SEA		
N.E. Atlantic A-1 (4,920m)	-	-
N.E. Atlantic A-2 (2,880m)	0.1	+
COASTAL		
N.E. Atlantic A-3 (158m)	1.0	+

- not detected

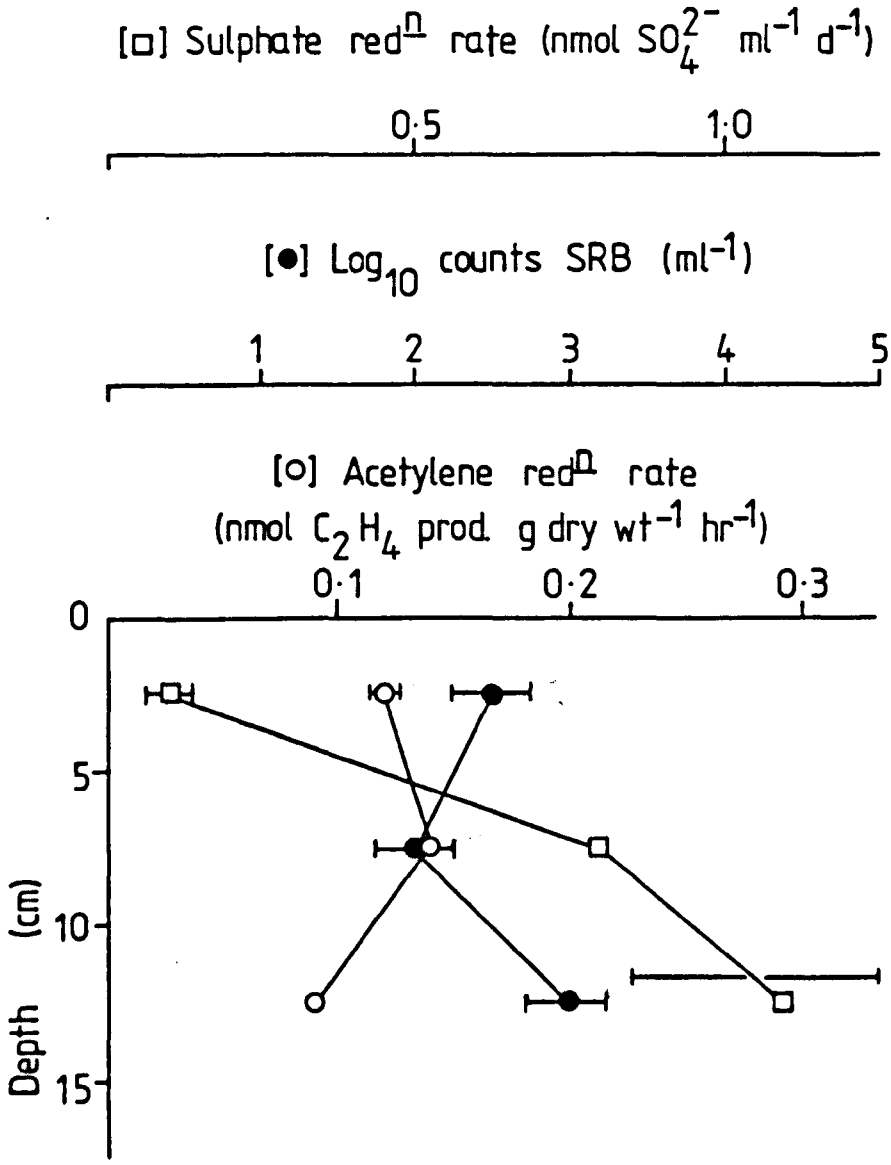

Fig.2.2 Lynn of Lorne LY-1, profile of sulphate reduction rate, counts of sulphate reducing bacteria and acetylene reduction rate with depth. Bar markers where shown indicate standard error.

reduction in E-24 sediment was measured in the top 5 cm section with a rapid decrease with depth down to 10 cm. However, the sulphate reduction activity at greater depths (35-40 cm) was of a similar order as that encountered in the 5-10 cm sediment section. $1.2x10^3$ sulphate reducing bacteria ml^{-1} of 0-5 cm sediment were recorded. In E-70 sediment the greatest number of sulphate reducers were found at a sediment depth of 5-10 cm with numbers of a same order of magnitude at 5 cm intervals either side of this band and counts below 15 cm some ten times lower. A very high sulphate reduction rate was measured in the surface 5 cm of the sediment (147.5 nmol $SO_4^{2-}ml^{-1}d^{-1}$) which was the highest rate of sulphate reducing activity found in this study. Again activity fell rapidly over the top 10 cm of E-70 sediment. Maximum values of the relatively low acetylene reduction rates recorded were found in the surface 5 cm of sediment (0.27 nmol C_2H_4 prod. g dry wt.$^{-1}hr^{-1}$) with activity gradually decreasing with increasing depth.

LOCH CRERAN

Maximum counts of sulphate reducing bacteria on sediment from sampling station CR-1 (fig.2.4) were measured at a depth of 15-20 cm down the sediment core ($3.3x10^4$ ml^{-1}), with the numbers of sulphate reducers above this depth fluctuating somewhat between around 10^3 ml^{-1} in the top 10 cm of sediment to around 10^2 ml^{-1} at a core depth of 10-15 cm. Throughout the top 20 cm of the sediment sulphate reduction remained steady at between 3.5-4.0 nmol $SO_4^{2-}ml^{-1}d^{-1}$. Maximum acetylene reduction occurred in the surface 5 cm of sediment and this fairly high rate of 0.74 nmol C_2H_4 prod. g dry wt.$^{-1}hr^{-1}$ was the

TABLE 2.2 MOST PROBABLE NUMBERS OF SULPHATE REDUCING BACTERIA AND SULPHATE REDUCTION RATES IN LOCH EIL E-24 SEDIMENT

Sediment section	Sulphate Reducing Bacteria (ml^{-1} sediment)	Sulphate Reduction Rate (nmol $SO_4^{2-} ml^{-1} d^{-1}$)
0-5 cm	1.2×10^3	15.5
5-10 cm	n.d.	1.5
35-40 cm	n.d.	2.0

n.d. not determined

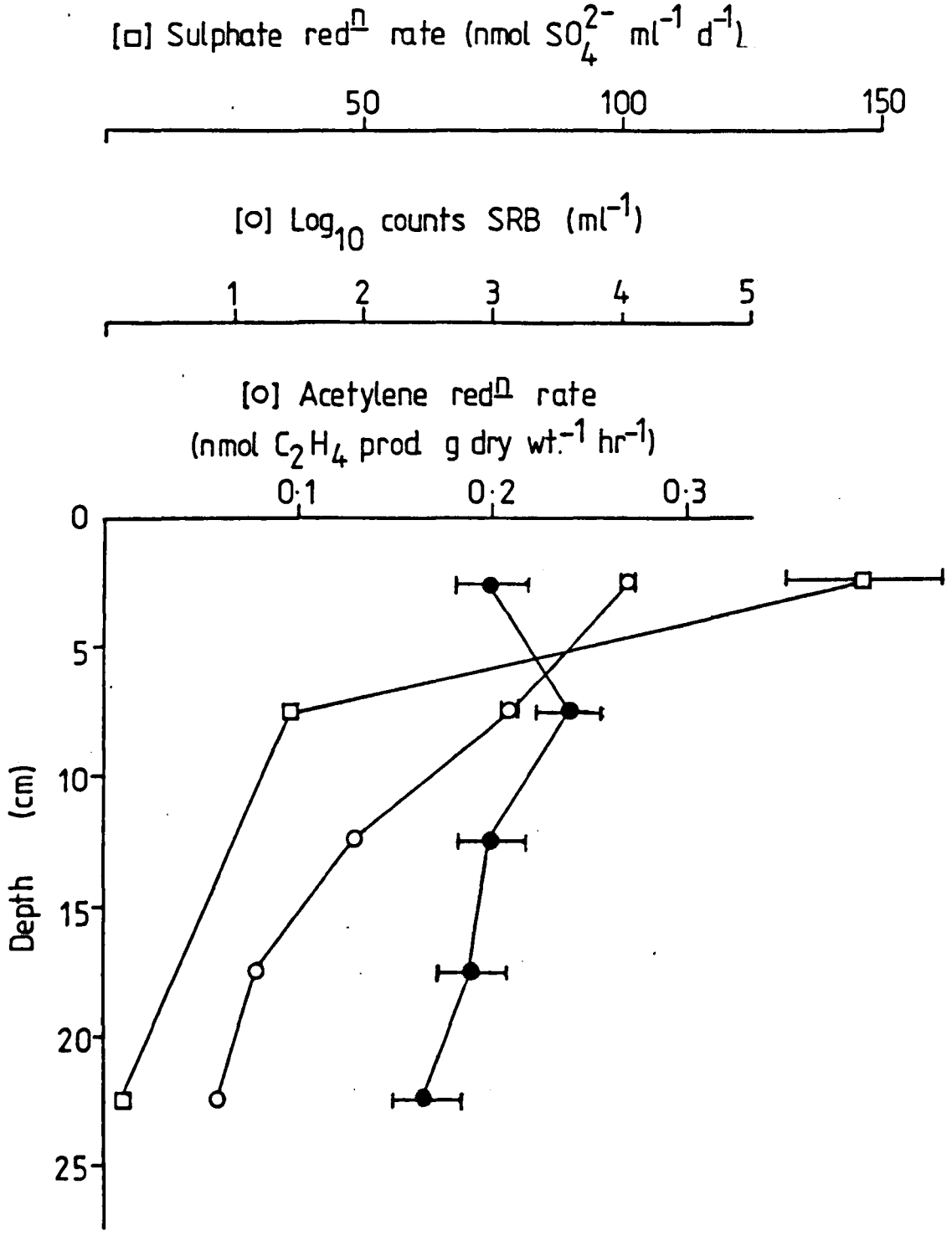

Fig.2.3 Loch Eil E-70, profile of sulphate reduction rate, counts of sulphate reducing bacteria and acetylene reduction rate with depth. Bar markers where shown indicate standard error.

highest activity encountered during the course of this work. As in E-70 sediment the activity decreased with depth down the sediment core. In CR-2 sediment highest counts of sulphate reducing bacteria were recorded in the 0-5 cm sediment section with numbers tending to fall off with depth (fig.2.5). Sulphate reduction showed peaks of activity at depths of 0-5 cm and 10-15 cm with rates of around 14 nmol $SO_4^{2-}ml^{-1}d^{-1}$ being measured. The acetylene reduction rate was low and constant over the surface 10 cm of sediment and decreased to a very low level of 0.01 nmol C_2H_4 prod. g dry wt.$^{-1}hr^{-1}$ at a depth of 15-20 cm.

SHETLAND

Results obtained for the two Shetland mainland sampling stations are shown in table 2.3 and fig.2.6. In D-4 sediment the number of sulphate reducing bacteria recorded decreased rapidly with depth from a maximum of $4.9x10^3ml^{-1}$ in the surface 5 cm of sediment. Sulphate reduction occurred at a very high rate in the 0-5 cm sediment section (103.3 nmol $SO_4^{2-}ml^{-1}d^{-1}$) and as in Loch Eil E-70 sediment, rates decreased rapidly with depth. Acetylene reduction was also at a maximum in the surface 5 cm of sediment and the rate of 0.29 nmol C_2H_4 prod.g dry wt.$^{-1}hr^{-1}$ was similar to that found in E-70 surface sediment. Likewise, activity decreased on increased depth. The numbers and distribution of sulphate reducing bacteria in sediment from sampling station CF-1 were very similar to the situation found at station D-4, with a maximum count of $3.3x10^3$ ml^{-1} in the surface sediment. However, sulphate reduction occurred at a lower rate (39.3 nmol $SO_4^{2-}ml^{-1}d^{-1}$) with activity again decreasing over the top 10 cm of sediment.

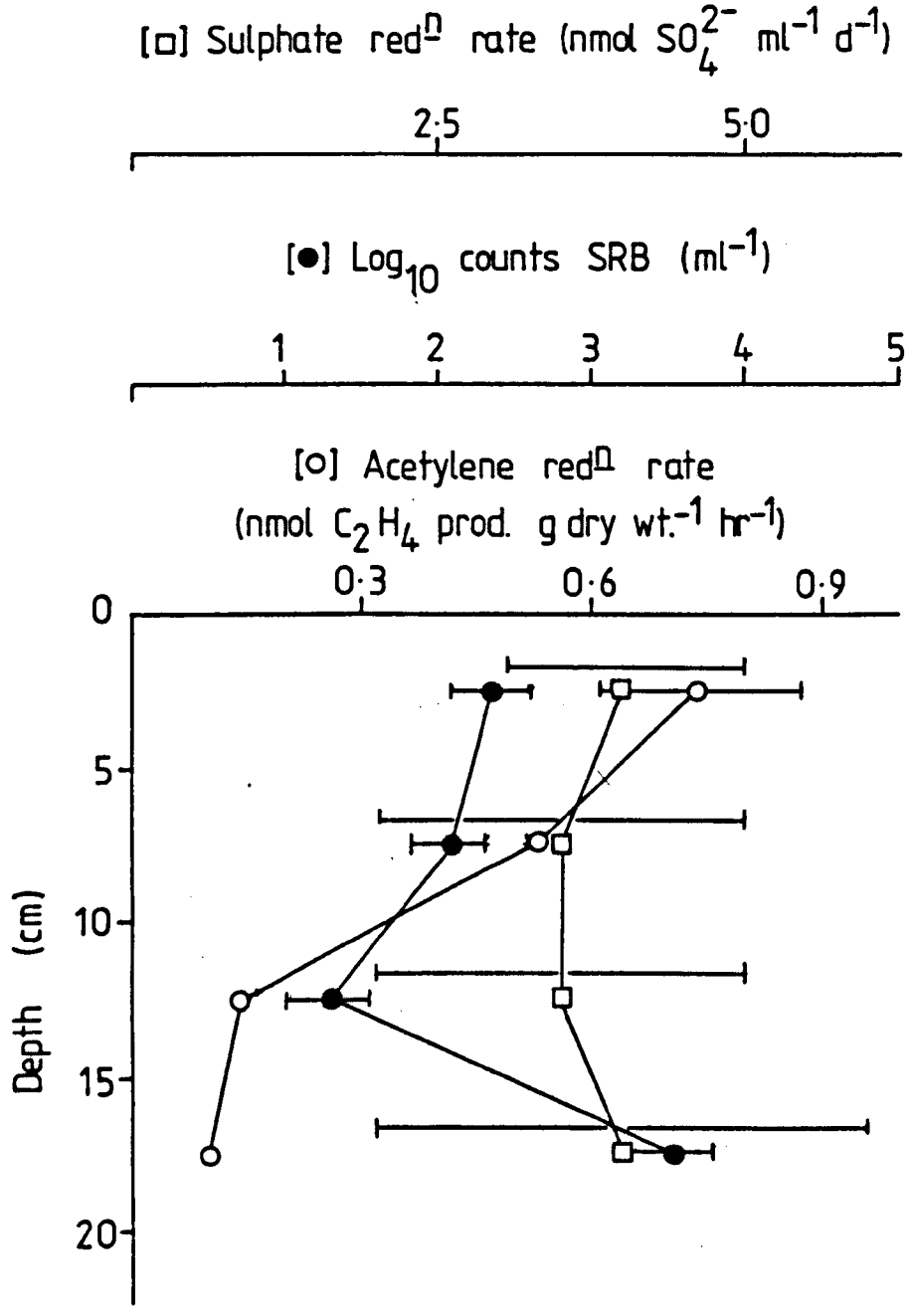

Fig.2.4 Loch Creran CR-1, profile of sulphate reduction rate, counts of sulphate reducing bacteria and acetylene reduction rate with depth. Bar markers where shown indicate standard error.

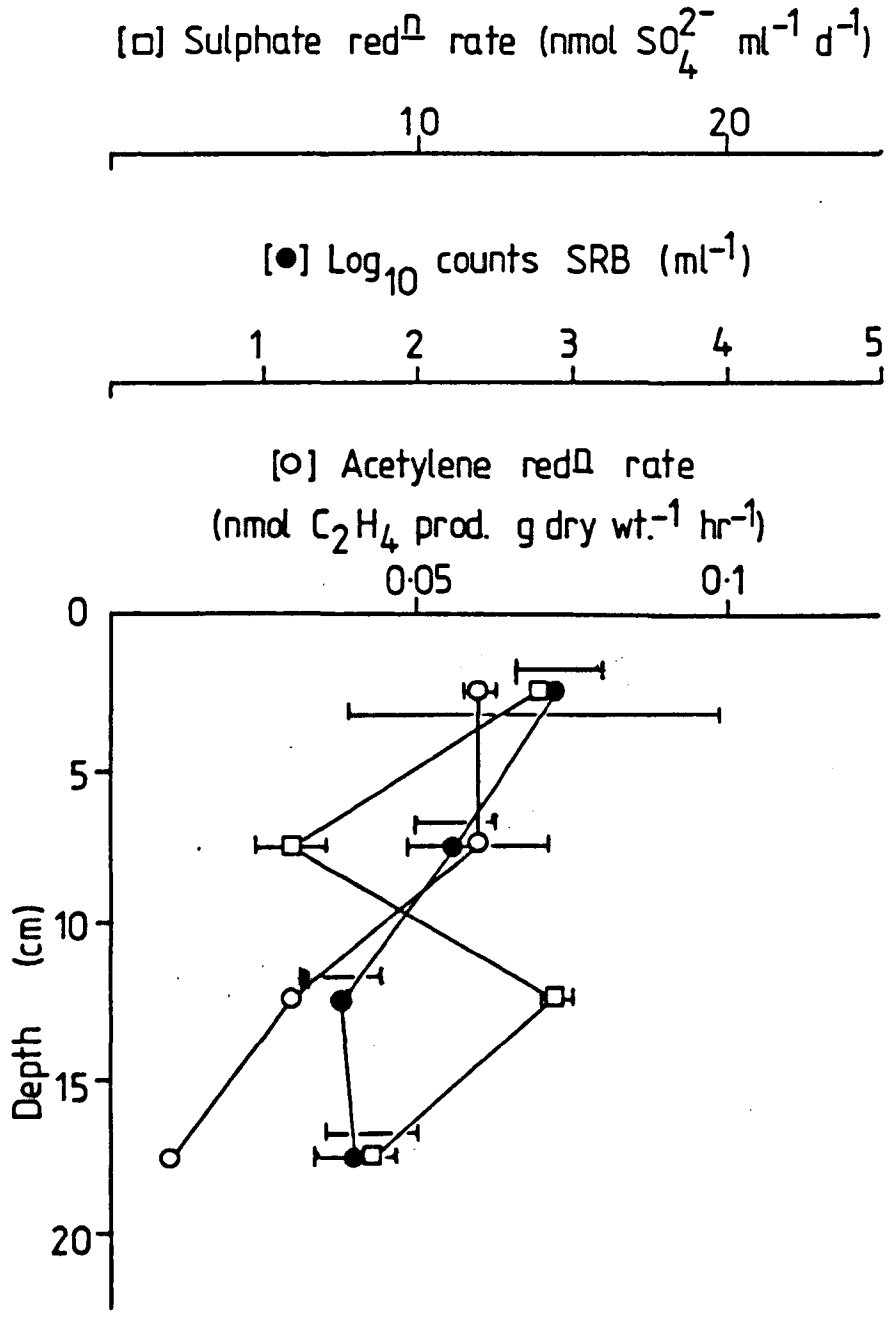

Fig.2.5 Loch Creran CR-2, profile of sulphate reduction rate, counts of sulphate reducing bacteria and acetylene reduction rate with depth. Bar markers where shown indicate standard error.

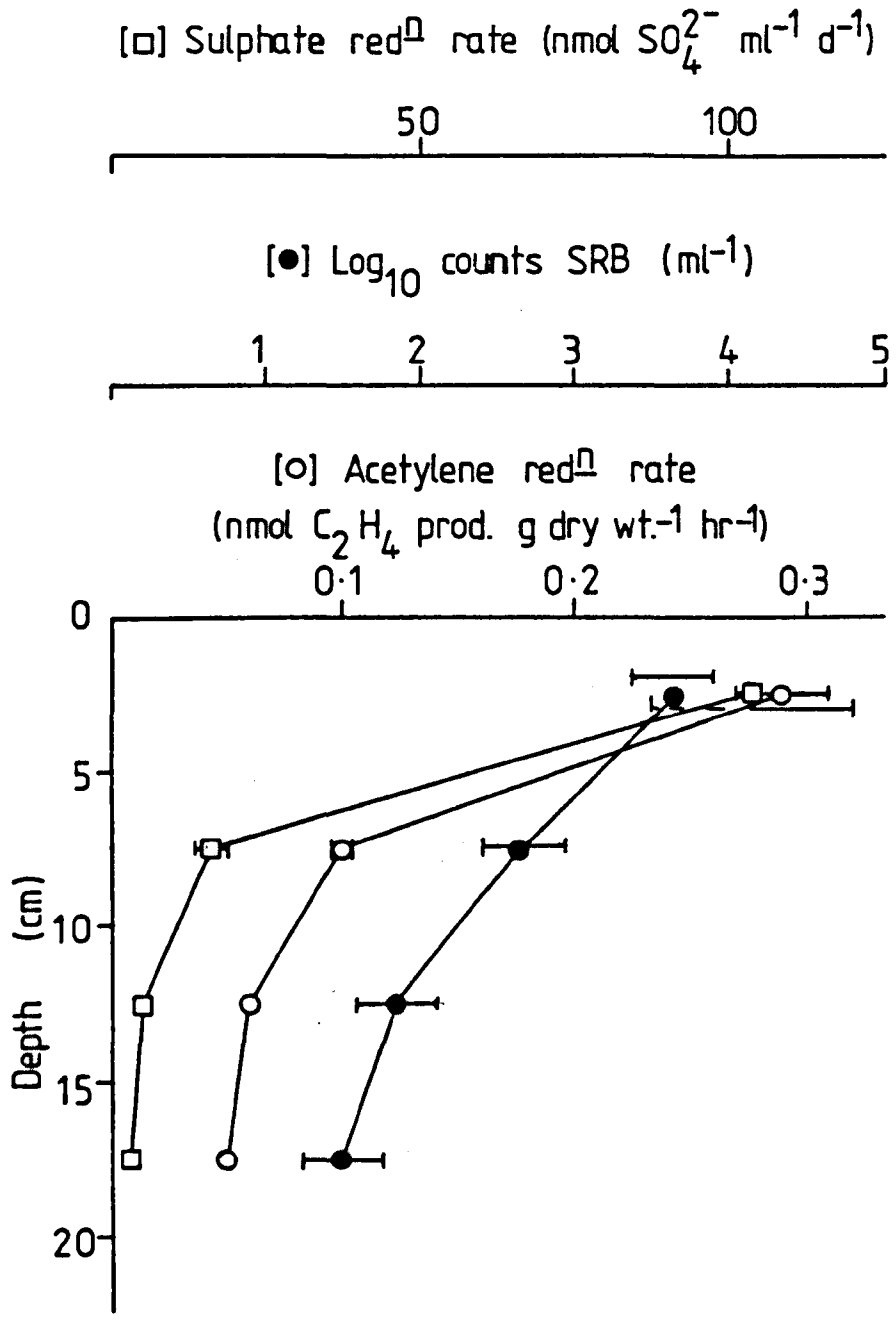

Fig.2.6 Sullom Voe D-4, profile of sulphate reduction rate, counts of sulphate reducing bacteria and acetylene reduction rate with depth. Bar markers where shown indicate standard error.

TABLE 2.3 MOST PROBABLE NUMBERS OF SULPHATE REDUCING BACTERIA AND SULPHATE REDUCTION RATES IN COLLA FIRTH SEDIMENT

Sediment Section	Sulphate Reducing Bacteria (ml^{-1} sediment)	Sulphate Reduction Rate (nmol $SO_4^{2-} ml^{-1} d^{-1}$)
0-5 cm	3.3×10^3	39.3
5-10 cm	7.0×10^2	27.3

SULPHATE REDUCING BACTERIA ENRICHMENTS

The results of a series of enrichments on different electron donors of sulphate reducing bacteria from the surface 5 cm of sediments from the N.E. Atlantic, Loch Eil and Loch Creran are presented in table 2.4. On no occasion were sulphate reducing bacteria detected in N.E. Atlantic A-1 abyssal sediment (depth 4,920m) despite enrichment attempts on three separate cruises. Sulphate reducing bacteria from the other four sampling stations could all use acetate, ethanol, hydrogen, lactate,propionate, pyruvate and succinate as electron donors for dissimilatory sulphate reduction. Enrichments of sulphate reducers from A-2 sediment (2,880m) could utilise only these compounds (in the main organic acids), whilst cultures from the continental shelf station A-3 were able in addition to use butyrate, glycerol and propanol as sources of reducing power. Sulphate reducing bacteria from the two sea-loch sampling stations used the widest range of compounds tested as electron donors and could utilise both butanol and formate. None of the sediments examined yielded sulphate reducing bacteria capable of using valerate and of the sediments investigated, none could utilise benzoate as an electron donor. The use of glucose as an electron donor was rarely encountered with weak growth only being shown in enrichment cultures from station CR-1 in Loch Creran.

STRAIN CHARACTERISTICS

The results of the characterization tests performed on the six strains of sulphate reducing bacteria isolated from station E-70 in Loch Eil are listed in table 2.5.

TABLE 2.4 ENRICHMENTS ON DIFFERENT ELECTRON DONORS OF SULPHATE REDUCING BACTERIA FROM LOCH CRERAN, LOCH EIL AND THE N.E. ATLANTIC

Electron Donor (0.1%w/v Concn.)	CR-1	E-70	A-1	A-2	A-3
Acetate	+	+	-	+	+
Benzoate	n.d.	n.d.	-	-	-
Butanol	+	+	-	-	-
Butyrate	+	+	n.d.	n.d.	+
Ethanol	+	+	-	+	+
Formate	+	+	n.d.	n.d.	n.d.
Glucose	±	-	-	-	-
Glycerol	+	+	-	-	+
Hydrogen	+	+	-	+	+
Lactate	+	+	-	+	+
Propanol	+	+	-	-	+
Propionate	+	+	-	+	+
Pyruvate	+	+	-	+	+
Succinate	+	+	-	+	+
Valerate	-	-	-	-	-

+ growth; - no growth; n.d. not determined.

TABLE 2.5 CHARACTERISTICS OF THE SIX STRAINS OF SULPHATE REDUCING BACTERIA ISOLATED FROM LOCH EIL E-70 SEDIMENT

Character	Strain					
	7	9	14	15	18	19
Form	V	V	V	V	V	V
Spores	-	-	-	-	-	-
Desulfoviridin	+	+	+	+	+	+
Growth with:						
lactate plus sulphate	+	+	+	+	+	+
pyruvate minus sulphate	+	+	+	+	+	+
choline minus sulphate	+	-	+	+	-	+
malate plus sulphate	+	+	+	+	+	+
formate plus sulphate	+	-	-	+	+	+
acetate plus sulphate	-	-	-	-	-	-
glucose plus sulphate	±	±	-	-	-	-
Growth with:						
0% w/v NaCl	-	-	-	-	-	-
0.5 " "	-	-	-	-	-	-
1.0 " "	+s	+	+s	+	+	+
1.5 " "	+	+	+	+	+	+
2.0 " "	+	+	+	+	+	+
2.5 " "	+	++	++	+	+	+
3.0 " "	++	++	++	+	++	++
Thermophily	-	-	-	-	-	-

V vibrio; +s slow growth; ++ rapid growth

Morphology

All the strains were curved rods (vibrios) in young cultures, although pleomorphy was common in old cultures, with the cells being present as vibrios, straight rods singly or in chains, or as coccoid forms. All the strains examined showed motility.

Sporulation

None of the strains showed evidence of sporulation under phase-contrast microscopy and were unable to grow after exposure to 80°C for 5 minutes.

Identification of pigment

All of the strains examined possessed desulfoviridin (a bisulphite reductase) and showed a red fluorescence at alkaline pH under ultraviolet light due to release of the pigment chromophore.

Substrate utilization

All of the isolates tested could grow on lactate or malate in the presence of sulphate, whilst none of the strains could utilise acetate as an electron donor. All of the strains could dismute pyruvate in the absence of sulphate and four of the strains choline chloride. Formate was utilized by four of the strains and two strains (7 and 9) appeared to be able to use glucose as an electron donor for sulphate reduction.

NaCl relations

A salt concentration of 1.0% w/v or more was required for growth of the six strains with the optimum concentration being at a value of 3.0% w/v NaCl in most cases. As the requirement

for NaCl was not above 1.0% w/v, all six isolated strains were recorded as not having a diagnostic NaCl requirement (Postgate, 1979).

Thermophily

None of the strains could grow at a temperature of 60°C and were therefore not thermophillic.

USE OF DIFFERENT ELECTRON DONORS BY ISOLATED STRAINS

The range of compounds utilized by the six strains of sulphate reducing bacteria isolated from Loch Eil is shown in table 2.6. In the main the strains were restricted to organic acids, especially those of the tricarboxylic acid cycle and to ethanol and its homologues. None of the strains examined were capable of growth on acetate, benzoate, butyrate, glutamate, propionate or valerate. Butanol and glycerol were utilized by five of the six strains, formate by four strains and succinate by three strains. Strains 7 and 9 appeared to use glucose as a source of reducing power, though these results were borderline. All of the strains examined could utilise ethanol, hydrogen, lactate, malate, propanol and pyruvate as electron donors for sulphate reduction.

PRELIMINARY GROWTH STUDIES ON STRAIN 19

i. EFFECT OF REDUCING AGENTS ON GROWTH

The growth of strain 19 in the presence of different reducing agents is shown in table 2.7. Maximum growth occurred when the media was poised with 0.01% w/v ascorbate, whilst growth in the presence of 0.01% w/v ascorbate and 0.01% w/v thioglycollate was

TABLE 2.6 GROWTH OF LOCH EIL E-70 SULPHATE REDUCING BACTERIA ON DIFFERENT ELECTRON DONORS

Electron Donor	Strain					
	7	9	14	15	18	19
Acetate	-	-	-	-	-	-
Benzoate	-	-	-	-	-	-
Butanol	+	+	+	-	+	+
Butyrate	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+
Formate	+	-	-	+	+	+
Glucose	±	±	-	-	-	-
Glutamate	-	-	-	-	-	-
Glycerol	+	+	-	+	+	+
Hydrogen	+	+	+	+	+	+
Lactate	+	+	+	+	+	+
Malate	+	+	+	+	+	+
Propanol	+	+	+	+	+	+
Propionate	-	-	-	-	-	-
Pyruvate	+	+	+	+	+	+
Succinate	-	-	+	+	+	+
Valerate	-	-	-	-	-	-

lower at $1.3x10^8$ bacteria ml^{-1}. A similar level of growth was attained in the presence of thioglycollate alone and represented a final population of around 80% of the maximum recorded in this experiment. No growth was observed in the presence of 5 mM Na_2S and this was assumed to be due to NaOH present in the Na_2S solution raising the media pH to 8.5.

ii. IRON REQUIREMENT

The effect of varying the iron concentration on the growth of strain 19 in 30mM lactate medium is shown in figure 2.7. Maximum growth was recorded at an iron concentration in excess of 2 μM Fe^{2+}. It can be seen that substantial growth occurred in the absence of any added iron. This was because the medium reagents and distilled water contained a significant amount of iron which was estimated to be at a maximum concentration of 7μM total iron (the majority of this originating from the yeast extract and NaCl components of the medium). The proportion of this iron which was present as Fe^{2+} was not known but the absolute Fe^{2+} requirement for maximum growth of strain 19 in the medium employed was >2μM with a maximum value of 9μMFe^{2+}.

iii. EFFECT OF 1mM CITRATE ON GROWTH

The data for this experiment are shown in table 2.8 and show that the presence of 1mM Na_3 citrate in the growth medium had no effect on the total growth of strain 19.

iv. SULPHATE REQUIREMENT

The effect of sulphate concentration on the growth of strain 19 in 30mM lactate medium is shown in figure 2.8. Sulphate was limiting for growth up to a concentration of 15mM.

TABLE 2.7 EFFECT OF REDUCING AGENTS ON THE GROWTH OF STRAIN 19

Reducing Agent	Growth (bacteria ml^{-1})	Final pH
0.01% w/v Ascorbate	$1.6x10^8$	7.6
0.01% w/v Thioglycollate	$1.3x10^8$	7.7
0.01% w/v Ascorbate + 0.01% w/v Thioglycollate	$1.3x10^8$	7.6
5mM Na_2S	No growth	8.5

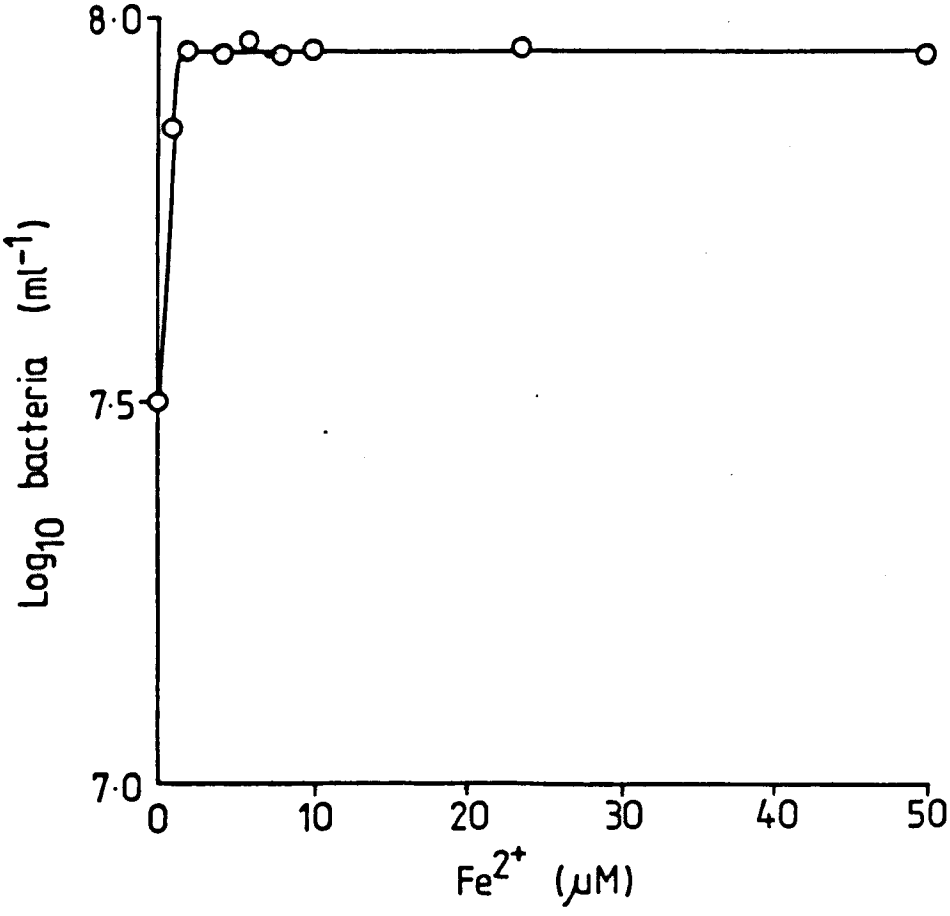

Fig.2.7 Effect of Fe^{2+} concentration on the growth of strain 19 in 30mM lactate medium.

TABLE 2.8 EFFECT OF 1mM CITRATE ON THE GROWTH OF STRAIN 19

	Total Growth (bacteria ml^{-1})
No citrate	1.9×10^8
1mM citrate	1.8×10^8

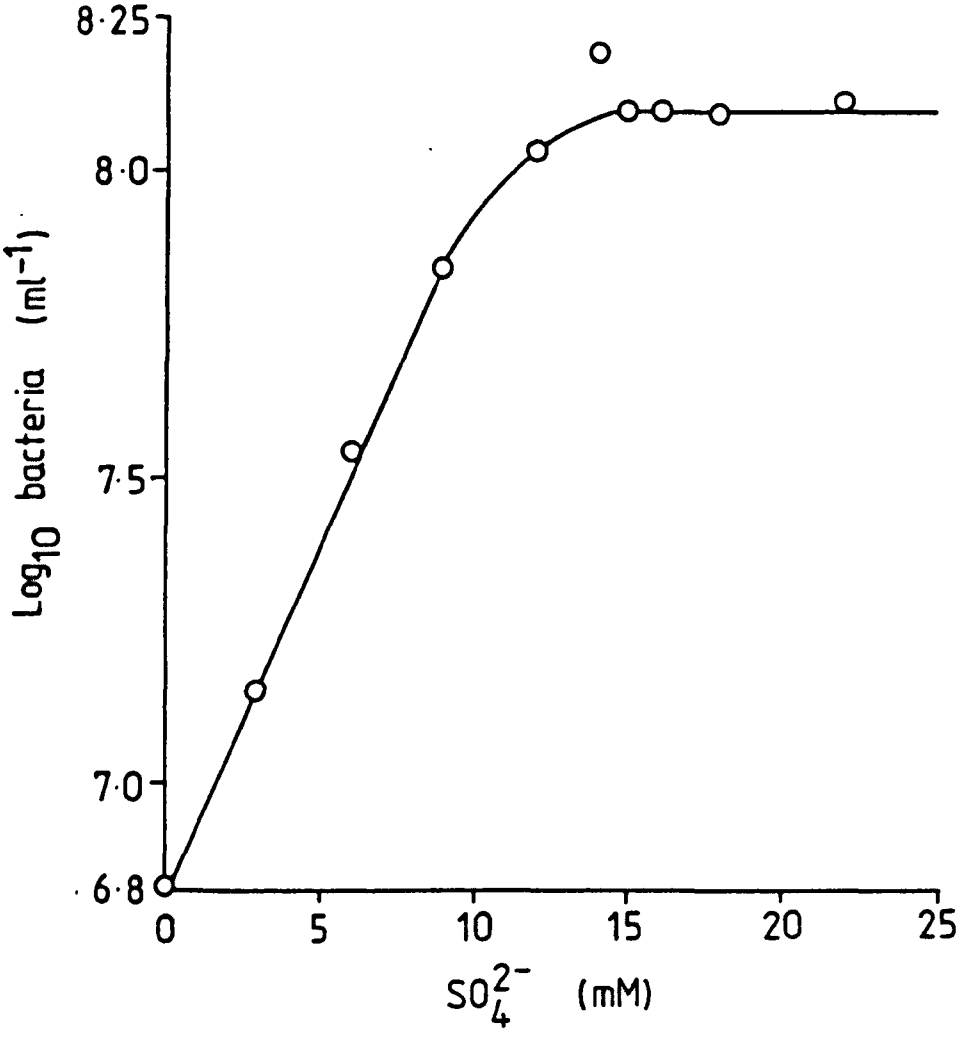

Fig.2.8 Effect of sulphate concentration on the growth of strain 19 in 30mM lactate medium.

MIXOTROPHIC GROWTH OF STRAIN 19

The data obtained in this experiment are presented in figure 2.9. Low growth was recorded on the basal medium alone and increased with increasing yeast extract concentration. A similar pattern was seen with 30mM butanol, ethanol and propanol as electron donors and, although the growth recorded was greater than that with the basal medium alone, yeast extract was essential for growth. Maximum growth, which was enhanced by yeast extract, occurred on 30mM lactate as electron donor with growth proceeding in the absence of yeast extract, in contrast to ethanol and its homologues.

SOURCES OF CELL CARBON ONLY FOR THE GROWTH OF STRAIN 19 UNDER A H_2/CO_2 ATMOSPHERE

The growth of strain 19 on possible sources on cell carbon only under a H_2/CO_2 atmosphere is shown in table 2.9. It can be seen that no appreciable growth or sulphate reduction (sulphide production) occurred in the presence of acetate, benzoate, butyrate, glucose, propionate or valerate at concentrations from 2.5 to 10.0mM. It must therefore be concluded that these compounds cannot act as sources of cell carbon during the mixotrophic growth of strain 19 on molecular hydrogen. Growth and sulphate reduction in the presence of lactate increased with increasing concentration to a maximum of 2.4×10^7 bacteria ml^{-1} and 5.4 mmol l^{-1} sulphide at a concentration of 10mM lactate. Growth and sulphate reduction also occurred at low levels on yeast extract and appeared to reach a maximum at 0.5% w/v yeast extract.

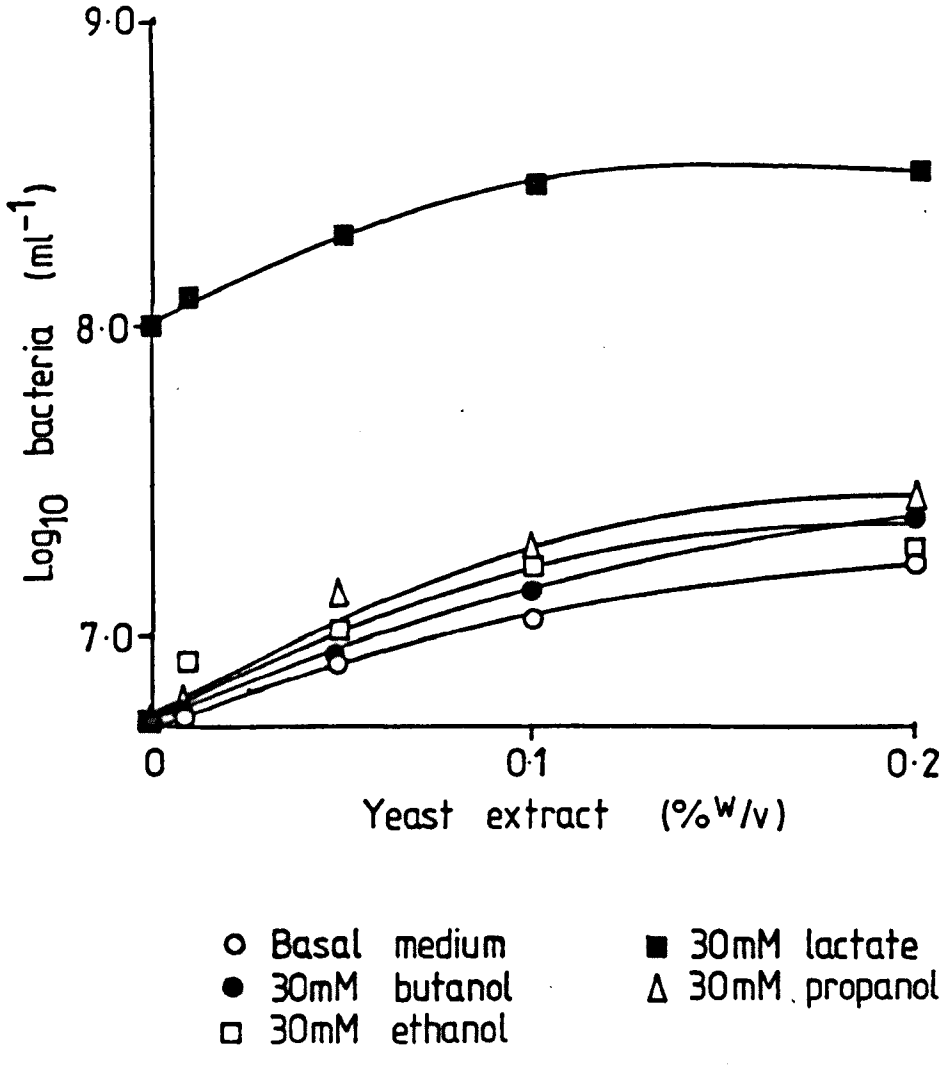

Fig.2.9 Effect of yeast extract concentration on the growth of strain 19 with 30mM substrate.

TABLE 2.9 GROWTH AND SULPHATE REDUCTION OF STRAIN 19 UNDER A H_2/CO_2 ATMOSPHERE ON POSSIBLE SOURCES OF CELL CARBON ONLY

Compound and Concentration		Growth (bacteria ml^{-1})	Final Sulphide Concentration (mmol l^{-1})
Acetate	0	-	0.8
	2.5mM	-	0.9
	5.0mM	-	0.9
	7.5mM	-	0.9
	10.0mM	-	0.9
Benzoate	0	-	1.1
	2.5mM	-	1.3
	5.0mM	-	1.2
	7.5mM	-	0.4
	10.0mM	-	0.8
Butyrate	0	-	0.9
	2.5mM	-	0.8
	5.0mM	-	0.9
	7.5mM	-	0.9
	10.0mM	-	0.8
Glucose	0	-	1.4
	2.5mM	-	1.4
	5.0mM	-	1.2
	7.5mM	-	1.3
	10.0mM	-	1.1
Lactate	0	-	1.2
	2.5mM	6.6×10^6	2.1
	5.0mM	1.6×10^7	3.2
	7.5mM	1.9×10^7	3.8
	10.0mM	2.4×10^7	5.4

- no growth

TABLE 2.9 (continued)

Compound and Concentration		Growth (bacteria ml^{-1})	Final Sulphide Concentration (mmol l^{-1})
Propionate	0	-	0.8
	2.5mM	-	0.9
	5.0mM	-	0.9
	7.5mM	-	0.8
	10.0mM	-	0.8
Valerate	0	-	0.8
	2.5mM	-	0.9
	5.0mM	-	0.7
	7.5mM	-	0.9
	10.0mM	-	0.9
Yeast extract	0	-	1.2
	0.5% w/v	$9.6x10^6$	2.0
	1.0% w/v	$7.6x10^6$	2.0
	2.0% w/v	$9.6x10^6$	3.2
	3.0% w/v	$7.6x10^6$	2.7

- no growth

EFFECT OF Mg^{2+} CONCENTRATION ON THE GROWTH OF STRAIN 19

The effect of Mg^{2+} on the maximum specific growth rate μm of strain 19 in modified medium E is shown in figure 2.10. Growth was exponential with the growth curve being of a similar shape to that in figure 2.15. It can be seen that the rate of growth was Mg^{2+} limited up to a concentration of 9mM $MgCl_2.6H_2O$. Low growth rates of 0.10 hr^{-1} were recorded with no Mg^{2+} addition and with 0.25mM Mg^{2+}. With increasing Mg^{2+} concentration having a marked effect on growth at higher concentrations. Mg^{2+} exerted a similar effect on the final population of cells at the end of logarithmic growth (figure 2.11) with the numbers of cells being Mg^{2+} limited up to a concentration of 9mM $MgCl_2.6H_2O$. In this instance however, although significant numbers of bacteria were recorded with no Mg^{2+} addition, the final population of cells with 0.25mM $MgCl_2.6H_2O$ was greater.

EFFECT OF Mg^{2+} CONCENTRATION ON THE GROWTH OF *D.DESULFURICANS* STRAINS IN POSTGATE'S MEDIUM C.

The effect of Mg^{2+} on the growth of *D.desulfuricans* strains Norway 4, Hossegor and Loch Eil 19 is shown in table 2.10. Growth when it occurred was exponential (see fig.2.15). With strain Norway 4 added Mg^{2+} had little effect on the maximum specific growth rate μm (approx.0.16 hr^{-1}), total growth (approx. 95 mg dry weight l^{-1}) or the total amount of sulphide present (approx.14 mmol l^{-1}). Added Mg^{2+} had even less effect on the growth on the Hossegor strain of *D.desulfuricans* with μm being around 0.22 hr^{-1} for each Mg^{2+} concentration, total growth at

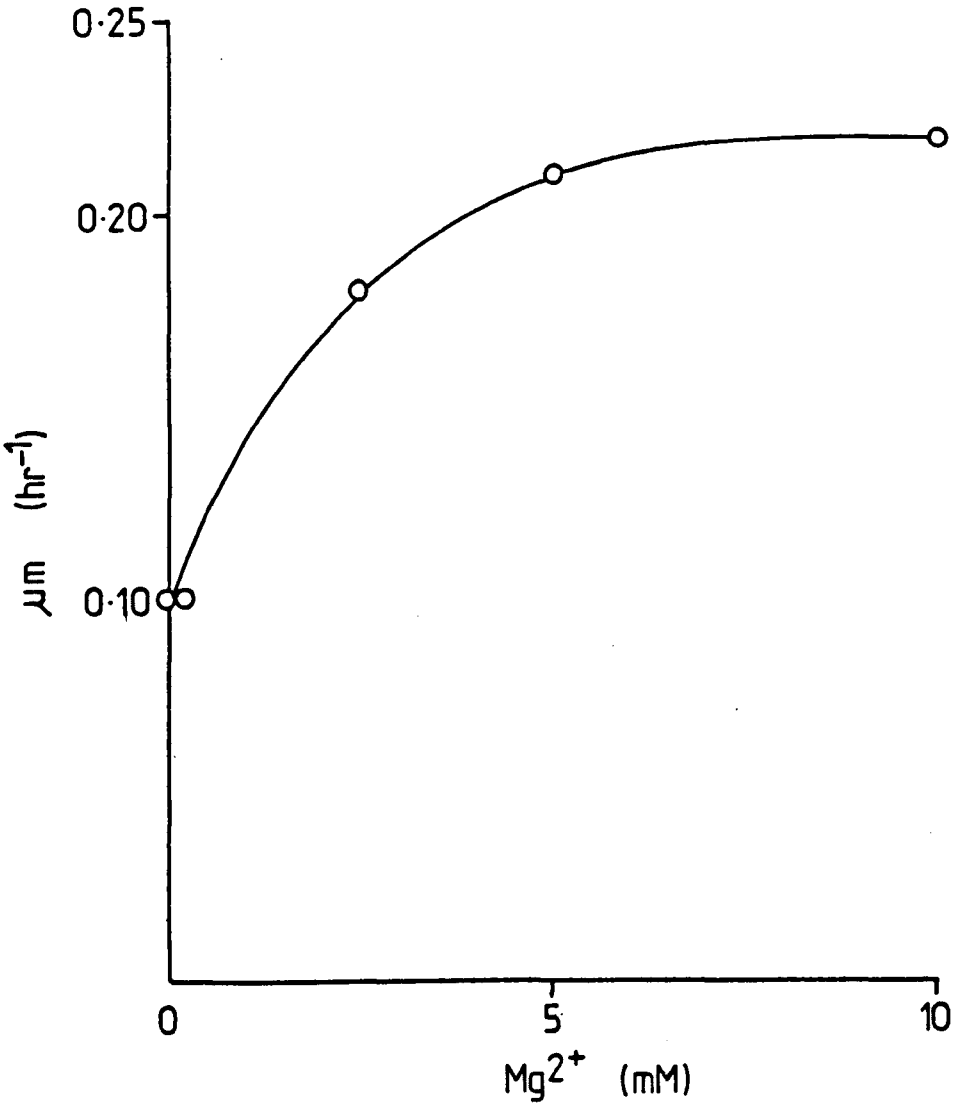

Fig.2.10 Effect of Mg^{2+} concentration on the maximum specific growth rate μm of strain 19 growin in modified medium E.

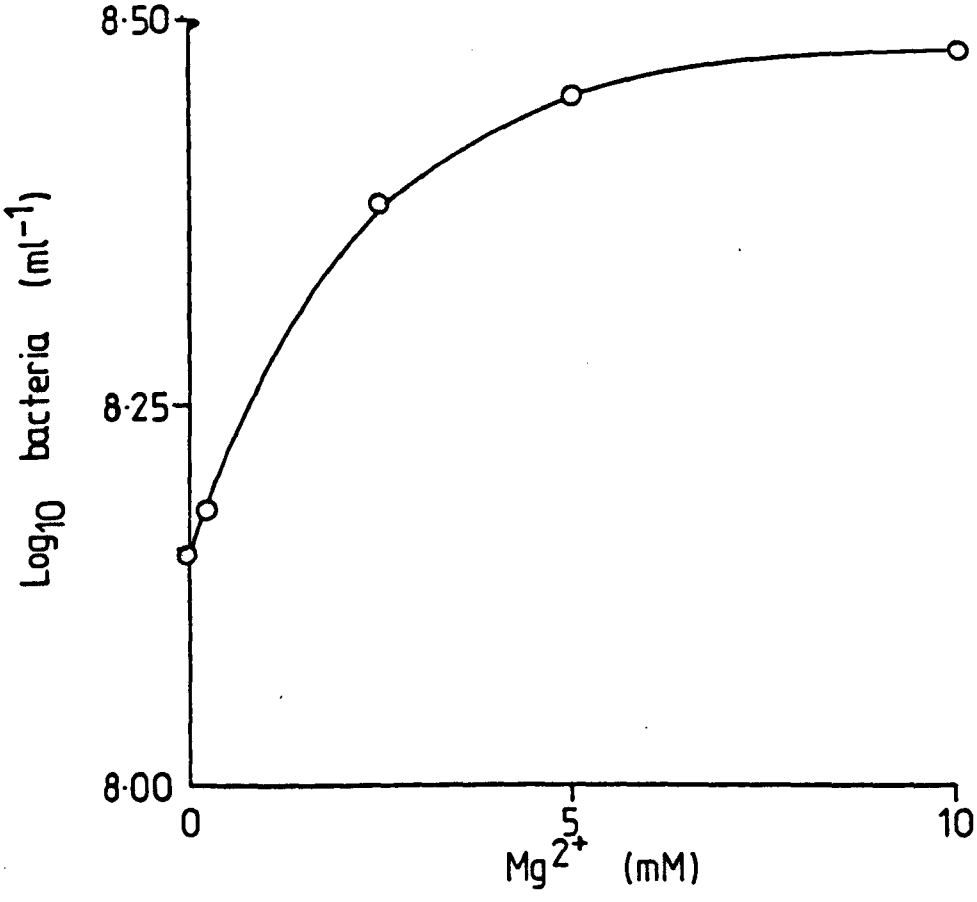

Fig.2.11 Effect of Mg^{2+} concentration on the final population of strain 19 growing in modified medium E.

around 138 mg dry weight l^{-1} and total amount of sulphide present at a level of around 14 mmol l^{-1}. With both strains μm, total growth and sulphide present after growth on no added Mg^{2+} was not significantly different from that with 10.0mM $MgCl_2.6H_2O$ addition. In contrast the growth of strain Loch Eil 19 in medium C was markedly affected by Mg^{2+} levels in the growth medium as is illustrated in figures 2.12, 2.13 and 2.14. As can be seen in figure 2.12 the rate of growth of strain 19 was Mg^{2+} limited up to a concentration of 10mM $MgCl_2.6H_2O$. A very low value of μm of 0.03 hr^{-1} was recorded with 0.25mM Mg^{2+} addition and this value was only slightly greater than that obtained with no Mg^{2+} added to the growth medium. The level of Mg^{2+} added to the medium appeared to have a similar effect on the total growth (fig.2.13) and total sulphide present (fig. 2.14) at the end of logarithmic growth. Although increasing Mg^{2+} concentration had a marked effect on these growth values upto a concentration of 5mM, above this value the effect was less than that on μm and it would appear from the two graphs that total growth and total sulphide present tended to reach a maximum at a Mg^{2+} concentration of <10mM.

EFFECT OF Mg^{2+} CONCENTRATION ON THE SULPHATE-FREE GROWTH OF STRAIN 19 ON PYRUVATE

The results of this experiment are shown in table 2.11. It can be seen that Mg^{2+} additions from 0.25 to 10.0mM had a negligible effect on the growth of strain 19 by the dismutation of 30mM pyruvate in the absence of sulphate, with total growth of around 50 mg dry weight l^{-1} being recorded in most instances.

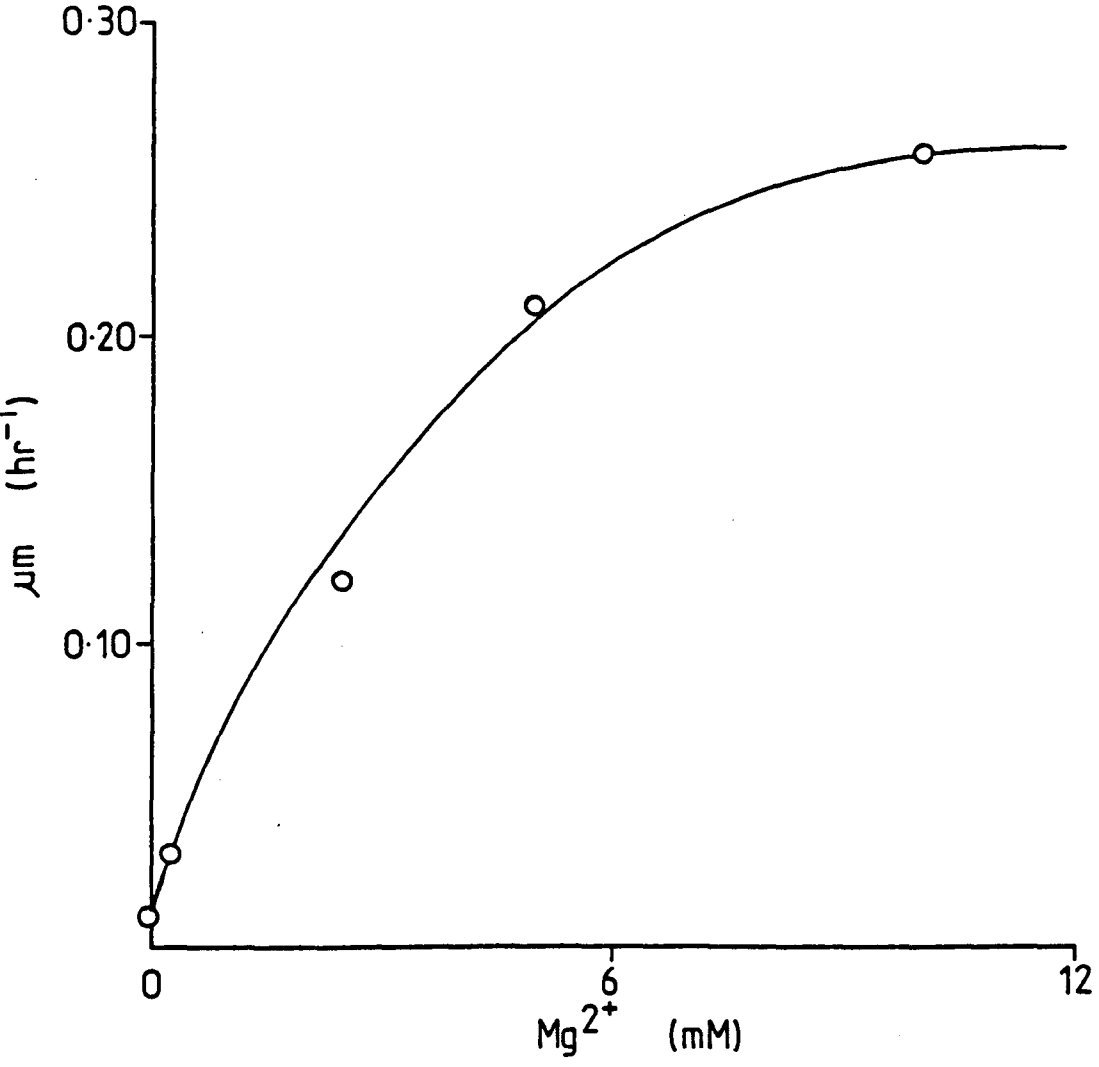

Fig.2.12 Effect of Mg^{2+} concentration on the maximum specific growth rate μm of strain 19 growing in medium C.

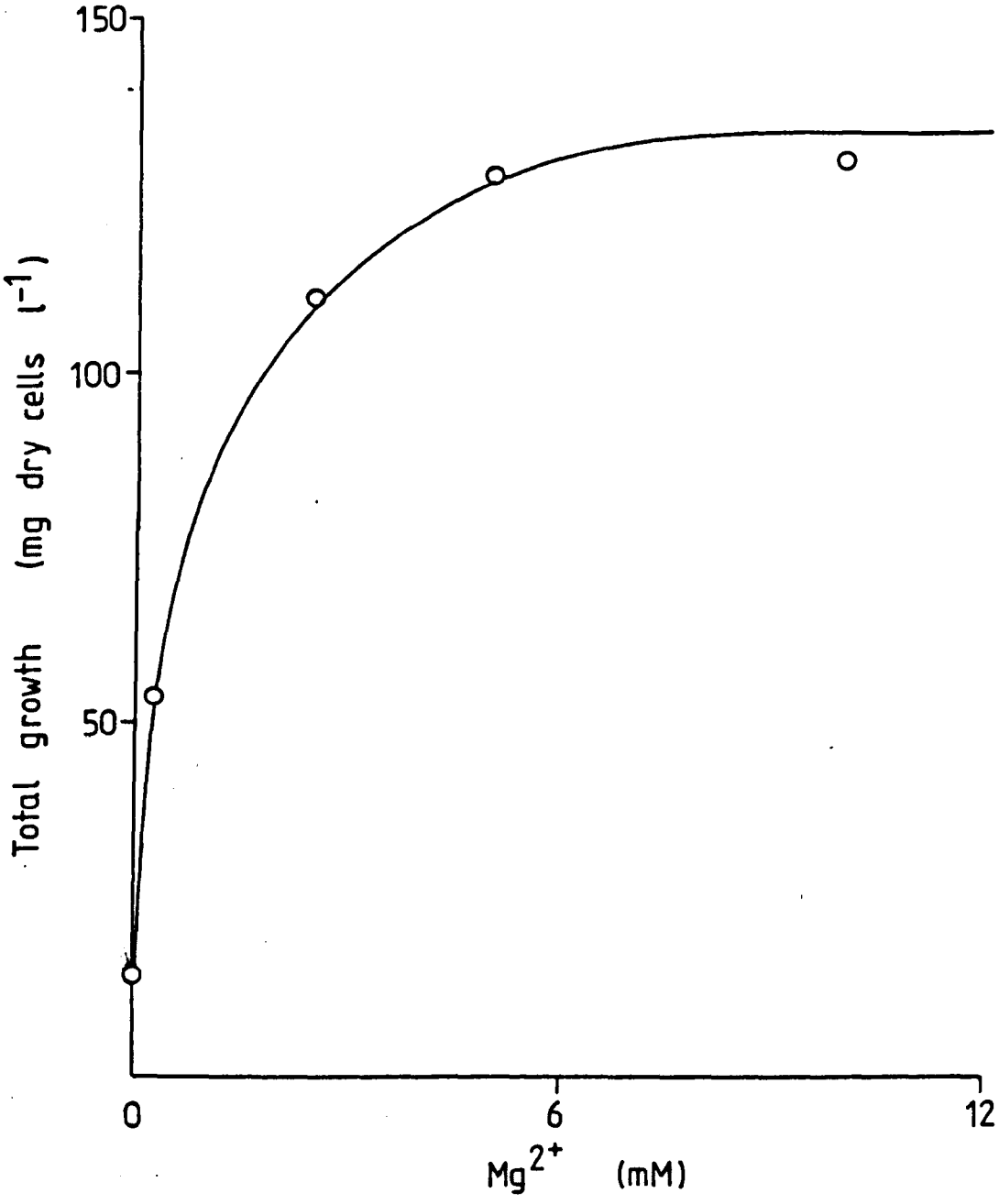

Fig.2.13 Effect of Mg^{2+} concentration on the total growth of strain 19 in medium C.

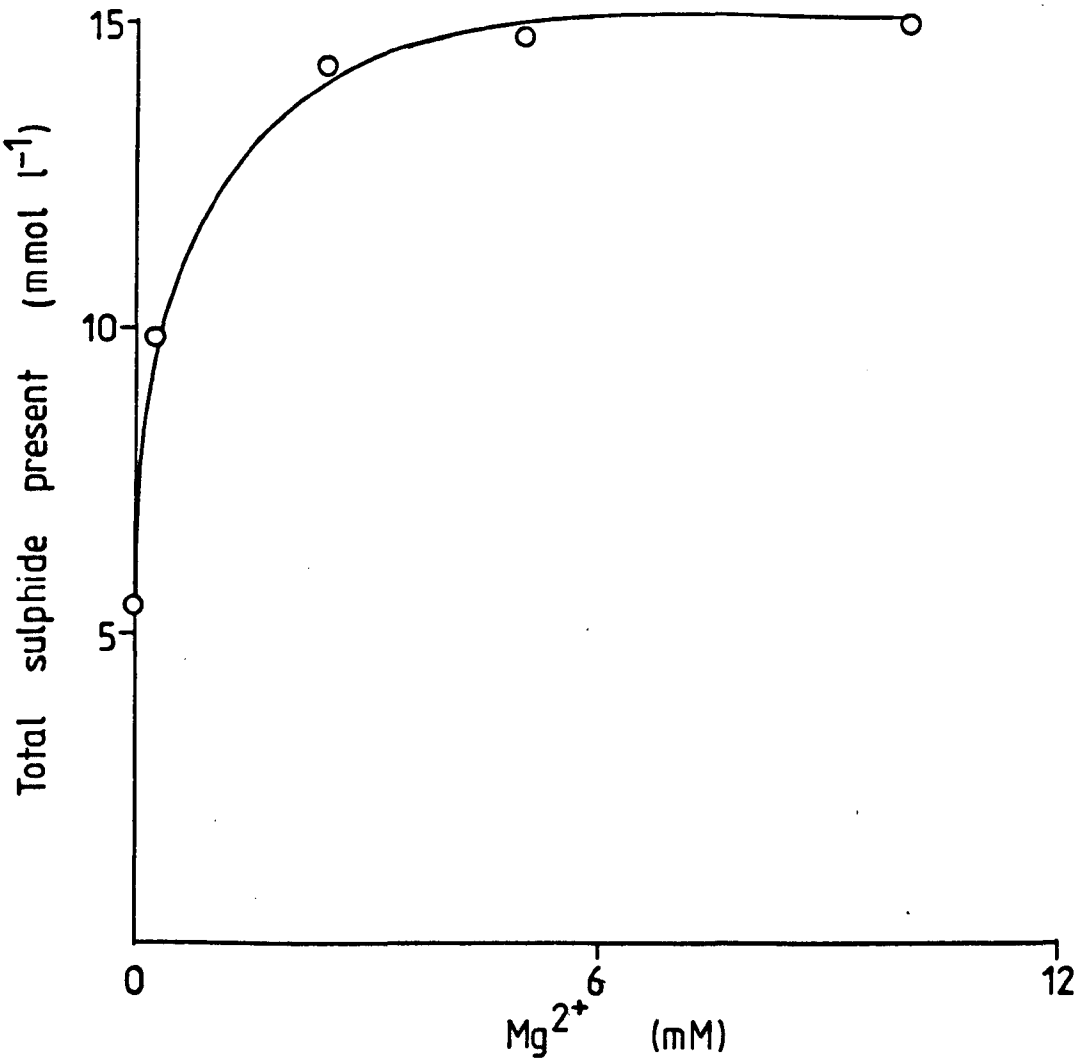

Fig.2.14 Effect of Mg^{2+} concentration on the total amount of sulphide present at the end of logarithmic growth of strain 19 in medium C.

TABLE 2.10 EFFECT OF Mg^{2+} ON THE GROWTH OF *D.DESULFURICANS* STRAINS IN POSTGATE'S MEDIUM C

Strain	Mg^{2+} Concentration (mM)	Maximum Specific Growth rate μm (hr^{-1})	Total Growth (mg dry weight l^{-1})	Total Sulphide Present (mmol l^{-1})
Norway 4	0	0.16	88.7	14.3
(NCIB 8310)	0.25	0.15	92.2	13.0
	2.5	0.16	102.6	14.1
	5.0	0.18	93.9	14.6
	10.0	0.17	97.4	13.4
Hossegor	0	0.22	131.9	14.7
(NCIB 8400)	0.25	0.21	135.0	14.4
	2.5	0.23	141.3	14.2
	5.0	0.23	142.9	14.2
	10.0	0.22	139.8	14.4
Loch Eil 19	0	0.01	14.3	5.5
	0.25	0.03	53.9	9.9
	2.5	0.12	110.0	14.3
	5.0	0.21	128.3	14.8
	10.0	0.26	131.2	15.0

TABLE 2.11 EFFECT OF $MgCl_2.6H_2O$ CONCENTRATION ON THE SULPHATE-FREE GROWTH OF *D.DESULFURICANS* LOCH EIL 19 ON PYRUVATE

$MgCl_2.6H_2O$ Concentration (mM)	Total Growth (mg dry weight l^{-1})
0	64.2
0.25	50.8
2.5	49.9
5.0	49.9
10.0	49.9

This level of growth represented 36% of that obtained in a later experiment in which energy was obtained by the respiratory oxidation of 30mM pyruvate in the presence of sulphate.

EFFECT OF Mg^{2+} AS CHLORIDE OR SULPHATE, AND OF Mn^{2+} ON THE GROWTH OF STRAIN 19

The data obtained in this experiment are presented in table 2.12. As in table 2.10 the addition of 0.25 and 10.0mM $MgCl_2.6H_2O$ to medium C produced a marked increase in the total growth of strain 19. The addition of the same concentrations of Mg^{2+} as $MgSO_4.7H_2O$ also increased the total growth which was somewhat greater than that obtained with Mg^{2+} present as the chloride. Total growth with 0.25 Mn^{2+}, at a value of 88.7 mg dry weight l^{-1}, was greater than that with the equivalent concentration of Mg^{2+}, although little growth occurred with 10.0mM $MnSO_4.4H_2O$. However, a white precipitate was observed in this tube and the lack of growth was attributed to the precipitation of excess Mn^{2+} out of solution and a possible lowering of the medium pH, inhibiting the growth of strain 19.

GROWTH OF STRAIN 19 IN POSTGATE'S MEDIUM C IN THE PRESENCE OR ABSENCE OF CITRATE, AND WITH Mg^{2+} OR Ca^{2+} SUPPLEMENTATION

The ability of strain 19 to grow in various formulations of Postgate's medium C is shown in table 2.13. As reported earlier no growth occurred in the medium as published, though supplementation with 10mM $MgCl_2.6H_2O$ resulted in good growth after 30-48 hours incubation. Supplementation with 10mM $CaCl_2.6H_2O$ also resulted in good growth after 30-48 hours

TABLE 2.12 EFFECT OF $MgCl_2 . 6H_2O$, $MgSO_4 . 7H_2O$ AND $MnSO_4 . 4H_2O$ CONCENTRATION ON THE GROWTH OF *D.DESULFURICANS* LOCH EIL 19

Divalent Cation Concentration (mM)		Total Growth (mg dry weight l^{-1})
None		17.2
$MgCl_2 . 6H_2O$	0.25	55.6
	10.0	129.8
$MgSO_4 . 7H_2O$	0.25	74.2
	10.0	130.6
$MnSO_4 . 4H_2O$	0.25	88.7
	10.0	10.0[p]

p white precipitate in culture tube

TABLE 2.13 GROWTH OF STRAIN 19 IN POSTGATE'S MEDIUM C IN THE PRESENCE OR ABSENCE OF CITRATE, AND WITH Mg^{2+} OR Ca^{2+} SUPPLEMENTATION

Medium Composition	Growth
Medium C (Postgate, 1979)	-
Medium C - citrate	+
Medium C + 10mM $MgCl_2.6H_2O$	+
Medium C + 10mM $CaCl_2.6H_2O$	+

incubation as did the omission of 1mM sodium citrate from the growth medium.

GROWTH AND SULPHATE REDUCTION OF STRAIN 19 ON LACTATE, MALATE OR PYRUVATE

The increase in cell density and concentration of sulphide with time in cultures of strain 19 growing on 30mM sodium lactate, malic acid or sodium pyruvate are shown in figures 2.15, 2.16 and 2.17 with the growth data calculated from these figures presented in table 2.14.

LACTATE

It can be seen from figure 2.15 that growth was exponential and closely mirrored by sulphide production, with growth ceasing after a period of 34 hours with the cessation of sulphate reduction (sulphide production). The highest values of μm and sulphate reduction rate were recorded during the growth of strain 19 on lactate and although μm was recorded during the early stages of logarithmic growth, the maximum rate of sulphate reduction occurred in the latter part of the log phase (approx.26 hours). The total growth attained on this substrate was similar to that recorded when pyruvate was the electron donor and somewhat greater than that with malate.

MALATE

Exponential growth and sulphate reduction occurred at a much slower rate on 30mM malic acid than with lactate as electron donor (figure 2.16) and ceased after 47 hours incubation. Although growth proceeded during the first 20 hours of

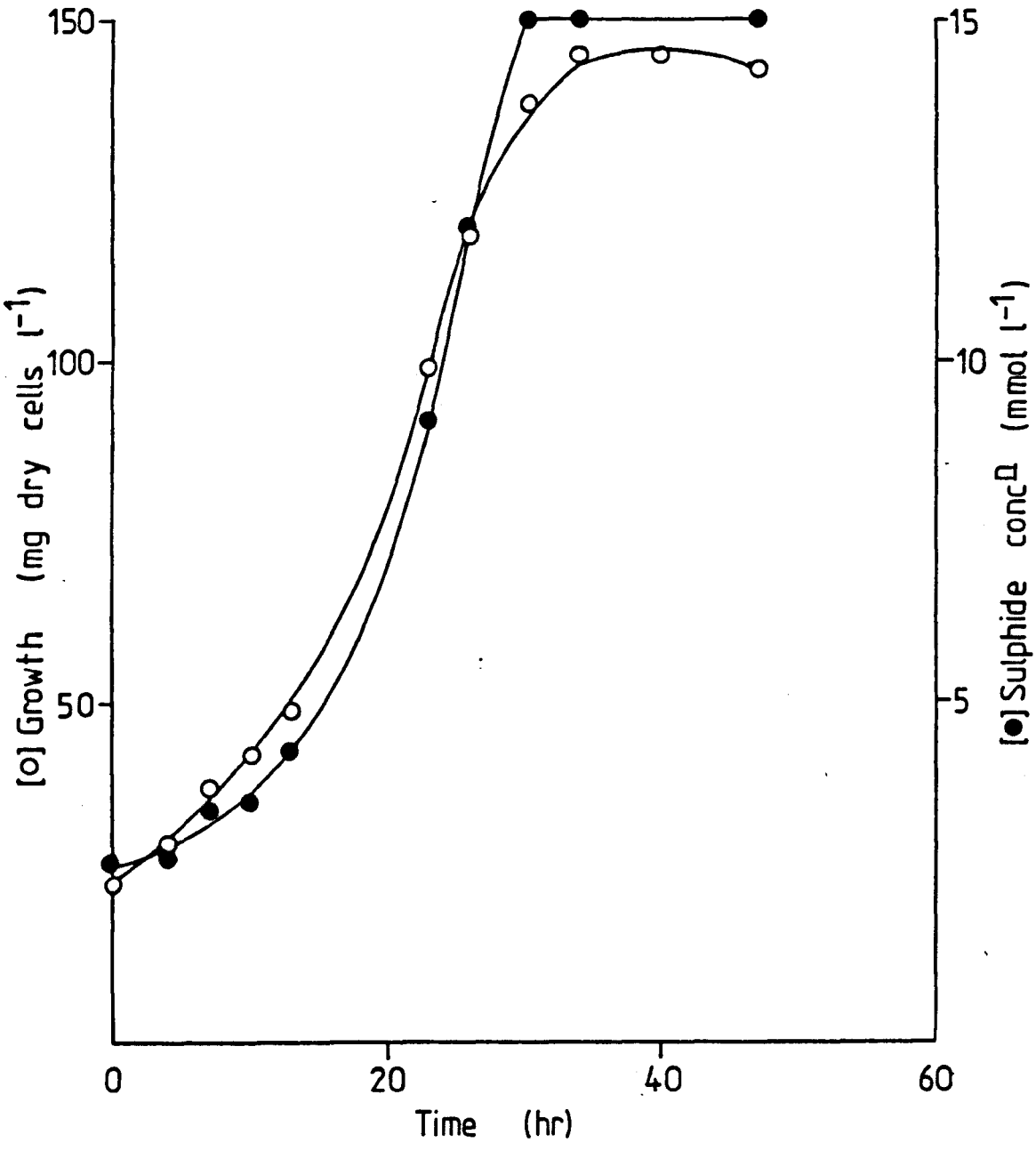

Fig.2.15 Growth and sulphide production of strain 19 growing on 30mM lactate.

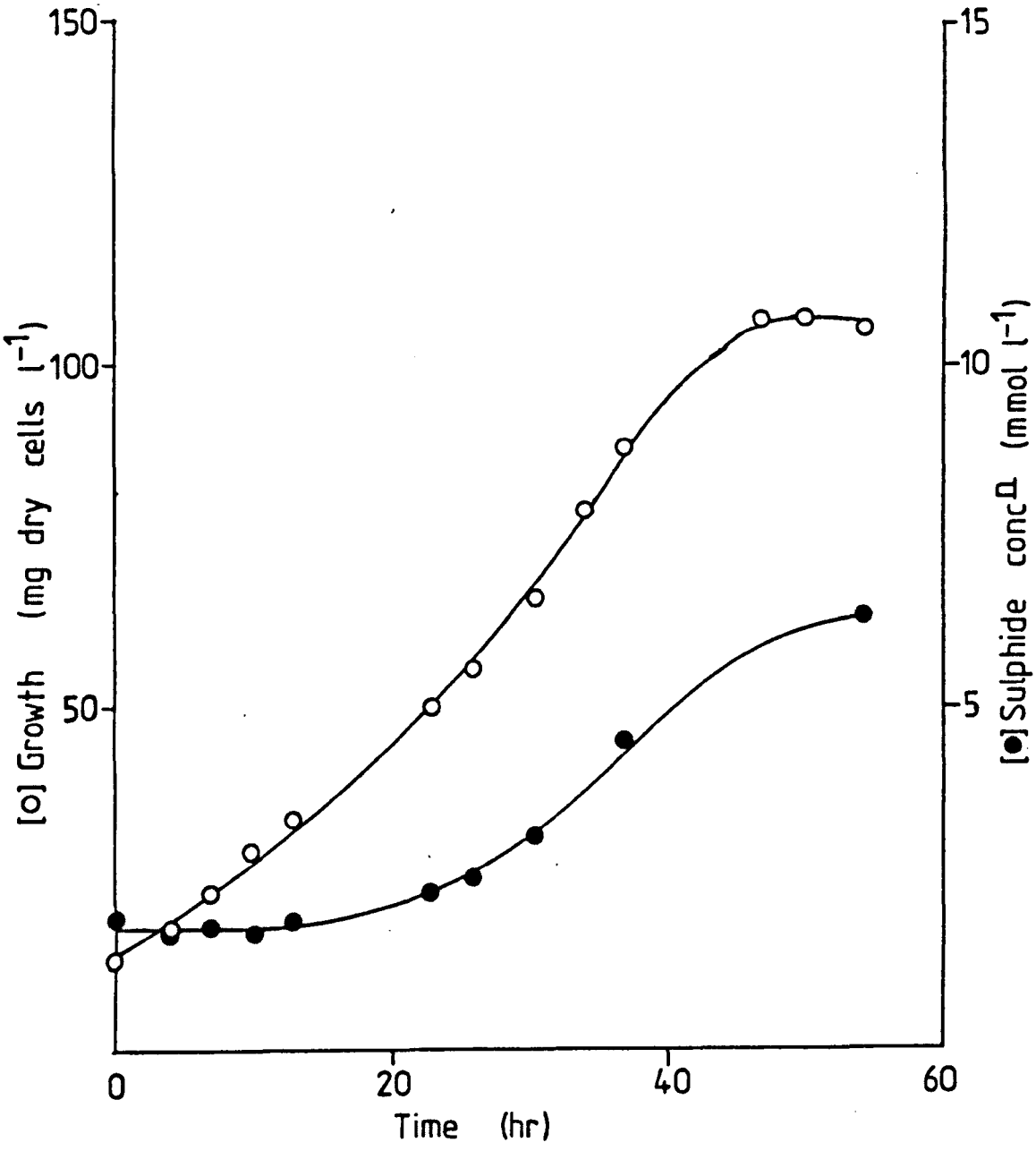

Fig.2.16 Growth and sulphide production of strain 19 growing on 30mM malate.

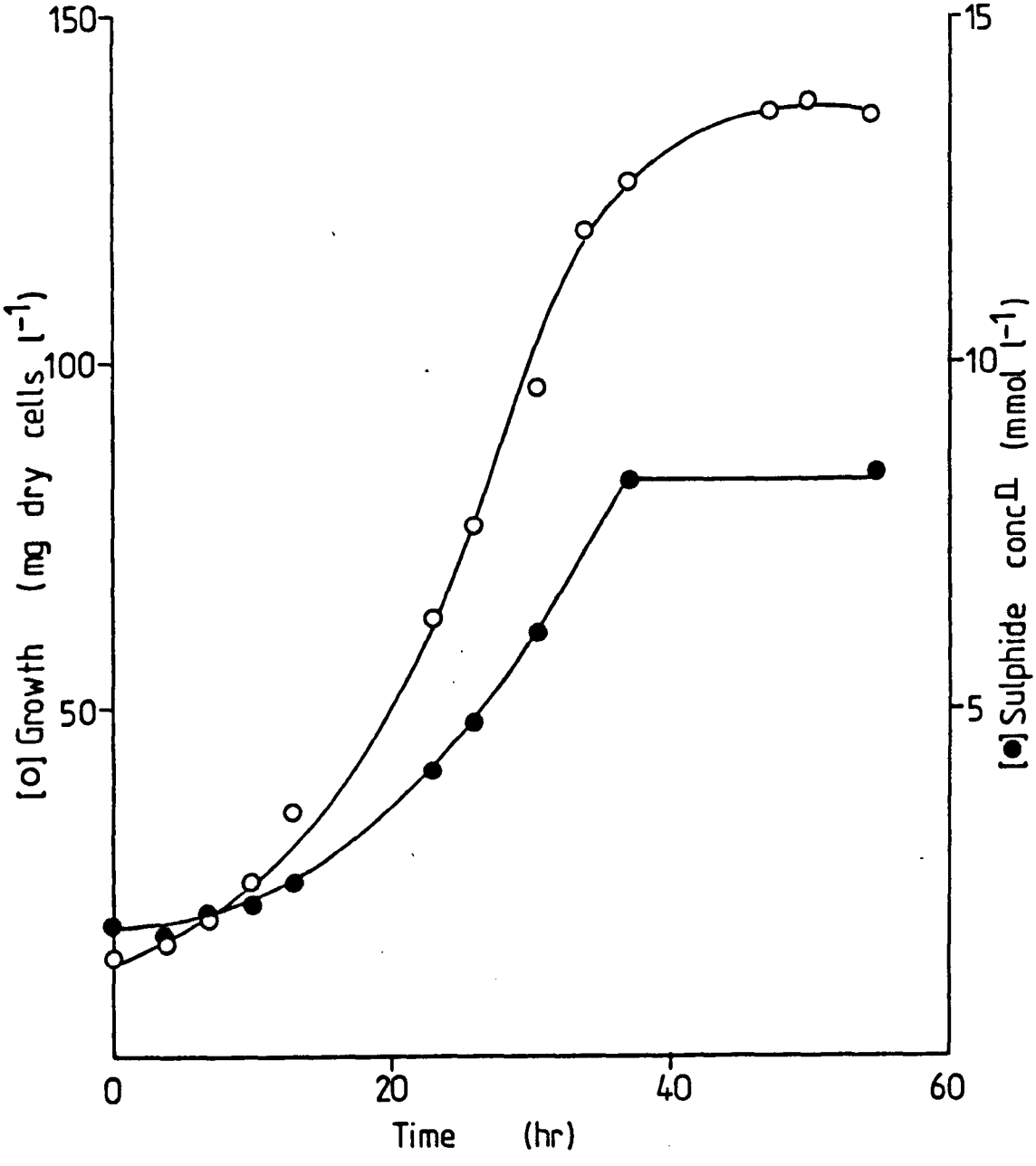

Fig.2.17 Growth and sulphide production of strain 19 growing on 30mM pyruvate.

TABLE 2.14 MAXIMUM SPECIFIC GROWTH RATE μm, TOTAL GROWTH AND MAXIMUM SULPHATE REDUCTION RATE OF STRAIN 19 GROWING ON 30mM LACTATE, MALATE OR PYRUVATE

Substrate	μM (hr^{-1})	Total Growth (mg dry weight l^{-1})	Maximum Sulphate Reduction Rate (μmol SO_4^{2-} $ml^{-1}d^{-1}$)
Lactate	0.20	122.0	24.8
Malate	0.08	93.0	5.0
Pyruvate	0.08	124.0	8.3

incubation there was a lag in sulphate reduction. However, as with growth on lactate, growth ceased with the termination of sulphate reduction. The maximum rate of sulphate reduction occurred once more in the latter portion of logarithmic growth after between 32 and 40 hours incubation. The maximum cell density of 93.0 mg dry weight l^{-1} attained was somewhat lower than that reached with lactate or pyruvate as electron donor.

PYRUVATE

The growth of strain 19 on 30mM sodium pyruvate is shown in figure 2.17 and it can be seen that growth was exponential and, like lactate, closely mirrored by sulphate reduction. Growth ceased after 46 hours incubation, after the cessation of sulphate reduction, with the maximum rate of sulphate reduction again occurring towards the end of logarithmic growth (between 33 and 37 hours). The total growth of strain 19 on pyruvate at 124.0 mg dry weight l^{-1} was similar to that obtained on lactate although μm and the maximum sulphate reduction rate were some 2½ to 3 times lower.

EFFECT OF NaCl CONCENTRATION ON THE GROWTH OF STRAIN 19

The influence of salt concentration on μm and the total growth of strain 19 in 30mM lactate medium is shown in table 2.15. No growth occurred at either end of the NaCl range examined whilst maximum values for μm and total growth were recorded at a salinity of 3.0% w/v. The cell density of 136.9 mg dry weight l^{-1} attained by strain 19 at this salinity was approximately twice that at 1.0% w/v NaCl and four times that at 5.0% w/v, with the maximum specific growth rates in both

TABLE 2.15 EFFECT OF NaCl CONCENTRATION ON THE GROWTH OF STRAIN 19

NaCl Concentration (% w/v)	μM (hr^{-1})	Total Growth (mg dry weight l^{-1})
0	-	-
1.0	0.07	61.5
3.0	0.20	136.9
5.0	0.12	32.5
7.0	-	-

- no growth

instances also being substantially lower. The growth at these three salinities was exponential with the growth curves obtained being of a similar shape to that presented in figure 2.15.

GROWTH OF STRAIN 19 ON DIFFERENT NITROGEN SOURCES

The ability of strain 19 to utilise various compounds as sole sources of nitrogen and as sources of nitrogen and electrons is shown in table 2.16. In the presence of lactate as electron donor NH_4^+, NO_3^- and a casein acid hydrolysate mixture of amino acids were utilized as sole nitrogen sources for growth. Growth occurred more rapidly with the amino acid mixture than with either of the inorganic sources of nitrogen. In the absence of 30mM lactate as electron donor the amino acid mixture could support growth either with or without NH_4^+ as nitrogen source.

USE OF NO_3^- AS A POTENTIAL ELECTRON ACCEPTOR FOR STRAIN 19

The results obtained in this experiment are presented in table 2.17. It can be seen that inorganic nitrate was unable to act as a terminal inorganic electron acceptor for lactate oxidation either with or without NH_4^+ as an alternative nitrogen source. As in the previous experiment, either compound could act as a nitrogen source for growth when SO_4^{2-} was available as the terminal electron acceptor.

TABLE 2.16 GROWTH OF STRAIN 19 ON DIFFERENT NITROGEN SOURCES

a) IN 30mM LACTATE MEDIUM:

Nitrogen Source	Growth
20mM NH_4Cl	+
20mM $NaNO_3$	+
20mM $NaNO_2$	-
1% w/v amino acid mixture	+

b) IN BASAL MEDIUM:

Nitrogen Source	Growth
1% w/v amino acid mixture	+
1% w/v amino acid mixture + 20mM NH_4Cl	+

+ growth; - no growth

TABLE 2.17 ABILITY OF STRAIN 19 TO USE NITRATE AS TERMINAL ELECTRON ACCEPTOR FOR LACTATE OXIDATION

Electron Acceptor	Nitrogen Source	Growth
20mM $NaNO_3$	20mM $NaNO_3$	-
20mM $NaNO_3$	20mM NH_4Cl	-
20mM Na_2SO_4	20mM $NaNO_3$	+
20mM Na_2SO_4	20mM NH_4Cl	+

+ growth; - no growth

D I S C U S S I O N

THE ACTIVITY OF SULPHATE REDUCING BACTERIA IN DIFFERING MARINE SEDIMENTS

The anaerobic oxidation of organic matter in the ten marine sediments examined *via* the respiratory activities of the sulphate reducing bacteria can be summarized by the equation:

$$2\ [CH_2O] + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad \text{(Berner, 1974)}$$

This reaction assumes that the reactive portion of the organic material oxidised by the sulphate reducers is at the oxidation state of carbohydrate. This is not necessarily the case as the organisms are generally restricted to organic acids and primary alcohols as electron donors for sulphate reduction and in the main do not utilize carbohydrates as reductants (Postgate, 1979). However, the stoichiometry for the overall reaction of two moles of carbon being oxidised by the reduction of one mole of sulphate, varies only slightly when other compounds are used as electron donors and the equation serves as a useful working model of sedimentary sulphate reduction. Assuming that sufficient sulphate reducing bacteria are present and that biophysical parameters such as temperature, pressure and Eh are within growth limits (factors that may however be important in the two deep sea stations A-1 and A-2, see below), it can be conjectured from the above equation that the activities of the sulphate reducing bacteria in the sediments examined will be rate limited by either electron acceptor (sulphate) or electron donor (organic carbon) availability.

In general the rate of sulphate reduction in marine

sediments is independent of electron acceptor (sulphate) concentration, although at low sulphate concentrations the process can become rate limited, exhibiting first-order kinetics with respect to sulphate. Although the exact values seem to vary (see Goldhaber and Kaplan, 1974) it would appear that sulphate reduction is only limited by electron acceptor availability at sulphate concentrations of below 2mM SO_4^{2-} (Benninger and Berner, cited in Goldhaber and Kaplan, 1974). Such a low level of sulphate is usually only encountered in marine sediments at great depth when the replenishment of sulphate is limited by the large diffusion distance. As a rule the rapid exchange of seawater from the overlying water to the surface sediment results in an interstitial concentration of sulphate similar to that in seawater (approx.30mM). As can be seen from the porewater sulphate concentrations presented in tables 4.2 to 4.6 in the appendix, levels of sulphate in the interstitial waters of the surface sediments were similar to those in the overlying waters and even at a depth of 60 cm in E-70 sediment (where the highest rates of sulphate reduction were recorded) sulphate was present at a concentration in excess of 10mM. Jørgensen (1977b) likewise found that the concentration of sulphate in the porewater of the surface 10 cm of a coastal sediment, a zone where 65% of the total sulphate reduction occurred, was very similar to that of the overlying water (27mM). Macrofaunal irrigation of the surface sediment can also increase the amount of sediment-overlying water interchange (Malcolm and Stanley, 1982) and has been reported in the inshore sediments examined (Stanley *et al*, 1978) and in coastal sediments (Grundmanis and Murray, 1977). Hence, it

was assumed that the activities of the sulphate reducing bacteria were not electron acceptor limited in any of the ten sediments examined.

It would therefore seem reasonable to suspect that sulphate reduction in the deep sea, coastal and inshore sediments examined was potentially carbon (*i.e.* electron donor) limited. Berner (1970) has demonstrated a linear relationship between organic carbon and the reduced sulphur compound pyrite (FeS_2, derived from sulphate reduction: $Fe^{2+} + HS^- \rightarrow FeS + H^+$; $FeS + S^o \rightarrow FeS_2$) in the surface 3 cm of coastal sediments, although this observation has been disputed by Jørgensen (1978c). However, Goldhaber and Kaplan (1974) have also shown a similar relationship using pooled data from published studies on a variety of marine sediments and concluded that the availability of organic carbon limited the rate of sulphate reduction and hence the sulphide pool and pyrite formation. Ramm and Bella (1974) have described the sulphate reduction rate in a model intertidal mud flat by a Michaelis-Menten equation in which the maximum reduction rate (R_{max}) was dependent on sulphate and available carbon (SOC, soluble organic carbon) concentration. As sulphate is generally in excess in surface marine sediments it follows that the activities of the sulphate reducing bacteria will be rate dependent on electron donor levels. That is, on organic carbon or more specifically, available (utilizable) carbon concentration.

There follows two consequences from this premise, namely, that the sediments in this study with high concentrations of organic material would tend to have a higher sulphate reduction activity and that this activity (sulphate and acetylene reduction

rates, bacterial numbers) would decrease with depth down the sediment as labile organic material was progressively degraded and made less utilizable. Activities in the ten sediments were compared using data obtained from the surface 0-5 cm of sediment only as activity is typically at a maximum in this surface layer, where transport from the sediment-water interface results in the highest concentrations of electron donors and acceptor (Nedwell, 1982). Such maxima have been reported in studies on sulphate reduction rates (*e.g.* Sorokin, 1962; Ivanov, 1968; Jørgensen *et al*, 1978; Sørensen *et al*, 1979; Aller and Yingst, 1980; Winfrey *et al*, 1981); counts of sulphate reducing bacteria (*e.g.* Sorokin, 1962; Hallberg, 1973; Ivanov, 1978; Marty, 1981) and on acetylene reduction rates (*e.g.* Marsho *et al*, 1975; Whitney *et al*, 1975; Teal *et al*, 1979). Hence, this method of comparing sulphate reducing bacteria activity between varied marine sediments was deemed to be reasonable and the data obtained were compared to one another and to those in other studies in tables 2.18, 2.19 and 2.21.

It can be seen from table 2.18 that there was a progression in the rate of sulphate reduction in the sediments examined with activity tending to increase with increasing organic content (see table i) and decreasing depth - a trend which in part reflected the quantity and quality of electron donors available to the sulphate reducing bacteria in the sediments. The absence of sulphate reduction in sediment from sampling station A-1 on the N.E. Atlantic abyssal plain and the low rate detected in A-2 sediment from the continental slope were however, probably caused by a combination of factors. Deep

sea sediments such as A-1 (depth 4,920m) and A-2 (2,880m) are typically low in organic carbon (approx.0.3%C) due to the efficient bacterial degradation of organic matter (either from land run-off, decay of dead organisms, algal extracellular products or animal excretory products) in the water column, which results in very little of it reaching a depth greater than 200 to 300m - that which does tends to be refractory, complex material (Riley and Chester, 1971). Obviously, this results in a lack of electron donors for dissimilatory sulphate reduction in A-1 and A-2 sediments and must be a major contributory factor to the low rates of sulphate reduction encountered. Cortecci (1975) concluded that the negligible sulphate reduction occurring in South Pacific sediments was due to the very small concentration of organic matter present, a conclusion also reached by Ivanov and co-workers (1980) on bottom sediments from the Indian Ocean. The low sedimentation rates in A-1 and A-2 sediments, together with the high dissolved oxygen concentration in the overlying water also results in oxidised sediments with Eh values in excess of +300mV in the surface 4 cm of sediment. However, although sulphate reducing bacteria require a redox potential of between -150 and -200mV before the initiation of sulphate reduction the process can occur within reduced microenvironments in otherwise oxidised marine sediments. Jørgensen (1977a) detected sulphate reduction within the oxidised (Eh + 250 to + 300mV) surface layer of a coastal sediment using a radiotracer method, together with sulphate reducing bacteria and the reduced sulphur compound pyrite. He demonstrated that sulphate reduction was occurring

TABLE 2.18 SULPHATE REDUCTION RATES IN THE SURFACE LAYERS OF MARINE SEDIMENTS

Location	Depth	Sediment Layer	Sulphate Reduction Rate nmol SO_4^{2-} ml^{-1} d^{-1}	Reference
DEEP SEA				
N.E. Atlantic A-1	4,920m	0-5 cm	0	This study
JOIDES Site 148	-	surface	$2x10^{-4}$	Tsou *et al.* 1973
Cariaco Trench	-	surface	0.1	Tsou *et al*, 1973
N.E.Atlantic A-2	2,880m	0-5 cm	0.1 *	This study
Black Sea	2,000m	surface	0.8-38 *	Sorokin, 1962
Gulf of California	3,260m	surface	6.0	Ivanov, 1978
Indian Ocean	1,820m	0-5 cm	7.0	Ivanov, 1978
COASTAL				
N.E.Atlantic A-3	158m	0-5 cm	1.0 *	This study
Pacific Ocean	140m	surface	3.0	Ivanov, 1978
Bay of Kiel	-	surface	8.2	Hartmann & Nielson, 1969
Black Sea	200m	surface	29-68 *	Sorokin, 1962

TABLE 2.18 (continued)

Location	Depth	Sediment Layer	Sulphate Reduction Rate nmol SO_4^{2-} ml^{-1} d^{-1}	Reference
INSHORE				
Lynn of Lorne LY-1	47m	0-5 cm	0.1 *	This study
Loch Creran CR-1	13m	0-5 cm	4.0 *	This study
" " CR-2	18m	0-5 cm	14.0 *	This study
Randers Fjord	0.5m	0-5 cm	12-52	Sørensen *et al*, 1979
Loch Eil E-24	30m	0-5 cm	15.5 *	This study
Limfjorden	4-12m	0-10 cm	25-200	Jørgensen, 1977b
Kysing Fjord	0.5m	0-5 cm	39-101	Sørensen *et al*. 1979
Colla Firth CF-1	24m	0-5 cm	39.3 *	This study
Brittany Coast	-	0-5 cm	65-480 *	Winfrey *et al*, 1981
Sullom Voe D-4	44m	0-5 cm	103.3 *	This study
Loch Eil E-70	49m	0-5 cm	147.5 *	This study
San Francisco Bay	-	surface	230 *	Oremland & Silverman, 1979
Aarhus Bay	-	surface	439-1013	Jørgensen, 1977b

* potential sulphate reduction rate

within reduced microsites (microniches) formed by the action of aerobic bacteria on small (50 to 200μm diam.) organic particles such as faecal pellets from marine animals. Similar observations have been made by Sørensen *et al*, (1979) on oxidised sediments in two other Danish fjords and it would appear that sulphate reduction was occurring in A-2 sediment within such anaerobic microniches. That these microenvironments exist in A-2 sediment can be inferred from the data of Hartwig and Stanley (1978) who found that the anaerobic process of heterotrophic nitrogen fixation in A-2 sediment occurred at the same low rate regardless of whether the sediment sample was incubated under an aerobic (80% Ar and 20% O_2) or an anaerobic atmosphere (Ar only).

As sediments from sampling stations A-1 and A-2 had the same low carbon content the question therefore arises as to why there was no detectable activity in A-1 sediments? One important constraint on sulphate reduction, or any bacterial activity, in the two sediments is that of pressure - 490 atmospheres (atm.) and 290 atm. at A-1 and A-2 respectively. Such pressures can inhibit bacterial growth by altering the activities of enzymes, diminishing cell permeability or the active transport of nutrients and by inhibiting macromolecular synthesis (Albright and Hardon, 1974). It could therefore be that pressure exerted an indirect effect on the sulphate reducing bacteria in A-1 sediment by depressing the activities of the heterotrophic microorganisms, causing a reduction in metabolites for sulphate reduction or a decrease in aerobic metabolism and hence anaerobic microniche formation. Many bacteria have a decreased growth rate at a hydrostatic pressure

of 200 atm. and are killed or fail to grow above 500 atm. (ZoBell and Johnson, 1949; Oppenheimer and ZoBell, 1952). This may account for the lack of activity in A-1 sediment compared with A-2 sediment whose microflora are under a pressure of some 200 atm. less than those from A-1. Alternatively, the high hydrostatic pressure at A-1 could exert a direct inhibition of dissimilatory sulphate reduction, although Wood (1965) has demonstrated that sulphate reduction in freshly isolated, shallow water sulphate reducing bacteria was not retarded by a pressure in excess of 500 atm. However, ZoBell and Morita (1957) found that an obligate barophillic sulphate reducer isolated from a depth of 7,000 to 10,000m required incubation for 10 months at 700 atm. and 4°C before growth was demonstrable. Hence, sulphate reduction in A-1 sediment may be proceeding too slowly for detection by the methods used in this study, although the use of the radiotracer ^{35}S is a technique of high sensitivity (Jørgensen, 1978a). Alternatively the rapid decompression on recovery of A-1 sediment cores (approx. 490 atm. in 90 minutes) may have disrupted any microenvironments present and affected the sulphate reducing bacteria and their rate of metabolism (Jørgensen, 1978b). It would therefore appear that the lack of sulphate reducing bacteria activity in N.E. Atlantic A-1 sediment was not solely a reflection of low carbon availability but of a combination of factors such as pressure, Eh and temperature.

The rate of sulphate reduction at station A-2 on the continental slope was within the general range of 10^{-4} to 10 n mol SO_4^{2-} $ml^{-1}d^{-1}$ for deep sea sediments, although it was

lower than the mean rate of 14 n mol SO_4^{2-} $ml^{-1}d^{-1}$ recorded by Sorokin (1962) at a comparable depth in the Black Sea. The Black Sea sediments, however, are much richer in organic material and are consequently more reduced than N.E. Atlantic ones, though Sorokin did find a similar difference in activity between the slope sediments and the bottom deposits, attributable to a lower concentration of assimilable organic material.

The highest sulphate reduction rate measured in the N.E. Atlantic sediments examined was from station A-3 on the continental shelf of the British Isles. Due to its shallower depth and vicinity to land, A-3 sediment receives less refractile organic matter at a higher sedimentation rate than either the slope or abyssal sediments and has a higher, though still low, organic carbon content (0.5%C). The resulting sulphate reduction rate was ten times that measured in A-2 slope sediment and was in agreement with the observation of Ivanov (1978) who calculated that whilst the total area of the continental shelf is only half that of the continental slope, the shelf sediments actually reduce more sulphate on an annual basis due to their higher activity (572×10^6 tons S yr^{-1} compared with 375.5×10^6 tons S yr^{-1} for the continental slope). Again, the measured rate of sulphate reduction at A-3 was at the lower end of the published range for coastal marine sediments ($10-10^2$ n mol SO_4^{2-} $ml^{-1}d^{-1}$). However, most of the published data for sulphate reduction rates in coastal and deep sea sediments are for areas of reduced sediment such as the Black Sea (see above), JOIDES Site 148 and the Cariaco Trench (anoxic basins). Likewise, the rate value for the Pacific Ocean station at a comparable depth to A-3 (Ivanov, 1978) is in fact a value for

an anoxic sediment (Eh<-100mV) of relatively high organic carbon content (3%C) which was in contrast to A-3 sediment (Eh>+365mV; 0.5%C). This serves to highlight the need for more information on the activities of the sulphate reducing bacteria in deep sea and coastal sediments in areas where their presence is not immediately obvious.

The inshore sediments examined were typically areas of higher organic content (3 to 20%C) than either the coastal or deep sea stations. This is due to their higher rates of primary productivity and inputs of terrestrial organic matter (especially river run-off) coupled with their shallower depth (13 to 49m), which results in less detrital mineralization in the water column. The incorporation of this less refractile organic material into the sediments and its subsequent break-down tends to result in sediments which are strongly reducing below a shallow, oxidised surface layer. As can be seen from table 1, of the inshore sediments examined, only stations LY-1 and CR-2 had a positive redox potential at a depth of 4 cm. It can be seen from table 2.18 that these sediments had the highest rates of sulphate reduction in this study, with most rates lying within the typical range for inshore marine sediments of 10 to 10^3n mol SO_4^{2-} $ml^{-1}d^{-1}$. The low activity measured at station LY-1 in the Lynn of Lorne was a reflection of its relatively low carbon content and its consequent oxidised nature (Eh+100mV at 4 cm) which had restricted sulphate reduction to anaerobic microniches within the sediment.

The relatively low sulphate reduction rate at station CR-1 in Loch Creran appears paradoxical since the sediment was highly reduced with an extremely high organic carbon content

(20%C) owing to its gross contamination by alginate waste effluent from the nearby Barcaldine factory. Bubbles of methane gas have been frequently observed emanating from sampling station CR-1 (close to the effluent dump) suggesting that a high rate of methanogenesis was occurring within the sediment. As sulphate reduction and methanogenesis tend to be mutually incompatable, either due to hydrogen competition or sulphide toxicity (see Nedwell, 1982) this would suggest that unlike most marine sediments methanogenesis was the predominant means of anaerobic oxidation in CR-1 sediment. However, this generally only occurs when sulphate is depleted to levels of less than 2mM SO_4^{2-} under conditions of high nutrient loadings and biochemical oxygen demand (Mountfort and Asher, 1981), and as can be seen from table 4.5 in the appendix, sulphate levels in CR-1 sediment were consistantly above 20mM. Appreciable levels of sulphide were also encountered in CR-1 sediment at the time of sampling (F.Drake, unpublished data) which suggests that active sulphate reduction was occurring. One possible explanation for the low measured sulphate reduction rate is that the low levels of the redox poising agent sodium thioglycollate in the reaction flasks could have inhibited the sulphate reducing bacteria present in the sediment samples. Thioglycollate has been reported to be harmful to many of the recently discovered genera of sulphate reducing bacteria (Pfennig *et al*, 1981) and if such organisms were present, could inhibit their growth and respiration. Two such organisms are *Desulfobulbus propionicus* (Widdel, 1980) which degrades propionate to acetate and CO_2, and *Desulfovibrio sapovorans* (Widdel, 1980) which can

degrade long-chain, odd-numbered, fatty acids to propionate and acetate. The activity of such organisms has recently been demonstrated in a marine sediment (Sørensen *et al*, 1981) and counts of upto 10^3 propionate-oxidising bacteria ml^{-1} have been recorded in sediment from the Ems-Dollard estuary in the Netherlands (Laanbroek and Pfennig, 1981). It is conceivable that under conditions of low sulphate concentration, which might occur within microenvironments of organic particles being degraded by heterotrophic and sulphate reducing bacteria, sulphate reducing bacteria such as *Desulfobulbus spp.* could be involved in the production of acetate with the concomitant use of hydrogen-utilizing methanogens as the electron acceptor (Mountfort and Asher, 1981). Recent calculations by Nedwell (1982) however, have suggested that the observed co-existence of sulphate reduction and methanogenesis in a U.K. saltmarsh is not due to sulphate-depleted microenvironments but rather to the methanogens persisting within the same environment, although only utilizing a negligible portion of the common environmental resource. However, methanogenesis was only occurring at some three orders of magnitude less than sulphate reduction and it was clear that methanogenesis in CR-1 sediment was in fact occurring on a large scale.

Intermediary sulphate reduction rates in the inshore sediments were measured in samples from stations CR-2 in Loch Creran, E-24 in Loch Eil and CF-1 in Colla Firth in the Shetland Islands. The sediments had increasing organic carbon levels and correspondingly decreasing redox potentials over the surface 4 cm of sediment (table i) which were reflected in their progressively increasing sulphate reduction rates.

Loch Creran CR-2 sediment was unaffected by any alginate effluent, whilst E-24 sediment from the head of Loch Eil, although not as polluted as E-70 sediment in the inner basin, was still subject to an elevated organic input *via* cellulose effluent from the pulp and paper mill at Annat Point (fig.ii). Colla Firth sediment was naturally rather high in organic carbon and was subjected to relatively high deposition rates of organic material through terrestrial run-off from the surrounding hills. The activities measured were of the same order as those recorded by Jørgensen (1977b) in similar fjordic sediments of comparable organic content (1 to 13%C).

The highest rates of sulphate reduction in this study were recorded in the surface sediments from the inner basins of two sea lochs (fjords), namely, station D-4 in Sullom Voe and E-70 in Loch Eil. The two sediments were in many respects similar, being in areas of high organic deposition, the resulting degradation of which resulted in reduced sediments with low Eh values of below -150mV at 4 cm depth. However, at the time of sampling (June, 1980) the bottom water in the inner basin of Sullom Voe was anoxic due to a combination of hydrographic and meteorological conditions (Stanley *et al*, 1981). This isolation of the overlying water and the high biological oxygen demand in the sediments resulted in oxygen depletion of the overlying water coupled with maximum reduction of the sediment - a situation that has been observed in other fjords (Jørgensen, 1977b). This anoxic environment and high organic carbon levels were reflected in the high sulphate reduction rate measured.

In contrast to the inner basin sediment of Sullom Voe, rapid renewal of the overlying water in Loch Eil has resulted

in water which is high in dissolved oxygen above E-70 sediment despite the extremely high organic loading. The station is situated approximately 2 km from the pulp and paper mill effluent discharge pipe and received a high organic matter input of effluent which was over 90% cellulose (Vance *et al*, 1979). The sediment was therefore highly reduced and had been shown by Duff (1981) to have a very high proportion of available (utilizable) organic matter, resulting in the very high sulphate reduction rate measured. This high rate of organic degradation in E-70 sediment has maintained the carbon content of the sediment at a steady level despite the high input of cellulose, an observation which is discussed in greater detail below.

As can be seen from table 2.19, sulphate reducing bacteria were recovered from both slope and shelf sediments in the N.E. Atlantic and as mentioned previously were assumed to be existing within reduced microniches in an otherwise oxidised sediment. Numbers of sulphate reducers of the order of 10^2 ml^{-1} have been demonstrated in such sediments (Tezuka, 1979; Jørgensen, 1977a) and it would appear that numbers of sulphate reducing bacteria were not a limiting factor for dissimilatory sulphate reduction in A-2 and A-3 sediments. No sulphate reducing bacteria could be detected in abyssal A-1 sediment for reasons already mentioned. Although the general trend of the published counts was to increase with decreasing depth, and presumably increasing sediment organic content, the numbers of sulphate reducing bacteria recovered from the inshore sediments examined in this study were all very similar at between $3x10^2$ and $7.9x10^3$ bacteria ml^{-1}. These values, whilst being comparable to those obtained by other

TABLE 2.19 COUNTS OF SULPHATE REDUCING BACTERIA IN THE SURFACE LAYERS OF MARINE SEDIMENTS

Location	Depth	Sediment Layer	Sulphate Reducing Bacteria (ml^{-1} sediment)	Reference
DEEP SEA				
N.E.Atlantic A-1	4,920m	0-5 cm	0	This study
N.E.Atlantic A-2	2,880m	0-5 cm	present	This study
Gulf of California	3,260m	surface	8.3	Ivanov, 1978
Indian Ocean	1,820m	0-5 cm	50	Ivanov, 1978
Oman Sea	4,010m	0-2 cm	$7x10^2$	Marty, 1981
Black Sea	2,000m	surface	$8x10^2$-$5x10^3$	Sorokin, 1962
Gulf of Oman	2,900	0-2 cm	$2.5x10^3$	Marty, 1981
Gulf of Aden	4,727m	0-2 cm	$5x10^3$	Marty, 1981
COASTAL				
N.E.Atlantic A-3	158m	0-5 cm	present	This study
Pacific Ocean	140m	surface	12.5	Ivanov, 1978
Futami Bay	50m	0-5 cm	$4.3x10^2$	Tezuka, 1979
Dutch Wadden Sea	-	surface	$5.7x10^3$-$7x10^4$	Vosjan, 1974
Gulf of Tadjoura	210m	0-2 cm	$8x10^3$	Marty, 1981
Black Sea	shelf	surface	10^4	Sorokin, 1962

TABLE 2.19 (continued)

Location	Depth	Sediment Layer	Sulphate Reducing Bacteria (ml^{-1} sediment)	Reference
COASTAL (cont.)				
Tokyo Bay	900m	0-2 cm	1.1×10^4	Tezuka, 1979
INSHORE				
Hiroshima Bay	3-32m	surface	10^2-10^5	Kimata *et al*, 1955a
Lynn of Lorne	47m	0-5 cm	3.0×10^2	This study
Futami Bay	40m	0-5 cm	4.3×10^2-7.5×10^2	Tezuka, 1979
Brittany Coast	-	0-5 cm	10^3	Winfrey *et al*, 1981
Loch Eil E-70	49m	0-5 cm	1.0×10^3	This study
Loch Eil E-24	30m	0-5 cm	1.2×10^3	This study
Loch Creran CR-1	13m	0-5 cm	2.3×10^3	This study
Colla Firth CF-1	24m	0-5 cm	3.3×10^3	This study
Kiel Bight	20m	surface	3.5×10^3-1.7×10^4	Bansemir & Rheinheimer, 1970 (cited in Schneider 1977)

TABLE 2.19 (continued)

Location	Depth	Sediment Layer	Sulphate Reducing Bacteria (ml^{-1} sediment)	Reference
INSHORE (cont)				
Sullom Voe D-4	44m	0-5 cm	4.9×10^3	This study
Loch Creran CR-2	18m	0-5 cm	7.9×10^3	This study
Limfjorden	4-12m	0-10 cm	4.3×10^4-9.3×10^4	Jørgensen, 1977b
Tokyo Bay	13-82m	0-2 cm	7.2×10^4-5.6×10^5	Tezuka, 1979

workers on similar sediments, did not reflect the differences in organic content or indeed sulphate reduction rate between, for example, LY-1 or E-70 sediments. A similar situation has been reported by Jørgensen (1977b) in Limfjorden sediments where the numbers of bacteria did not reflect a 60% increase in sulphate reduction rate or an increase in organic content between sampling stations. It would appear, therefore, that the numbers of sulphate reducing bacteria in the sediments examined indicated the potential sulphate reduction activity, with the actual activity depending on factors such as electron donor supply, Eh and pressure.

That differing organic loadings have tended to affect the activity and not necessarily the population of sulphate reducing bacteria in the surface 5 cm of the sediments examined can be demonstrated by calculating the specific rate of metabolism (sulphate reduction) for the enumerated bacteria. It can be seen from table 2.20 that sulphate reducing bacteria from sediments with low organic inputs such as LY-1 and CR-2 had a lower rate of metabolism than those organisms from areas of higher organic deposition such as D-4 and E-70. The specific rates of metabolism calculated in table 2.20 tend to lie within the range of values calculated by Jørgensen (1978c) for surface, coastal sediments, namely 10^{-12} to 10^{-11} mol SO_4^{2-} bacterium^{-1} d^{-1}. Jørgensen's calculations were made using data obtained from sediments of relatively high carbon content (*e.g.* Black Sea, Wadden Sea) which would account for the calculated rate for LY-1 bacteria lying outwith his range of values. These rates are upto a thousand-fold higher than those for sulphate

TABLE 2.20 CALCULATED SPECIFIC RATES OF METABOLISM OF SULPHATE REDUCING BACTERIA IN INSHORE MARINE SEDIMENTS

Location	Rate (mol SO_4^{2-} bacterium^{-1} d^{-1})	% Carbon
Lynn of Lorne LY-1	3.3×10^{-13}	2.4
Loch Creran CR-2	1.8×10^{-12}	2.0
Loch Creran CR-1	1.7×10^{-12}	20.0
Colla Firth CF-1	1.2×10^{-11}	8.0
Loch Eil E-24	1.3×10^{-11}	5.3
Sullom Voe D-4	2.1×10^{-11}	9.6
Loch Eil E-70	1.5×10^{-10}	6.2

reducing bacteria growing in pure culture under optimum conditions - a phenomenon which is discussed in greater detail elsewhere in this section.

As can be seen from table 2.20 the trend of increasing metabolic rate with increasing sediment organic content is only an approximate relationship, even ignoring the data for station CR-1 in Loch Creran. This is because dissimilatory sulphate reduction in marine sediments is dependent more on the availability (decomposability) of the organic material present and not on the overall carbon content of the sediment (Sorokin, 1962; Aller and Yingst, 1980). Lyons and Gaudette (1979) have demonstrated that sulphate reduction rate in a New Hampshire estuarine sediment was directly proportional to the availability of readily utilizable organic material in the sediment. No data was available on the levels of available carbon for sulphate reduction in most of the sediments examined so only approximate relationships between sulphate reducing bacterial activity and carbon input could be made. The organic carbon content of a marine sediment as indicated by % carbon data tends to reflect that material which is unavailable for sulphate reduction with the majority of the carbon being continuously mineralized (Jørgensen, 1978c). Hence, it was assumed that the organic carbon content of the sediments examined in this study reflected organic input and the levels of available carbon present in the sediment, though it seems clear that the measured organic carbon in stations such as the two Shetland ones included refractory material such as lignin, humic acids and in particular, peat (Pearson and Stanley, 1977; Stanley *et al*, 1981).

Recent calculations by Duff (1981) for E-70 sediment in the inner basin of Loch Eil have shown that over 70% of the organic matter in the sediment is capable of being metabolized by the sulphate reducing bacteria - a figure greater than that found in other comparable areas (*e.g.* Goldhaber and Kaplan, 1980). This may explain the observation that whilst high amounts of cellulose waste have been discharged into Loch Eil since 1966 (Pearson, 1981), there has been only a slight increase in cellulose levels and no net accumulation of cellulose in the loch (Stanley *et al*, 1978); although the redox potential of the sediments has reflected the levels of organic input (Pearson and Stanley, 1979). The cellulose is therefore being removed from the sediments by either resuspension and transportation out of the loch or by bacterial decomposition (Vance, 1977). The highly reduced nature of E-24 and E-70 sediments (table i) suggest the latter. That sulphate reducing bacteria are of great importance in the anaerobic mineralization of this cellulose input can be demonstrated as follows.

It has already been mentioned that the degradation of organic matter in inshore marine sediments occurs predominantly in the surface 10 cm (*e.g.* Sørensen *et al*, 1979). The average sulphate reduction rate over this depth in the effluent affected inner basin sediments at station E-70 in Loch Eil was 91.8 n mol SO_4^{2-} $ml^{-1}d^{-1}$ which when recalculated on an area basis gives a sulphate reduction rate of 0.29 g S $m^{-2}d^{-1}$. As the largest organic input into the sediment was cellulose and other structural carbohydrates (Pearson, 1982) it seems reasonable that the organic matter had an average elemental composition

of (CH_2O). Assuming that the oxidation of this material occurred according to the equation of Berner (1974) described earlier in which 1 g of carbon is oxidised for each 4/3 g of sulphate reduced (Goldhaber and Kaplan, 1974) then the equivalent of 4/3 x 0.29 = 0.392 g org.C $m^{-2}d^{-1}$ or 143 g org.C $m^{-2}yr^{-1}$. was oxidised by sulphate reducing bacteria. Vance (1977) working on Loch Eil sediment in experimental tanks has demonstrated that a wood pulp loading of 219 g $m^{-2}yr^{-1}$ resulted in an oxygen uptake rate of 766.5 g $m^{-2}yr^{-1}$. Assuming a respiratory quotient of 1.0 this value gives a respiratory carbon loss of 291 g C $m^{-2}yr^{-1}$ which is in good agreement with the calculated average organic input into Loch Eil of 290 g org C $m^{-2}yr^{-1}$ (Pearson, 1982). It therefore appears that input and decomposition rates in Loch Eil are balanced and that sulphate reducing bacteria are capable of degrading $\frac{143}{291}$ x 100 = 49% of the total carbon mineralized by the sediment each year. This figure may in fact be >49% as only the surface 10 cm of sediment was considered in the above calculation and upto 35% of the sulphate reducing activity in inshore sediments may occur below this depth (Jørgensen, 1977b). Nevertheless within the error of such estimations the value of 49% can be considered to be in good agreement with those published by other workers. Sulphate reduction has been shown to account for more than 50% of the total mineralization in a model estuarine sediment (Jørgensen and Fenchel, 1974) and for 53% in a Danish fjordic sediment (Jørgensen, 1977b). Lower contributions to carbon metabolism have been recorded in sediments from Long Island Sound (36%; Aller and Yingst, 1980) and from a Danish estuary (21%; Sørensen *et al*, 1979).

It can therefore be seen that sulphate reduction activity in Loch Eil E-70 has increased to metabolize a substantial proportion of the organic input, a fact further emphasized by the high specific rate of metabolism of its sulphate reducing microflora which was upto a hundred-fold greater than those calculated for the other inshore sediments examined. Such pulp mill effluent has been reported in the literature as increasing the activity of sulphate reducing bacteria in both coastal water (Kimata *et al*, 1955a) and in freshwaters (Sand *et al*, 1975). It is unclear as to what factor(s) limit sulphate reduction in a polluted, inshore sediment such as E-70 as supplies of electron donor and acceptor appear abundant. The high levels of sulphide present in the sediment (>600μM) may inhibit sulphate reduction, although in pure culture inhibition only occurs above a concentration of 10mM (Brown *et al*, 1973) and this question would obviously benefit from further research.

The acetylene reduction rates of the surface marine sediments investigated in this study lie towards the high end of the published range of rates (table 2.21). The rates show an increase in activity with increasing organic carbon content, although as mentioned above this does not necessarily reflect increasing amounts of available organic matter. However, the highest acetylene reduction rates recorded in this study of 0.27 to 0.74 n mol C_2H_4 prod.g dry $wt^{-1}hr^{-1}$ for the inner basin sediments of Loch Eil, Sullom Voe and from the alginate polluted site in Loch Creran were comparable to those obtained from intertidal sediments (Chesapeake Bay and Delaware Saltmarshes) which are typically areas of high productivity and

TABLE 2.21 ACETYLENE REDUCTION RATES IN THE SURFACE LAYERS OF MARINE SEDIMENTS

Location	Depth	Sediment Layer	Acetylene Reduction Rate (nmol C_2H_4 prod.g dry $wt^{-1}hr^{-1}$)	Reference
N.E.Atlantic A-3	158m	0-10 cm	5×10^{-4}	Hartwig & Stanley, 1978
N.E.Atlantic A-2	2,880m	0-1 cm	1.3×10^{-3}	Hartwig & Stanley, 1978
North Sea	16-50m	0-5 cm	0.01-0.1	Blake, 1980
Tampa Bay	Intertidal	0-5 cm	0.03-0.6	Zuberer & Silver, 1978
Tay Estuary	Intertidal	0-5 cm	0.05-0.14	Herbert, 1975
Tokyo Bay	30m	0-5 cm	0.06	Maruyama *et al.* 1974
Loch Creran CR-2	18m	0-5 cm	0.06	This study
Loch Etive	23-55m	0-5 cm	0.06-0.28	Blake & Leftley, 1977
Barbados	0.5m	surface	0.1	Patriquin & Knowles, 1975
Lynn of Lorne LY-1	47m	0-5 cm	0.12	This study
Chesapeake Bay	Intertidal	0-5 cm	0.2 - 12.5	Marsho *et al*, 1975
Loch Eil E-70	49m	0-5 cm	0.27	This study
Sullom Voe D-4	44m	0-5 cm	0.29	This study
Delaware	Intertidal	0-1 cm	0.4 - 10.2	Dicker & Smith, 1980a
Loch Creran CR-1	13m	0-5 cm	0.74	This study

detrital organic content (Nedwell, 1982). The acetylene reduction rate for Loch Creran CR-2 sediment was comparable to that obtained by Blake and Leftley (1977) in a similar, unpolluted Scottish west coast sea-loch. The activity recorded in the oxidised sediment from station LY-1 in the Lynn of Lorne was surprisingly twice that of the more reduced Loch Creran sediment and it was again thought that the essentially anaerobic process of heterotrophic nitrogen fixation was occurring within reduced microniches at LY-1. This has been shown to be the case in oxidised carbonate sand from the coast of Barbados (Patriquin and Knowles, 1975) and in N.E.Atlantic oxic sediments (Hartwig and Stanley, 1978).

The measured rates of acetylene reduction were assumed to be of heterotrophic bacterial origin as incubation was performed in the dark. Cyanobacteria have been shown to be capable of low rates of activity in the absence of light (Smith *et al*, 1971) but were considered to have made only a negligible contribution to the measured rates of acetylene reduction. Heterotrophic nitrogen fixation within marine sediments is thought to be primarily limited by a lack of oxidisable carbon owing to the low efficiencies (per unit energy substrate consumed) of the process and to the intense competition within the marine environment for the generally low concentrations of available organic matter (Stewart, 1969; Patriquin and Knowles, 1975). Hence, supplementation with organic substrates of many marine sediments has resulted in increased acetylene reduction rates (*et al*, Maruyama *et al*, 1974; Herbert, 1975). This may account for the elevated activities recorded in the examined inshore sediments which tended to have reasonably high organic

contents (table i) and have been shown in the case of Loch Eil E-70 sediment to have high proportions of metabolizable organic matter. In fact, supplementation of E-70 sediment with glucose resulted in no stimulation of acetylene reduction activity (results not shown) which was in contrast to sediments with presumably lower available carbon contents (see Maruyama *et al*, 1974; Herbert, 1975; Marsho *et al*, 1975).

It has already been stated that sulphate reducing bacteria are thought to be the predominant heterotrophic, nitrogen fixing bacteria under marine (*i.e.* 0.4M NaCl) conditions (Herbert, 1975; Blake and Leftley, 1977). They have been recovered in fixed nitrogen-free media from a variety of marine sediments in appreciable numbers (Herbert, 1975; Patriquin and Knowles, 1975; Blake, 1980) and were capable of contributing around 50% of the acetylene reduction activity in a Delaware salt marsh, although only present in relatively low numbers (Dicker and Smith, 1980b). Although sulphate reducing bacteria were not enumerated on fixed nitrogen-free media in this study many strains of *Desulfovibrio* are capable of fixing atmospheric nitrogen (Postgate, 1979), and Blake *et al*, (1982) have concluded that most of the sulphate reducing bacteria present in similar fjordic sediments were capable of fixing nitrogen. It was therefore assumed that as acetylene reduction (nitrogen fixation) activity in marine sediments is not controlled by bacterial numbers (Dicker and Smith, 1980b) and as between 10^2 and 10^3 sulphate reducers ml^{-1} were present in the sediments examined (table 2.19) that acetylene reduction was not limited by bacterial numbers. This assumption was confirmed somewhat by the observation that there was no clear

relationship between the counts of sulphate reducing bacteria in the sediments studied and the measured acetylene reduction rates. A similar lack of correlation has been reported in other marine sediments (Blake and Leftley, 1977; Dicker and Smith, 1980b) and implied that some other factor influenced the acetylene reducing activity.

The reduction of one molecule of nitrogen to two molecules of ammonia requires on average twelve molecules of adenosine-5'-triphosphate (Jones, C.W., 1982) and as already mentioned this often results in heterotrophic nitrogen fixation in marine sediments being energy limited, as indicated by substrate supplementation experiments. It would therefore seem reasonable to assume that if sulphate reducing bacteria were the predominant heterotrophic nitrogen fixers in the sediments examined there would be a close correlation between sulphate reduction (*i.e.* respiration) rates and acetylene reduction rates. This in fact appeared not to be the case with sediments such as E-70 and D-4 having a thousand-fold the respiratory activity of LY-1 sediment but only twice the acetylene reduction rate. This lack of correlation is explicable in two possible ways - either other bacteria were the predominant nitrogen fixers or that activity was inhibited in sediments such as E-70 and D-4.

Much of the evidence for the participation of sulphate reducing bacteria in marine, heterotrophic nitrogen fixation has come from the work of Herbert (1975) on Tay Estuary sediments. He found that of the bacteria isolated on fixed nitrogen-free media (*Desulfovibrio*, *Azotobacter*, *Klebsiella*,

Enterobacter and *Clostridium spp.*) only *Desulfovibrio* and *Clostridium spp.* could fix nitrogen under marine (salinity 23‰)conditions and as the former were numerically the predominant organisms concluded that sulphate reducing bacteria were the principle nitrogen fixers in the sediments. A similar conclusion was reached by Jones, K. (1982) during studies on estuarine, intertidal sediments in Lancashire. However, in addition to *Desulfovibrio spp.* he also implicated salt-tolerant *Klebsiella pneumoniae* as important contributors to the measured nitrogen fixing activity of the sediments. *Azotobacter spp.* have been isolated from several marine, estuarine and intertidal regions and show a preferred salinity of 25 to 30‰ for optimum growth and acetylene reduction (Lakshmanaperumalsamy *et al*, 1975; Dicker and Smith, 1981). Werner *et al* (1974) have isolated nitrogen fixing *Klebsiella* and *Enterobacter spp.* from the Oregon coast which were active at salinities up to 23‰. Active acetylene reducing activity has also been found in *Klebsiella sp.* isolated from marine areas in Canada which were affected by pulp and paper mill effluent (Knowles *et al*, 1974), a situation similar to that found in Loch Eil. That bacteria other than sulphate reducers may be contributing to heterotrophic nitrogen fixation in inshore sediments can be inferred from the published substrate supplementation experiments. These typically use compounds such as glucose, sucrose and mannitol as added energy sources (*e.g.* Maruyama *et al*, 1974; Marsho *et al*, 1975; Dicker and Smith, 1980a) which are preferred growth substrates for nitrogen fixing organisms such as *Azotobacter, Aerobacter, Bacillus* and *Clostridium spp.* (Stanier *et al*, 1977) and are not widely used by *Desulfovibrio*

spp. (Postgate, 1979). Paradoxically, known electron donors for sulphate reduction such as lactate, malate and succinate were more stimulatory under aerobic and not anaerobic conditions in a Florida intertidal sediment (Zuberer and Silver, 1978). However, the lag periods common in such experiments may result in the fermentation of sugars such as glucose into utilizable substrates for sulphate reduction, resulting in increased acetylene reduction activity.

An alternative nitrogen reducing microflora may perhaps be an explanation for the high acetylene reduction rate of 0.75 n mol C_2H_4 prod. g dry $wt^{-1}hr^{-1}$ in the surface 5 cm of Loch Creran CR-1 sediment. High levels of methane production have been observed in this sediment and methanogenic bacteria were assumed to be present. *"Methanobacterium omelianskii"* a methanogenic nitrogen-fixer has been reported to be present in marine sediments (Barker, 1940), although it is now known that the organism is in fact an association between an anaerobic bacterium and a methanogen (Stanier *et al*, 1977). Nonetheless, such consortia of bacteria may have contributed to the high acetylene reduction rate recorded in CR-1 sediment.

As was mentioned in the first section of this thesis the inshore sediments examined had characteristically high levels of interstitial ammonia. This was thought to originate either from dissimilatory nitrate reduction or from the decomposition of organic matter (ammonification). Additionally, sulphate reduction itself can result in large amounts of ammonia being released into the sediment porewater as shown by the following equation for the oxidation of marine organic matter of "average composition" (Richards *et al*, 1965):

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 53\ SO_4^{2-} \rightarrow 39CO_2 + 67HCO_3^- + 16NH_4^+ + HPO_4^{2-} + 53HS^- + 39H_2O$$

These processes have resulted in the surface 5 cm sediments having average ammonia levels of 53μM (CR-2), 21μM (LY-1), 677μM (E-70), 380μM (D-4) and 102μM (CR-1). That is, sediments with higher organic inputs such as E-70 and D-4 tending to have higher ammonia levels than sediments with lower organic loadings (*e.g.* LY-1). Ammonia has been reported to inhibit bacterial nitrogenase activity (Stewart, 1969) at interstitial water concentrations of around 200μM NH_4^+ in marine sediments (Teal *et al*, 1979). As a consequence, the higher levels of sulphate respiration measured in the organically rich E-70 and D-4 sediments (380 to 677μM NH_4^+) would not necessarily result in vastly greater acetylene reduction rates than those recorded in sediments of lower respiratory activity and organic loading (*e.g.* LY-1, 21μM NH_4^+). Furthermore, ammonium ions are the conventional nitrogen source for sulphate reducing bacteria (Postgate, 1979) and if present in the inshore sediments examined would be used in preference to dissolved nitrogen for the following reasons. Senez (1962) has demonstrated that the molar growth yield and exponential growth rate of a nitrogen fixing *Desulfovibrio sp.* growing on N_2 as the sole nitrogen source were about half the values obtained during growth on ammonium chloride as nitrogen source. However, the cellular rate of catabolic activity (quantity of carbon substrate metabolized per g of cells per hour) was unaffected and Senez concluded that the organisms produced energy and ATP at a constant rate regardless of the rate at which these

compounds were required for biosynthetic activity. Hence, when sulphate reducing bacteria are fixing N_2 their growth is limited by nitrogen availability which results in energy being wasted as heat. In addition, it has been shown that in marine carbonate sand there is an inverse relationship between the efficiency of nitrogen fixation and substrate concentration, which may limit nitrogen fixation rates (Patriquin and Knowles, 1975). Hence, in the highly competative sediment environment, even ones such as E-70 and D-4 which are organically (*i.e.* energy) rich, it is energetically more favourable for sulphate reducing bacteria to utilize fixed nitrogen sources such as ammonia than to fix nitrogen.

The question therefore arises as to why any acetylene reduction activity at all was measured in ammonia rich sediments such as those in the inner basins of Loch Eil and Sullom Voe? It has been reported that many nitrogen fixing bacteria occur with other heterotrophs on or in suspended particles in the aquatic environment and utilize the components of these particles as nutritional sources (Kawai and Sugahara, 1972). The dependence of sulphate reducing bacteria on other heterotrophic bacteria for the supply of electron donors within marine sediments is well known (*e.g.* Vacelet, 1971; Cahet, 1975) and it was possible that such aggregations of sulphate reducing and heterotrophic bacteria could have resulted in ammonia deficient microenvironments where heterotrophic nitrogen fixation could have occurred. Jones *et al*, (1980) have suggested that carbon supplementation in an English freshwater lake resulted in the activation of nitrogenase by the stimulation of bacterial growth and the concomitant utilization of ammonia.

THE ACTIVITY OF SULPHATE REDUCING BACTERIA WITH DEPTH IN INSHORE MARINE SEDIMENTS

Loch Eil E-70

It can be seen from figure 2.3 that there was a rapid decrease in metabolic activity with increased sediment depth, a feature that is common to many marine sediments (Aller and Yingst, 1980). The decrease in the observed sulphate reduction rate by four-fold in the surface 10 cm of sediment was probably due to carbon availability as factors such as sulphate supply, Eh and numbers of sulphate reducing bacteria would not appear to be rate limiting. The organic detritus reaching the sediment is rapidly degraded by heterotrophic bacteria in the surface layers with the result that the organic content of the sediment decreases with increasing depth and consists of more recalcitrant molecules as labile compounds are progressively metabolized. Data for this station reported by Duff (1981) have shown that the concentration of metabolizable carbon was a function of sediment depth with the organic carbon as a whole decreasing to a constant value of 1% dry wt. at a depth of around 30 cm. This 1% is presumably carbon that is inert to sulphate reducing bacteria and would account for the low sulphate reduction rate of 4.0 nmol SO_4^{2-} $ml^{-1}d^{-1}$ recorded at a depth of 20 to 25 cm in this sediment. As heterotrophic bacteria also tend to decrease with increasing sediment depth (Rittenberg *et al*, 1955) the observed decrease in sulphate reducing activity may have been due additionally to a depression in the conversion of organic matter into suitable electron donors for dissimilatory sulphate reduction (Nedwell and

Floodgate, 1972). This has been suggested by Sorokin (1962) as a possible explanation for the negligible sulphate reduction rates measured in the deeper strata of Black Sea sediments.

The observed rapid decrease in sulphate reduction rate with depth that occurred in E-70 sediment has been shown in a number of marine sediments. Jørgensen (1977b) demonstrated that activity showed a large decrease with depth in Danish fjordic sediments with an average of 65% of the sulphate reduction being recorded in the surface 10 cm of sediment. Winfrey *et al*, (1981) working on inshore sediments from the Brittany coast found that sulphate reduction rates were highest in the upper 5 to 10 cm and fell rapidly with depth. The authors attributed this to the availability of organic matter (*i.e.* substrate quality) as did Aller and Yingst (1980), who in studies on surface sediments from Long Island Sound (USA) in a comparable water depth, demonstrated that sulphate reducing activity (as determined by sulphate depletion in incubated samples) decreased exponentially from the sediment surface to a depth of 10 cm. Similar decreases over the surface 10 cm of sediment have also been reported by Nedwell and Abram (1978) in samples from an English saltmarsh.

Most probable number estimations of sulphate reducing bacteria also showed a decrease with sediment depth, although to a lesser extent than their respiratory activity. This again served to illustrate that electron donor (available carbon) had a greater effect on metabolic activity than on bacterial biomass, resulting in the fact that although both values decreased with depth there was no correlation between

them. This has been observed in other marine sediments (see Nedwell, 1982) and can be demonstrated by calculating the specific rates of metabolism for the bacteria at the three different depths for which data was available. The rates obtained are $1.5x10^{-10}$, $7.2x10^{-12}$ and $1.3x10^{-11}$ mol SO_4^{2-} $bacterium^{-1}d^{-1}$ at 0 to 5, 5-10 and 20-25 cm depths, respectively, which while lying within the range calculated by Jørgensen (1978c), show no correlation. The observed decrease in bacteria however, has been reported in many marine sediments. Sorokin (1962) found that the numbers of sulphate reducing bacteria in Black Sea sediments fell rapidly over the surface 5 to 10 cm. A similar trend has been reported in sediments from the Gulf of Aden and Oman Sea (Marty, 1981) which was mirrored by a decrease in total heterotrophic bacteria (see above). The annual mean counts of sulphate reducing bacteria in two saltmarsh sediments also decreased with depth with no significant correlation between bacterial counts and activities being observed (Nedwell and Abram, 1978).

The maximum rate of acetylene reduction activity occurred in the surface 5 cm of E-70 sediment where presumably there was the highest organic carbon availability. Maximum fixation has been observed over this depth in many marine sediments (*e.g.* Teal *et al*, 1979) with Whitney and co-workers (1975) reporting that around half of the total fixation in a Long Island saltmarsh occurred in the top 4 cm of sediment. The decrease in acetylene reduction rate with depth may have again been due to decreased carbon availability. However, the depth profile of activity tended to be a reverse of that of interstitial ammonia concentration suggesting that the increasing levels of

fixed nitrogen in the sediment porewater (271 to 1192 μM NH_4^+) were in addition inhibiting heterotrophic nitrogen fixation (see table 4.1 appendix and above). Similar decreases in acetylene reduction activity have been reported in intertidal sediments from Chesapeake Bay (Marsho *et al*, 1975), the Great Sippewissett Marsh (Teal *et al*, 1979) and from Long Island (Whitney *et al*, 1975) and have been attributed to a decrease in organic energy sources with depth in sediments where the interstitial ammonium concentrations did not exceed 50μM (Teal *et al*, 1979). Again there was no correlation between sulphate reduction and acetylene reduction rate and this was assumed to have been caused by an inhibition of nitrogenase synthesis by porewater ammonia.

LOCH EIL E-24 AND COLLA FIRTH CF-1

As with Loch Eil E-70 sediment, sediment from these two stations was highly reduced below a shallow oxidised zone enabling maximum rates of sulphate reduction to occur over the surface 5 cm. As with E-70 sediment there was a decrease in activity over the top 10 cm of sediment, with a reduction in E-24 sulphate reducing activity by a factor of ten, and just below one and a half times in Colla Firth sediment. As sulphate reducing bacteria were present in both sediments at around $10^3 ml^{-1}$ in conditions of low redox and sulphate abundance, it was thought that the observed decrease in activity with depth was due to decreasing levels of electron donors as discussed above.

SULLOM VOE D-4

As with E-70 sediment, the maximum rates of sulphate and acetylene reduction and highest counts of sulphate reducing bacteria in D-4 sediment were recorded in the surface sediment (fig.2.6). Both sediments are inner basin sediments of high organic content and corresponding low redox potentials, enabling anaerobic processes to occur in the organically richer surface layers. In addition the overlying water at station D-4 was stagnant and oxygen depleted which contributed to the reduced nature of the surface 5 cm of sediment. The rate of decrease of the three measured values at D-4 over the top 10 cm of sediment was greater than that measured in Loch Eil E-70 sediment and this may have been a reflection of the observed lack of bioturbation in D-4 sediment, due to the oxygen depletion in the overlying water column restricting the activities of macrobenthic organisms (Stanley *et al*, 1981). In addition, D-4 sediment was assumed to have lower levels of metabolizable carbon than E-70, with the high organic content of the sediment (9.6%C) probably consisting of refractory eroded peat particles (Pearson and Stanley, 1977). The processes of organic decomposition in the inner basin sediments of Sullom Voe also result in a steady increase in interstitial ammonia concentration with depth (Stanley *et al*, 1981) which may have again exerted an inhibitory effect on the nitrogenase of sulphate reducing bacteria.

LYNN OF LORNE LY-1

In contrast to the situation encountered in the reduced Loch Eil and Sullom Voe sediments, respiratory activity in LY-1

sediment increased with depth from a very low rate of 0.1 nmol SO_4^{2-} $ml^{-1}d^{-1}$ in the surface 5 cm. The sediment was low in carbon and highly oxidised (table i) and it was thought that the increase in sulphate reduction rate was due to a decrease in redox potential with depth. A similar situation has been reported by Sørensen *et al*, (1979) who observed that sulphate reduction in an oxidised fjordic sediment was negligible above 3 cm depth and was restricted to reduced microsites or larger, reduced patches within the oxidised zone (surface 10 cm). Jørgensen (1977a) has also demonstrated a similar increase in activity with increasing sediment depth in oxidised (+320 to 0mV) sediment from the Limfjorden, Denmark during his work on sulphate reduction in reduced microniches and concluded that sulphate reduction was occurring within reduced faecal pellets. The numbers of sulphate reducing bacteria were at a maximum at a depth of 10 to 15 cm which was again probably an indication of increased sediment reduction. Sandkvist (1968) recovered the maximum numbers of sulphate reducers at a depth of 10 to 30 cm in tidal mudflats where the redox potential was <-100mV. As in this study, the counts in the surface 5 cm of the sediment were a factor of ten less and were most likely a reflection of the more oxidised conditions ($Eh \geqslant +70mV$). Acetylene reduction activity was fairly constant with depth at around 0.1 nmol C_2H_4 prod. g dry $wt^{-1}hr^{-1}$ and the reason for this was unclear but may have been due to a combination of the high Eh, low carbon and low fixed nitrogen levels encountered in the surface 15 cm of this sediment.

LOCH CRERAN CR-1

Due to its proximity to the alginate factory effluent dump, sediment from station CR-1 in the main basin of Loch Creran was very fluid and heterogeneous as indicated by the wide spread in measured sulphate reduction rates (fig.2.4). Respiratory activity was low throughout the sediment column which as already mentioned may have been caused by the method of estimation employed. Alternatively, a similar depth profile has been obtained in a seven month old model estuarine sediment by Jørgensen and Fenchel (1974) who suggested that the negligible activity was due to the presence of recalcitrant organic substances such as lignin, which reduced the rate of heterotrophic metabolism and hence sulphate reduction. As with E-70 and D-4 sediment the numbers of sulphate reducing bacteria recovered decreased over the surface 15 cm of sediment although the reason for the increase below this depth was unclear. Acetylene reduction activity was at a maximum on the top 5 cm of sediment as in the Loch Eil and Sullom Voe sediments with the high rate recorded decreasing rapidly with depth. Whether sulphate reducing bacteria were responsible for this high activity was unclear (see above), although the increase in porewater ammonia levels to above 200µM below 5 cm depth would imply that the rapid decrease in nitrogen fixation was due to an inhibition of bacterial nitrogenase by ammonia, as was thought to occur in E-70 and D-4 sediments. In conclusion it must be stated that due to the unique conditions in Loch Creran CR-1 sediment, resulting from its gross pollution by alginate effluent the activity and control of sulphate reducing bacteria activity was

only poorly understood with no comparable sediment having been described in the literature. The fluidity and heterogeneous nature of the surface sediments meant that the variation between replicate samples may have masked any significant trends in activity.

LOCH CRERAN CR-2

Although sediment from sampling station CR-2 in the main basin of Loch Creran was unaffected by the localized alginate dump, the sulphate reducing activity again showed a peculiar depth profile (fig.2.5). As with D-4 and E-70 sediment there was a decrease in sulphate reduction rate over the surface 10 cm but then an increase between 10 and 15 cm, followed by a further decrease. As can be seen from table i, the surface 4 cm of CR-2 sediment were oxidised, having a redox potential range from +175 to +50mV. The requirement of low (<-100mV) Eh values for dissimilatory sulphate reduction has already been discussed and one possible explanation for the observed depth profile is that a decrease in microniche sulphate reducing activity with decreasing carbon availability occurred over the surface 10 cm of sediment, followed by a slight increase in activity below this depth as redox conditions became more favourable. However, as available carbon levels became even lower below 15 cm sediment depth, this redox effect was again negated by electron donor availability. As would be expected, the numbers of sulphate reducing bacteria were relatively unaffected by these factors, with the maximum population being again recorded in the surface 5 cm of sediment and declining with increasing sediment depth. Acetylene reducing activity

was very low and showed a decrease with depth which was again thought to be due to decreasing available carbon levels and increasing interstitial ammonia concentrations. The activity at 15 to 20 cm sediment depth was only 0.01 nmol C_2H_4 prod. g dry $wt^{-1}hr^{-1}$ which was in agreement with the findings of Whitney *et al* (1975) who reported that below 20 cm depth nitrogen fixation was undetectable in a Long Island saltmarsh and as can be seen from fig.2.5 the apparent trend was for acetylene reducing activity to decrease to zero below this depth.

ELECTRON DONORS FOR SULPHATE REDUCTION

The absence of sulphate reducing bacteria in sediment samples from station A-1 on the N.E. Atlantic abyssal plain (table 2.4) has already been discussed and it is thought that organic mineralization by sulphate reduction was minimal under the conditions prevailing at this station. Electron donors utilized by sulphate reducing bacteria from the remaining Atlantic and sea-loch sediments were generally typical of those reported for *Desulfovibrio spp.*, consisting in the main of simple organic acids, hydrogen, glycerol and primary alcohols (Postgate, 1979). Surprisingly, all these sediments contained a sulphate reducing flora which was capable of growth on acetate, butyrate or propionate - compounds which cannot support the growth of *Desulfovibrio spp.* which incompletely oxidise most substrates to only the level of acetate, which accumulates in the growth medium (Wake *et al*, 1977). Selwyn and Postgate (1959) have likewise found that similar marine enrichment cultures could use acetate as an electron donor for sulphate reduction and Jørgensen (1977b) has suggested that this organic acid is of importance as a reductant for sulphate reduction in a fjordic sediment. The observed growth on acetate, butyrate or propionate by the sedimentary sulphate reducing bacteria may be in part due to a syntrophy between *Desulfovibrio spp.* and other heterotrophic bacteria as has been demonstrated by Kimata *et al* (1955e) in Hiroshima Bay (Japan) samples and by Keith *et al* (1982) in Tay Estuary sediments. Alternatively the compounds may have been utilized as electron donors for sulphate reduction *per se*. Until recently the only organism

capable of reducing sulphate at the expense of acetate was thought to be the spore-forming organism *Desulfotomaculum acetoxidans* (Widdel and Pfennig, 1977) and as none of the acetate enrichments in this study contained spore-forming bacteria it was concluded that growth on acetate was not due to this organism. In addition it is now thought that *Desulfotomaculum acetoxidans* is primarily an intestinal organism which is introduced into manure-polluted waters (Widdel and Pfennig, 1981). However, as already mentioned, the recently published work of Widdel (1980) has demonstrated the existence of novel genera which are capable of utilizing acetate, butyrate and propionate with the concomitant reduction of sulphate to sulphide. Such organisms have been recovered from N.E. Atlantic and Loch Eil sediments (see tables 3.1 and 3.3, next section) and from marine sediments (Laanbroek and Pfennig, 1981; Keith *et al*, 1982) suggesting that the observed growth of enrichment cultures on supposed unutilizable substrates in this study was in fact partly or wholly due to the action of these new types of sulphate reducing bacteria.

All of the sediments examined contained organisms which were capable of utilizing molecular hydrogen (H_2) as an electron donor for sulphate reduction. H_2 is a common end-product of many heterotrophic fermentation reactions - the principal source of substrates for sedimentary sulphate reduction. Under the conditions of low H_2 tension that exist in many marine sediments (Nedwell, 1982) the fermenting organism can achieve a small energy gain by reoxidising its reduced coenzyme $NADH_2$ by hydrogen transfer to a H_2-scavenging sulphate reducer *via*

the reaction $NADH_2 \rightarrow NAD + H_2$ (Abram and Nedwell, 1978a). This reaction only occurs at, and is one reason for, the low H_2 tensions in many sediments and it has been suggested by Nedwell (1982) that this process is of importance in sediments of low available carbon content such as those from stations A-2 and A-3 on the continental slope and shelf of the British Isles (see below). It is unclear as to what extent any of these electron donors were utilized *in situ*. Little published data exists on the naturally occurring levels of low molecular weight organic acids in marine sediments (Goldhaber and Kaplan, 1974) despite the ample evidence for the ubiquity of the fermentative bacteria that produce them (*e.g.* ZoBell, 1938; Wood, 1965; Vance *et al*, 1982). Recent data does exist for station E-70 in Loch Eil with acetate, butyrate, lactate, propionate, succinate and valerate being recorded in the surface 6 cm of sediment (Miller *et al*, 1979). All of these compounds, with the exception of valerate, were utilized as electron donors in sediment enrichment cultures and it can be concluded that these compounds are used as electron donors *in situ* by the E-70 sulphate reducing bacterial population. It may be significant that the maximum peak in lactate concentration found by Miller and co-workers (21.4 to 13.7 $\mu g\ g^{-1}$ dry wt^{-1}, over the surface 6 cm of sediment) was at the same depth as the maximum rate of sulphate reduction measured in this study (fig.2.3). As would be expected, lactate has been shown to increase the rate of sulphate reduction in many marine sediments (*e.g.* Sorokin, 1962; Vosjan, 1974; Nedwell and Abram, 1979; Oremland and Silverman, 1979), suggesting that lactate is an important electron donor for sulphate reduction in marine sediments. It has been suggested by Nedwell (1982) that reduced

fermentation end-products such as lactate are used preferentially to H_2 by sulphate reducing bacteria in slurries of saltmarsh sediment. Such reduced compounds are less likely to be produced if H_2 transfer occurs, for reasons discussed earlier in this section, and the process of H_2-scavenging by sulphate reducers may be of more importance in carbon limited environments. Hence, all subsequent pure culture work was performed on bacteria isolated from a lactate enrichment from the region of highest activity, namely the surface sediment of station E-70 in the polluted, inner basin of Loch Eil.

LOCH EIL E-70 SULPHATE REDUCING BACTERIA ISOLATES

The six strains of sulphate reducing bacteria isolated from a lactate enrichment of Loch Eil surface sediment were all very similar (table 2.5). All the isolates were Gram negative, nonsporulating, obligatory anaerobic, mesophillic vibrios which were motile and possessed the pigment desulfoviridin, and therefore belonged to the genus *Desulfovibrio* (Postgate and Campbell, 1966). The absence of any members of the genus *Desulfotomaculum* was consistant with reports that the majority of marine sulphate reducing bacteria are *Desulfovibrio spp.* (Trüper *et al*, 1969; Goldhaber and Kaplan, 1974). A comparison of the characteristics of the six isolates with the working classifications of Postgate and Campbell (1966) and Postgate (1979) indicated that all the strains shared several features with *D.desulfuricans* — being capable of growth on pyruvate and choline in the absence of sulphate and on malate in the presence of sulphate. As none of the strains had a

salt requirement above 1% w/v NaCl they were not assigned to the subspecies *aestuarii*. This predominance of *D.desulfuricans* as the lactate oxidising sulphate reducing bacteria in a marine sediment has also been reported in estuarine sediments (Laanbroek and Pfennig, 1981) and is consistent with the view of Skyring *et al* (1977) who after an extensive taxonomic study of marine and freshwater sulphate reducing bacteria, proposed that *Desulfovibrio* species with lower DNA base ratios (% GC 46.1 to 58.6) were probably more important in saline environments than those species with higher base ratios (%GC 61.7 to 64.8). As the reported DNA base composition of *D.desulfuricans* is 55.3±1% G+C (Postgate and Campbell, 1966) the preponderance of such sulphate reducing bacteria in Loch Eil sediment would not necessarily be unusual.

As was mentioned previously in the materials and methods section, the media used to evaluate the potential electron donors for the six *D.desulfuricans* isolates contained yeast extract. As this substance can act as an addition growth substrate (Khosrovi and Miller, 1975) a very low concentration was used (0.001% w/v) in conjunction with no carbon source blanks. Yeast extract was retained in the growth media because of its stimulatory effect on growth, due partly to its constituent amino acids (Butlin *et al*, 1949; Khosrovi and Miller, 1975) and to its ability to decrease the number of cells required to initiate growth (Pankhurst, 1967; cited in Pankhurst, 1971). This permitted the use of smaller inocula with less sulphide carry-over and less initial media blackening (a criterion for growth). In addition it has been reported by Postgate (1979)

that growth of *Desulfovibrio spp.* on an unfamiliar carbon source may require a metabolic adjustment (*e.g.* derepression of enzyme formation, permeability change) which is assisted by the presence of yeast extract.

All six isolates demonstrated the classical limited range of substrates for growth and were confined mainly to simple C_2— to C_4— compounds, with no growth occurring on acetate, butyrate or propionate (table 2.6). Although growth on these compounds in enrichment cultures was probably due to novel sulphate reducing bacteria, the disruption of any syntrophic relationships in pure culture cannot be ruled out entirely. Published data on the range of electron donors utilized by *D.desulfuricans* strains from marine sediments is scarce with the one large scale survey on marine isolates by Trüper *et al* (1969), concentrating only on electron donors of diagnostic importance. The substrates utilized by the six E-70 isolates were in agreement with those which supported growth of nine *D.desulfuricans* strains isolated from the Ems-Dollard estuary by Laanbroek and Pfennig (1981) - namely: hydrogen, formate, lactate and malate (all strains); and ethanol (seven strains). Growth on hydrogen was due to mixotrophic growth in which an inorganic or non-assimilable organic molecule provided the reducing power for sulphate reduction, whilst a different compound (in this case yeast extract) acted as a source of cell carbon (Mechalas and Rittenberg, 1960). This type of metabolism would appear to be advantageous to the *D.desulfuricans* strains within Loch Eil sediment if these organisms were indeed acting as hydrogen scavengers (see above). As the six Loch Eil isolates were so similar in character, all future work was concentrated on strain 19 which was representative of the whole.

PRELIMINARY GROWTH EXPERIMENTS AND THE DEVELOPMENT OF MODIFIED MEDIUM E

None of the six isolates from Loch Eil E-70 were capable of growth on the recommended medium for growth studies - Postgate's medium C (Postgate, 1979). To the author's knowledge this has never been reported previously despite the medium being widely used in research on sulphate reducing bacteria (*e.g.* Pankhurst, 1971). The series of preliminary growth experiments initiated were intended to determine the cause of the observed failure of growth and if necessary, to lead to the suitable formulation of an alternative low iron (Fe^{2+}) medium for growth studies.

The redox poising agents of Postgate's medium B (0.01% w/v sodium ascorbate and thioglycollate) were used throughout these studies. Despite sodium thioglycollate depressing the final population of strain 19 by around 20% compared to ascorbate alone (Table 2.7), its increased reduction of the medium (Eo' <-100mV at 0.05% w/v; Costilow, 1981) was deemed to be important for the small inocula that are used in growth studies. Although Khosrovi and Miller (1975) have suggested that sulphide is a preferable reductant to thioglycollate in growth studies (final population with thioglycollate 74% of that with S^{2-}), the problems of pH increase due to NaOH contamination associated with the use of sodium sulphide meant that it was not used in this study.

Members of the genus *Desulfovibrio* have a very high requirement for ferrous iron (Fe^{2+}) which is required for the iron-rich proteins ferredoxin and cytochrome C_3 (Postgate, 1979).

Under conditions of iron limitation the organisms become deficient in cytochromes and can only lose electrons *via* fermentation (Vosjan, 1975), which could possibly limit the growth of an Fe^{2+} starved organism on a low iron medium such as medium C (10 μM Fe^{2+}). As high levels of Fe^{2+} upto 170 μM have been recorded in the surface 2 cm of Loch Eil sediment (Duff, 1981), it was originally suspected that the failure of the strains to grow in medium C was due to an abnormally high Fe^{2+} requirement. However, the maximum absolute iron requirement of strain 19 was found to be 9 μM (Fig.2.7) which was in close agreement with the value of 10μM reported by Postgate (1956) for the El Agheila Z strain of *D.desulfuricans* and was less than the concentration of Fe^{2+} in medium C. This iron requirement was obtained in a growth medium based on Postgate's medium E, which unlike medium C, does not contain sodium citrate. This chelating agent is added to medium C at a concentration of 1mM in order to obtain repeatable exponential growth curves by increasing iron solubility (Postgate, 1965; 1979). In the absence of chelation the iron would be present mainly as solid FeS and growth would be limited by the rate of dissolution of this solid phase. In the presence of citrate however, although the actual concentration of Fe^{2+} in solution is decreased by chelation, its availability is increased due to the instantaneous adjustment of the equilibrium (Spencer, 1957):

$$\text{complexed } Fe^{2+} \rightleftharpoons \text{citrate} + Fe^{2+}$$

The question therefore arose as to whether citrate was inhibiting strain 19 at low iron concentrations? It was found that in fact 1mM sodium citrate had no effect on the total

growth of the organism and as the use of citrate is recommended in growth studies (Postgate, 1965) it was retained in all subsequent media.

During growth in 30mM lactate medium, cultures of strain 19 were found to be sulphate limited for concentrations up to 15 mM. This figure was in agreement with the reaction equation for the oxidation of lactate by sulphate:

$$2CH_3CHOHCOOH + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + S^{2-} + 2H_2O$$

As sulphate reducing bacteria in the marine sediments examined were generally electron donor, as opposed to electron acceptor limited (see above), the sulphate level in the growth experiments on strain 19 was kept in excess of this minimum value of 1 lactate : 2 SO_4^{2-}. Hence, a value of 20mM SO_4^{2-} was chosen which represented the average sulphate concentration in E-70 sediment at the time of enrichment of the parent culture.

As the cause of the lack of growth in medium C remained as elusive as ever, these preliminary growth experiments were used as a basis for a low iron medium as detailed in the materials and methods section. In essence this was an electron donor limited medium which contained a low level of chelated iron.

MIXOTROPHIC SUBSTRATES

The mixotrophic growth of strain 19 on molecular hydrogen as electron donor and yeast extract as a source of cell carbon has already been discussed. Similarly, other substrates such as isobutanol have been shown to act as sources of H_2 for the mixotrophic growth of *D.desulfuricans* strains (Mechalas and

Rittenberg, 1960). Compounds such as ethanol and its homologues butanol and propanol are thought to be on the border line of being mixotrophic substrates for sulphate reducing bacteria (Wake *et al*, 1977; Postgate, 1979) and this was confirmed by the noticeably poor growth of strain 19 on these compounds, which was qualitatively comparable to that on hydrogen itself. Such "incomplete" substrates can be detected by the observation that yeast extract is always a requirement for growth, whilst it has only a moderate stimulatory effect with complete substrates such as lactate, malate or pyruvate. As was seen from Fig.2.9 growth of strain 19 on butanol, ethanol and propanol was indeed mixotrophic in nature, being greater than that on the basal medium alone, with an obligate requirement for a source of assimilable carbon. In contrast, growth on lactate occurred in the absence of yeast extract although small amounts did augment growth. These mixotrophic reactions are thought to be of wide importance in the ecology of *Desulfovibrio* (Postgate, 1965) and would enable the *D.desulfuricans* strains present in Loch Eil to utilize a wider range of substrates for sulphate reduction than would be immediately obvious from data such as those in table 2.6.

SOURCES OF CELL CARBON FOR MIXOTROPHIC GROWTH ON H_2

In addition to mixotrophic growth on hydrogen with yeast extract as carbon source, other compounds can provide cell carbon. For example, sulphate reducing bacteria isolated from an oil bed have reportedly grown mixotrophically on H_2 or formate in the presence of low concentrations of acetate and CO_2

(Sorokin, 1966). Growth rates and yields have been reported for a *D.vulgaris sp.* growing on H_2 as electron donor and acetate + CO_2 as carbon sources (Badziong and Thauer, 1978) which provided 70% and 30% of the cellular carbon respectively (Badziong *et al*, 1978). As can be seen from table 2.9, strain 19 could not reduce sulphate at the expense of acetate, butyrate, propionate, valerate, benzoate or glucose. High concentrations of the former four compounds have been recorded in E-70 sediment (Miller *et al*, 1979) and it was wondered whether these compounds could act as carbon sources and support sulphate reduction and/or growth with the known mixotrophic substrate H_2, in a similar way to yeast extract. The lack of either growth or sulphate reduction on any of these compounds demonstrated that they cannot act as sources of cell carbon for the mixotrophic growth of *Desulfovibrio spp.* on H_2 in Loch Eil sediment. As yeast extract is approximately 75% amino acids by weight (Bridson and Brecker, 1970) a possible source of cell carbon for such growth *in situ* may be free amino acids. Amino acids are an important component of the dissolved organic matter in the marine environment and are a good substrate for marine heterotrophic bacteria (Amano *et al*, 1982). Recent work has shown that the most important source of dissolved free amino acids (alanine, glycine, glutamic acid, ornithine and serine) in a shallow Danish estuary appeared to be the sediment (Jørgensen, N, 1982). As all of these amino acids (except ornithine) occur in yeast extract at concentrations of 2.3 to 6.7 g/100 g (Bridson and Brecker, 1970), it would appear that strain 19 was utilizing sources of cell carbon which may in fact be present

in situ. Free amino acids are thought to occur in appreciable levels in the surface layers of similar, anoxic fjordic sediments to Loch Eil (S.Stanley unpub. data) and are reportedly utilized by sulphate reducers in eutrophic lake sediments (Smith and Klug, 1981).

Growth with yeast extract as carbon source increased with increasing concentration and levelled out at 0.5% w/v. It has been calculated that 0.01% w/v yeast extract is capable of providing enough cell carbon for the production of 100 mg cell l^{-1} of a *Desulfovibrio sp.* (Khosrovi and Miller, 1975). Assuming that the average weight of a single sulphate reducer is 3.13×10^{-13}g (Postgate, 1979), this cell yield approximates to 3×10^{8} bacteria ml^{-1}. Hence, 0.5% w/v yeast extract was more than enough to provide cell carbon for mixotrophic growth of strain 19 on H_2 and the levelling off of growth at a final population of only around 8×10^{6} bacteria ml^{-1} would appear to be due to limitation by electron donor (H_2) availability. Once again lactate acted as both a carbon and electron source with growth and sulphate reduction increasing with increased concentration as would be expected.

THE EFFECT OF MAGNESIUM ON THE GROWTH OF *D.DESULFURICANS* STRAINS

The failure of the Loch Eil sulphate reducing bacteria isolates to grow in Postgate's medium C has already been discussed. During the progress of the work described in this section, the first descriptions of the newly isolated sulphate reducing bacteria appeared in the literature. In a paper describing the acetate oxidising organism *Desulfobacter*

postgatei, Widdel and Pfennig (1981) reported that the growth of marine strains was significantly retarded at magnesium concentrations of below 6 mM Mg^{2+}. A much earlier paper by Kimata *et al* (1955c) had likewise reported that Mg^{2+} was essential for the development of a marine *Desulfovibrio sp.* Magnesium is present in abundance in the marine environment, occurring predominantly as dissolved Mg^{2+} at an average concentration of 53mM in seawater of 35‰salinity (Riley and Chester, 1971). It was known that isolate 19 grew well in modified Postgate's medium E, which contained Mg^{2+} at a concentration of 9.8mM (2.0 g $litre^{-1}$ $MgCl_2.6H_2O$). However, medium C had a low Mg^{2+} level of only 0.24 mM Mg^{2+} (0.06 g $litre^{-1}$ $MgSO_4.7H_2O$) and it was suspected that Mg^{2+} limitation was the reason for the lack of growth of isolate 19 and the other Loch Eil isolates.

This hypothesis was substantiated by the data for strain 19 presented in figures 2.10 and 2.11. At a Mg^{2+} concentration similar to that in medium C (0.25mM) the maximum specific growth rate (μm) and the final bacterial population were only slightly greater than the values recorded with no $MgCl_2.6H_2O$ addition. Growth was Mg^{2+} limited up to a concentration of 9mM which indicated that in modified medium E magnesium was present in excess. The growth recorded with no added magnesium was attributed to Mg^{2+} carry-over in the inoculum and to magnesium present in the original bacteria. This accounted for the observation that growth of the Loch Eil isolates sometimes occurred on first subculture into medium C, with growth failing to occur on subsequent subculturing.

As medium C (supplemented with 0.4M NaCl) is a recommended

medium for growth studies on marine sulphate reducing bacteria (Postgate, 1979), it was decided to investigate whether Mg^{2+} had any effect on the growth of two marine type strains of *D.desulfuricans* in medium C. NCIB strain 8400 had been originally isolated in seawater from Hossegor, S.W. France; whilst NCIB strain 8310 had been isolated in seawater from Oslo Harbour, Norway. As was expected the supplementation of medium C with magnesium had a negligible effect on the growth of the two type strains - with μm, total growth attained and amount of sulphate reduced being very similar with either zero or 10mM Mg^{2+}. These findings support the use of medium C when growth studies are being performed on type strains of sulphate reducing bacteria.

However, the growth of strain 19 was again markedly affected by the concentration of Mg^{2+} in the growth medium. μm was notably limited by Mg^{2+} levels, being very low at 0.25mM Mg^{2+} with a value of 0.03 hr^{-1}, which represented a much slower rate of growth than that obtained at the same magnesium concentration in modified medium E. A possible explanation for this observation is discussed elsewhere in this section. Again the growth rate was Mg^{2+} limited up to a concentration of between 9 and 10mM. The total growth attained and the amount of sulphate reduced by isolate 19 were also Mg^{2+} limited, although this effect was somewhat less than that on the maximum specific growth rate. That the observed effects were due to Mg^{2+} and not to some other component of the added $MgCl_2.6H_2O$ can be seen from table 2.12. The stimulatory effect on growth was essentially the same when magnesium was added either in the form of chloride or as sulphate - the

difference in total growth being probably due to the difficulty in weighing the very deliquescent magnesium chloride crystals. The question therefore arose as to how a deficiency in Mg^{2+} was affecting the growth of isolate 19 (and presumably the other Loch Eil isolates)?

Magnesium is essential to all bacteria and is the most abundant intracellular divalent cation. It is involved in many cellular processes, being predominantly associated with the structure of the ribosomes and to a lesser extent with the cell membrane and cell wall (Jasper and Silver, 1977). The element can also activate many enzymes, particularly those which are associated with phosphate group transfer or hydrolysis (Hughes, 1975). Such enzymic reactions are of great importance in dissimilatory sulphate reduction and were therefore suspected of having some role in the observed effect of magnesium concentration on growth.

It was known that isolate 19, in common with other *D.desulfuricans* strains, was capable of growth on pyruvate in the absence of sulphate (table 2.5). Under such conditions pyruvate is fermented to yield one ATP per mole pyruvate by substrate level phosphorylation (Vosjan, 1970). As can be seen from the data presented in table 2.11, the addition of Mg^{2+} to the growth medium of isolate 19 had no effect on the total growth attained. This was in contrast to the effect of added Mg^{2+} during respiratory growth on sulphate as terminal electron acceptor (figs.2.11 and 2.13) and suggested that the lack of Mg^{2+} was affecting some portion of the dissimilatory sulphate metabolism.

The dissimilatory reduction of sulphate to sulphide by

Desulfovibrio spp. can be summarized by the equations (Peck, 1962; Jones, C.W., 1982):

$$ATP + SO_4^{2-} \rightleftharpoons APS^{2-} + PPi \quad (1)$$

$$PPi + H_2O \longrightarrow 2Pi \quad (2)$$

$$APS^{2-} + 2e \rightleftharpoons AMP^{2-} + SO_3^{2-} \quad (3)$$

$$SO_3^{2-} + 6e + 6H^+ \longrightarrow S^{2-} + 3H_2O \quad (4)$$

It can be seen from equation (1) that sulphate must be activated to adenosine phosphosulphate (APS) prior to reduction. This is necessary as the standard redox potential of the $APS/AMP^{2-} + SO_3^{2-}$ couple is 420mV higher than the SO_4^{2-}/SO_3^{2-}couple, enabling sulphate to act as an electron acceptor (Jones, C.W.,1982). This activation of sulphate *via* the enzyme sulphate adenylytransferase is a reversible reaction with the equilibrium constant in fact lying to the left, favouring ATP and SO_4^{2-}. The reaction is "pulled" to the right by an inorganic pyrophosphatase which hydrolyses the pyrophosphate (PPi) formed (equation 2).

Both of these enzymes in *D.desulfuricans* are known to have a Mg^{2+} requirement for activity. Akagi and Campbell (1962) reported that a sulphate adenylytransferase from a *D.desulfuricans* strain required in excess of 3.3mM Mg^{2+}. The same workers have also reported that an inorganic pyrophosphatase from the same organism required 7mM Mg^{2+} for optimum activity (Akagi and Campbell, 1963). A lower figure of 3mM was obtained by Ware and Postgate (1971) as the Mg^{2+} requirement for optimum activity of an inorganic pyrophosphatase purified from *D.desulfuricans* strain Berre-sol (NCIB 8388). The divalent cation Mn^{2+} was also reported as being able to substitute for Mg^{2+} in the above inorganic pyrophosphatases and has often been

used as a metal ion probe in the investigation of Mg^{2+} in biological systems - the ion having similar chemical properties to Mg^{2+} (Hughes, 1975). As can be seen from table 2.12, Mn^{2+} in the form of $MnSO_4.4H_2O$, could substitute for Mg^{2+} in isolate 19, indicating that Mn^{2+} was being bound at the same site as Mg^{2+} in the organism. This suggested that the observed lack of growth of isolate 19 was due to Mg^{2+} limitation lowering the activity of its inorganic pyrophosphatase and hence preventing the activation of sulphate *via* sulphate adenylytransferase. It is thought that in nature many inorganic pyrophosphatases are controlled by chemical species involved in metabolism and biosynthesis (Klemme and Gest, 1971). In *D.desulfuricans* strain Berre-sol the enzyme is activated by reducing agents and inactivated when the environment is aerobic-conserving ATP when growth cannot occur (Ware and Postgate, 1971).

Is there therefore a reason for a shut-off of this enzyme under conditions of low magnesium concentration in sulphate reducing bacteria from Loch Eil? Although, magnesium is abundant in seawater it can be removed by burial of interstitial water, ion exchange, carbonate formation and by exchange for iron in anaerobic sediments (such as Loch Eil E-70) according to the equation (Drever, 1974):

$$2Fe_{(clay)} + 3Mg_{(soln)} + 4[S] \rightarrow 3Mg_{(clay)} + 2Fe\,S_{2\,(pyrite)}$$

- where [S] is reduced sulphur such as HS^- or H_2S. However, these processes do not reduce the levels of magnesium significantly. Data for a similar anoxic, sea-loch to Loch

Eil (Loch Etive) have shown that the porewater Mg^{2+} content from the inner basin decreased only slightly from 46mM at the sediment-water interface to 42mM at a depth of 80 cm (Malcolm, 1982). Obviously from a consideration of the previous data magnesium could not be a rate-limiting nutrient *in situ*.

The isolation of strain 19 from a habitat of high Mg^{2+} concentration suggested that it may possess an inefficient Mg^{2+} uptake system. Although in most bacteria magnesium uptake is an energy-requiring process it has been reported to occur by passive equilibration in some Gram negative organisms (see Jasper and Silver, 1977). The presence of 1mM citrate as a chelating agent in both media C and E has already been discussed and this compound is known to bind with divalent cations such as Mg^{2+} (Meynell and Meynell, 1970). The absence of this chelating agent from medium C enabled isolate 19 to grow normally (table 2.13) and as this medium is low in magnesium (0.24mM) this result demonstrated that the organism did in fact possess an active Mg^{2+} uptake system.

From these observations it would appear that the failure of isolate 19 (and the other Loch Eil isolates) to grow in Postgate's medium C was due to the citrate binding with Mg^{2+} so as to render it unavailable to the organism. Once a metal such as Mg^{2+} is within the bacterial cell it is bound by the appropriate natural ligands in accordance with its chemical reactivity (Hughes, 1975). It is known that many cell constituents do not bind metals as tightly as synthetic chelating agents and have correspondingly lower formation (stability) constants - a measure of the tightness of binding between the

different substances and the metal magnesium (Albert, 1958). It was thought that citrate interfered with the binding of Mg^{2+} within isolate 19 by the possession of a stability constant for Mg^{2+} which was greater than that of the binding sites in the cell. Whether these sites were on the enzyme inorganic pyrophosphatase was not known. The lack of toxicity of citrate towards the two *D.desulfuricans* type strains examined in this study would therefore be due to the stability constant of citrate being less than those for their binding sites. This explanation has also been postulated by Albert (1958) for the reported variation in inhibitory levels of chelating agents and emphasises the warning of Bridson and Brecker (1970) for a testing of the effects of a certain concentration of chelate on all organisms that may be grown in a particular culture media. If the strains of sulphate reducing bacteria isolated from Loch Eil were not a peculiarity of this sediment then the use in ecological studies of a medium such as Postgate's C with chelated iron (*e.g.* Keith *et al*, 1982) should be approached with care. In fact recent reports by Pfennig *et al*, (1981) suggest that media with EDTA (ethylenediamine tetra-acetic acid)-chelated iron should be avoided in sulphate reducing bacteria enrichments and isolation as they are harmful for some species.

Whether the toxicity of citrate towards isolate 19 was due to an unusual inorganic pyrophosphate was not known. Inorganic pyrophosphatase can be present in *Desulfovibrio spp.* as either one or two enzymes (Ware and Postgate, 1971) and a novel form in isolate 19 cannot be ruled out. Obviously this matter would benefit greatly from further studies on the

purified inorganic pyrophosphatase of isolate 19 as described by Akagi and Campbell (1963) and Ware and Postgate (1971).

Such an inhibition of a sulphate reducing bacteria by a relatively low concentration of chelating agent has been rarely reported in the literature. A very recent report has mentioned that chelating agents such as nitriloacetic acid and EDTA have inhibitory effects on some strains (Webster, 1982), although as with Pfennig *et al* (1981) no concentrations were given. Much higher concentrations of chelates have been reported to inhibit growth, with EDTA inhibiting the growth of *D.vulgaris* (NCIB 8303) in a similar lactate, sulphate, yeast extract, reduced medium at a concentration >3.4mM (Saleh *et al*, 1964). However, such a high concentration of chelating agent is not normally used in culture media, although its toxic effects are thought to result from the EDTA binding Mg^{2+} and Ca^{2+} so that they are no longer available to the bacteria (Bridson and Brecker, 1970).

As most of the enzymes involved in dissimilatory sulphate reduction do not require calcium ions (Roy and Trudinger, 1970) and as Ca^{2+} does not activate inorganic pyrophosphatase in *D.desulfuricans* (Akagi and Campbell, 1963), it was thought that the growth of isolate 19 on Postgate's medium C supplemented with 10mM $CaCl_2.6H_2O$ (table 2.13) was due to Ca^{2+} binding with citrate, either within the culture medium or the cell, to free Mg^{2+} for the organism. This would also explain the higher values of μm at low Mg^{2+} concentrations in modified medium E (4.6mM Ca^{2+}) than in medium C (0.3mM Ca^{2+}).

Citrate could not be omitted from the growth medium as

it was essential for repeatable, exponential growth curves (Khosrovi and Miller, 1975). However, the replacement of 0.24mM $MgSO_4.7H_2O$ in Postgate's medium C by 10mM $MgCl_2.6H_2O$ enabled isolate 19 to grow exponentially and reproducibly as can be seen from Figures 2.15 to 2.17.

GROWTH AND SULPHATE REDUCTION ON DIFFERENT ELECTRON DONORS

Although ten of the electron donors examined in this study could support the growth of *D.desulfuricans* strain 19, the rates and levels of growth were generally low. The mixotrophic nature of butanol, ethanol, H_2 and propanol has already been discussed and as growth on formate was low it was thought that it too was an "incomplete" substrate (Postgate, 1979). Slightly higher levels of growth were obtained with succinate and glycerol (19 and 32 mg dry wt. l^{-1}, respectively) although the rate of sulphide production, as indicated by blackening, appeared very slow. Hence, growth experiments were concentrated on the organic acids lactate, malate and pyruvate.

As can be seen from figures 2.15, 2.16 and 2.17 exponential (and reproducible) growth curves were obtained, which supported the retainment of the chelating agent citrate in the growth medium to increase iron solubility (Postgate, 1965, 1979; Khosrovi and Miller, 1975). Growth occurred most rapidly on lactate as the growth substrate with a maximum specific growth rate of 0.20 hr^{-1}. This value was in good agreement with the published range of values of 0.20 to 0.23 hr^{-1} for type strains of *D.desulfuricans* growing on lactate (Senez, 1962; Postgate, 1965; Alico and Liegey, 1966) and is identical to that obtained by Leban *et al* (1966) for a *Desulfovibrio sp.* isolated from San Francisco Bay. Growth was noticably slower on pyruvate and malate with the maximum specific growth rate on pyruvate being less than that recorded by Senez (1962) for *D.desulfuricans* (NCIB 8393). However, as has been published

elsewhere (Chemistry Research, 1951; Senez, 1962) the growth attained on equimolar amounts of lactate or pyruvate was approximately the same at around 120 mg dry wt l^{-1} despite their differing values of μm.

Sulphate reduction (sulphide production) on lactate and pyruvate closely mirrored growth as has been reported by Brown *et al* (1973). This and the cessation of growth with the end of sulphate reduction underlined the relationship between biosynthesis and sulphate reduction. The final sulphide concentrations recorded during growth on lactate or pyruvate were very close to the theoretical maxima of 15 and 7.5 mmol l^{-1} respectively, assuming a ratio of 2 mol lactate or 4 mol pyruvate: 1 mol SO_4^{2-} reduced (Wake *et al*, 1977), and it was concluded that the cessation of sulphate reduction and growth was due to electron donor depletion. The sulphide production curve with malate as electron donor showed a lag period, although growth again ceased with the cessation of sulphate reduction. In addition the final sulphide concentration was well below its theoretical maximum of 15 mmol l^{-1} (2 malate: 1 SO_4^{2-} reduced; Wake *et al*, 1977) indicating that the termination of sulphate reduction was not due to the depletion of the electron donor. The reasons for this and the lag period when growth was occurring were unclear.

The maximum rate of sulphate reduction recorded in this study was with lactate as electron donor and was three to five times greater than that with pyruvate or malate. With all three substrates the maximum rate of sulphate reduction was recorded during the latter stages of the exponential phase

which was in contrast to the maximum specific growth rate, μ_m. This was probably due to the dependence of sulphide formation on the bacterial density in addition to the growth rate (Leban *et al*, 1966). That is, on sulphide formation through the metabolism of existing cells and through the formation of new ones. Hence, the sulphate reduction rates of the three cultures could be better compared by a comparison of the rate of sulphate reduction per bacterium (the specific rate of sulphate reduction).

Assuming a bacterial dry weight of 3×10^{-13} g cell^{-1} (Postgate, 1979) resulted in specific rates of sulphate reduction for the three cultures over the period of maximum sulphate reduction as follows:

lactate	4.7×10^{-14} mol SO_4^{2-} bacterium^{-1}d^{-1}
malate	2.4×10^{-14} mol SO_4^{2-} bacterium^{-1}d^{-1}
pyruvate	2.2×10^{-14} mol SO_4^{2-} bacterium^{-1}d^{-1}

The specific reduction rate on lactate as electron donor was twice that on pyruvate or malate and demonstrated that the nature of the electron donor had a marked effect on the rate of sulphate reduction. Similar work on *D.vulgaris* by Kaplan and Rittenberg (1964) has demonstrated that H_2 gave the fastest sulphate reduction rate, with the rate on lactate or ethanol as electron donor being around one-half and one-tenth of this rate, respectively. The high specific rate of sulphate reduction on lactate as electron donor recorded for a *D.desulfuricans* strain 19 would suggest that lactate is its preferred substrate *in situ* in Loch Eil E-70 sediment. As

this strain was representative of the sulphate reducing flora isolated from E-70 surface sediment it would appear that lactate is an important electron donor for sulphate reduction in this inshore, organically rich sediment. This conclusion is also supported to some extent by the maximum in sulphate reduction rate in E-70 sediment being recorded at a depth where lactate concentration peaked (Miller *et al*, 1979). In addition, it has already been mentioned that the addition of lactate to several marine sediments has produced a stimulation in sulphate reduction rates. It would also appear from the calculated specific sulphate reduction rates for strain 19 that pyruvate and malate may also be important substrates for sulphate reduction by *Desulfovibrio spp.* in Loch Eil, although the presence of appreciable levels of pyruvate in marine sediments is questionable (Nedwell, 1982).

It can be seen from a comparison of the published specific rates of sulphate reduction (table 2.22) that the rates recorded with strain 19 were in general higher than those reported elsewhere. This would indicate that this isolate was well suited to growth and sulphate reduction on the organic acids lactate, malate and pyruvate. Despite these high rates, a comparison with table 2.20 showed that the metabolic rate per bacterium growing under good cultural conditions on either lactate, malate or pyruvate was several thousand-fold lower than the metabolic rate per sulphate reducing bacterial colony in Loch Eil E-70 surface sediment. As it was unlikely that the rate of sulphate reduction recorded was this much in error, it was concluded that the number of sulphate reducing bacteria recovered were under-

Table 2.22 SPECIFIC RATES OF SULPHATE REDUCTION BY *DESULFOVIBRIO SPP.*

Specific Rate of Sulphate Reduction (mol SO_4^{2-} bacterium^{-1} d^{-1})	Substrate	Organism	Reference
3.6-5.1 x 10^{-15}	lactate	*D.vulgaris*	Kaplan and Rittenberg (1964)
0.3-3.8 x 10^{-15}	ethanol	*D.vulgaris*	Kaplan and Rittenberg (1964)
0.2-1.1 x 10^{-14}	H_2	*D.vulgaris*	Kaplan and Rittenberg (1964)
1.3 x 10^{-15}	lactate	*D.desulfuricans*	Kaplan and Rittenberg (1964)
1.8 x 10^{-14}	lactate	*Desulfovibrio sp.*	Leban *et al* (1966)
5 x 10^{-14} *	lactate	*D.desulfuricans*	Edwards (1967) (cited in Ramm and Bella, 1974)
1.2 x 10^{-14}	lactate	*D. vulgaris*	Brown *et al* (1973)
0.2-0.4 x 10^{-15}	pyruvate, lactate	*Desulfovibrio sp.*	Ishimoto *et al* (1954) (cited in Jørgensen 1978c)
0.2-1.0 x 10^{-14}	pyruvate, lactate	*Desulfovibrio sp.*	Senez (1954) (cited in Jørgensen 1978c)

* calculated assuming a bacterial density of approx. 10^8 bacteria ml^{-1}

estimated to a large extent. Similar results have been found by Jørgensen (1977b) and this gross underestimation of the true population *in situ* occurs often in viable counts on heterotrophic bacteria (Jørgensen, 1978c). The MPN method used in this study however, was found to give a high recovery (approximately 100%) of *D.desulfuricans* strains in pure culture (Postgate, 1963). One possible explanation for the low numbers recovered may have been due to a failure of the shaking of diluent bottles to release adsorbed sulphate reducing bacteria from sediment particles. Sulphate reduction in *Desulfovibrio spp.* is often confined to surfaces in the marine environment (Wood, 1965) and such absorption by the bacteria and insufficient homogenization would result in lower numbers of bacteria being recovered on sediment dilution. It was thought that the organisms were not killed during dilution, as this process was carried out in reduced diluent under an anaerobic N_2 atmosphere. Alternatively, the enumeration method employed in this study may have failed to recover types of sulphate reducers which were incapable of growth on a lactate-rich medium, such as Postgate's medium B. The presence of such types of sulphate reducer in E-70 sediment has already been discussed and the newly described genera of *Desulfobacter* and *Desulfobulbus* (Widdel, 1980) are not capable of growth on the medium employed for enumeration due to substrate unsuitability or thioglycollate toxicity. The numbers of these novel sulphate reducing bacteria in Loch Eil E-70 sediment are given in the proceeding section, together with a discussion of their contribution to the observed specific rate of metabolism of the sulphate reducing flora in the surface sediment.

THE EFFECT OF NaCl ON GROWTH

As has previously been described, the growth of *D.desulfuricans* strain 19 and the other Loch Eil E-70 isolates required the presence of 1% w/v NaCl in the growth medium (table 2.5). This salt requirement was similar to that reported by Trüper *et al* (1969) for several marine strains of *D.desulfuricans* isolated from deep sea and coastal sampling sites. The data presented in table 2.15 indicated that isolate 19 grew optimally at a salinity of 3% w/v NaCl, with both the maximum specific growth rate and total growth attained being greater than that with either 1% or 5% w/v NaCl. The failure of growth to occur with 7% w/v NaCl indicated that this organism behaved towards salinity in a similar way as the "intermediate" saltwater *D.desulfuricans* strains described by Littlewood and Postgate (1957) and Ochynski and Postgate (1963). Such strains grew optimally at between 2 and 3% w/v NaCl and showed a tailing-off in growth at salinities in excess of 6% w/v.

The salinity of the overlying water at station E-70 in the inner basin of Loch Eil varies between 27.7 and 32.0‰ (Stanley *et al*, 1978). These values for salinity however, are a measure of the concentration of the major ions in the seawater sample (*e.g.* Na^+, Cl^-, SO_4^{2-}, Mg^{2+}, Ca^{2+}, K^+, etc) and are not solely an indication of NaCl concentration. Such a salinity value (S‰) is generally estimated by conductivity measurements (see Wilson, 1975) and can often lead to difficulties when comparing field salinity measurements with laboratory data for sulphate reducing bacteria, where salinity and NaCl

concentration are synonymous. Measurement of the growth medium in this study (Postgate's C + 3% w/v NaCl) using a combined salinity-conductivity-temperature meter (Yellow Springs Instruments, Ohio) yielded a salinity of S=36‰ which was in contrast to the salinity with respect to NaCl of 30‰ There may have been some error in the determination of S‰ as the instrument functions on the basis of the solution tested having the ionic composition of seawater. However, as sea-salt is approximately 85% NaCl by weight and as "salt requiring" *D.desulfuricans* strains require Na^+ (Trüper *et al*, 1969) it was concluded that the optimum growth of isolate 19 at 3% w/v NaCl was a reflection of its adaption to the prevailing salinity *in situ* at station E-70 in Loch Eil.

This conclusion was supported somewhat by the data of Kimata *et al* (1955b) for a marine *Desulfovibrio sp.* isolated from Hiroshima Bay, Japan. The salinity in this area was on average 33‰(S‰ = chlorinity of 18.1‰ x 1.80655; Wilson, 1975) and the organism showed maximum sulphate reduction at 3% w/v NaCl. As in the case of isolate 19, activity was negligible below 1% w/v NaCl and at concentrations in excess of 6% w/v.

GROWTH ON DIFFERENT NITROGEN SOURCES

The growth of isolate 19 on NH_4Cl as the sole nitrogen source was expected, as NH_4^+ is the principal source of nitrogen in most of the media employed in the cultivation of sulphate reducing bacteria (Postgate, 1979). The high level of NH_4^+ in the interstitial water of Loch Eil E-70 surface

sediment (approx. 680μM) has already been discussed and it was thought that this compound was utilized as a nitrogen source *in situ* by *D.desulfuricans* strains such as isolate 19. The use of NO_3^- as a nitrogen source was unusual, as although its utilization by sulphate reducing bacteria has been reported, it is generally thought to be inhibitory (Postgate, 1979). However, nitrate is utilized by sulphate reducing bacteria isolated from the Tay Estuary in N.E. Scotland (R.Herbert, pers.comm.), although its use as a nitrogen source in Loch Eil was thought to be unlikely due to the low levels of NO_3^- in the sediment porewater (table 4.1, appendix). Good growth occurred with the casein hydrolysate amino acid mixture and appeared more rapid than with either of the inorganic nitrogen sources. A similar observation has been reported by Senez (1962) and has been attributed to the presence of preformed amino acids and carbon skeletons in the casamino acids.

The amino acid mixture was also capable of supporting growth in the absence of the usual electron donor lactate, both with and without an alternative fixed nitrogen source. Whether this growth occurred on fermentable compounds in the casein hydrolysate or whether the mixture was acting as a source of electrons for dissimilatory sulphate reduction was unclear. However, the amounts of blackening in the test bottles suggested that the latter case was true. Smith and Klug (1981) have reported that a ^{14}C-amino acid mixture was mineralized by sulphate reducing bacteria in the surface sediments of an eutrophic lake with isolates also utilizing casamino acids for growth. The presence of amino acids in

anoxic, marine sediments has already been discussed and it would seem reasonable to assume that amino acids can serve as both sources of nitrogen and electrons in Loch Eil sediment.

The inability of inorganic NO_3^- to act as an alternative electron acceptor to sulphate in the oxidation of lactate by isolate 19 was in accordance with similar published studies.

Nitrate has been reported as being incapable of supporting the growth of a marine sulphate reducing bacterium on lactate in the absence of sulphate (Hata, 1960). Similarly, it was not utilized as an electron acceptor for H_2-oxidation by the Marburg and Madison strains of *D.vulgaris* (Badziong *et al*, 1978). The absence of an alternative electron acceptor to sulphate in isolate 19 is consistent with the observation that sulphate is never rate-limiting for dissimilatory sulphate reduction over a depth of at least 60 cm in Loch Eil inner basin sediments.

SECTION 3

THE ACTIVITIES OF ACETATE, LACTATE AND PROPIONATE OXIDISING SULPHATE REDUCING BACTERIA IN TWO CONTRASTING MARINE SEDIMENTS

INTRODUCTION

The importance of dissimilatory sulphate reduction in the anaerobic oxidation of organic matter in marine sediments was discussed in the previous section and was a reflection on the abundance of the terminal electron acceptor sulphate. Sulphate reducing bacteria were demonstrated to be capable of oxidising over 49% of the total carbon degraded per year by the surface sediment in the polluted inner basin of Loch Eil. This figure was in general agreement with other published values for inshore marine sediments (see Aller and Yingst, 1980) and demonstrated the general predominance of the sulphate reducers in anaerobic mineralization. However, the *Desulfovibrio spp.* isolated from Loch Eil showed the classically narrow range of electron donors for sulphate reduction, although this was expanded somewhat by mutualism in enrichment cultures. As a rule members of the genus *Desulfovibrio* incompletely oxidise organic substrates to the level of acetate, which due to their lack of a functional tricarboxylic acid cycle, accumulates in the growth medium. Hence, within marine sediments they are incapable of performing a complete oxidation of organic matter to CO_2. It has been calculated that this would limit their mineralization to only one third of the organic matter present in the sediment even if the heterotrophic bacteria present in the sediment quantitatively fermented this material to the few organic acids and alcohols that they can use (Jørgensen, 1977b). This tends to suggest that if sulphate reducing bacteria are responsible for around half of the carbon degradation in Loch Eil, then there must be organisms present which are capable of oxidising acetate with the concomitant reduction of sulphate to sulphide. Until recently such

organisms, with the exception of *Desulfotomaculum acetoxidans* (see below), were thought not to exist and the use of acetate as an electron donor for sulphate reduction was considered thermodynamically unfavourable (Wake *et al*, 1977).

Baars (1930, cited by Postgate, 1979) described an acetate utilizing species *Desulfovibrio rubentschickii* which, although subsequently lost, appeared to support the report of acetate oxidising sulphate reducing cultures by Rubentschik (1928, cited in Pfennig *et al*, 1981). However, attempts by Selwyn and Postgate (1959) to re-isolate the species failed despite the authors' recovery of acetate utilizing marine enrichment cultures. The discovery in 1976 by Pfennig and Biebl of the sulphur reducing, acetate oxidising organism *Desulfuromonas acetoxidans* appeared to solve a portion of the paradox of anaerobic degradation by sulphate reducing bacteria. The organism was isolated from anaerobic sediment in the Antarctic Ocean and reduced elemental sulphur to H_2S with the concomitant oxidation of acetate to CO_2. Postgate (1979) speculated that this organism could remove the acetate produced by *Desulfovibrio spp.*, using elemental sulphur derived from their H_2S *via* anoxygenic photosynthesis by green sulphur bacteria. The isolation of *Desulfotomaculum acetoxidans* in 1977 by Widdel and Pfennig appeared to solve the acetate "problem" - the organism being a true acetate oxidising sulphate reducing bacterium which was present in the marine environment. However, Jørgensen's (1977b) reservation on the importance of this organism *in situ* has recently been substantiated by a report that the organism is normally of intestinal origin (optimum growth temperature 36 ^{o}C) which is introduced into natural

waters by faecal pollution (Widdel and Pfennig, 1981).

During the course of this study however, the first descriptions of truely marine, acetate oxidising sulphate reducing bacteria were reported by Widdel (1980) and Widdel and Pfennig (1981). By the use of modified culture conditions the authors isolated a number of new morphological and physiological types of sulphate reducing bacteria which could oxidise acetate and other fatty acids (C_1- to C_{14}-) through to CO_2. These organisms were recovered from marine brackish and freshwater environments in novel media which utilized Na_2S as the reducing agent and a CO_2-bicarbonate buffer system. In addition, sodium thioglycollate and EDTA-chelated iron were not used (cf. Pankhurst, 1971) as they proved inhibitory to some species. Enrichment and isolation of several species of these sulphate reducers also used sodium dithionite as an additional reductant and compounds such as isobutyric acid, valeric acid and caproic acid as growth stimulating factors. The preparation and use of these media are described in much fuller detail by Pfennig *et al*, (1981).

One well described acetate oxidising organism is *Desulfobacter postgatei* (Widdel and Pfennig, 1981), a Gram negative, non-sporing sulphate reducing organism which was isolated from marine sediment with acetate as the sole electron donor. This organism performs a complete oxidation of acetate to CO_2 using sulphate, sulphite or thiosulphate as electron acceptors with a requirement for growth of 6mM Mg^{2+}. Other sulphate reducing bacteria which have been reported to completely oxidise acetate and other fatty acids are *Desulfococcus multivorans* (C_1- to C_{14}-), *Desulfonema limicola* (C_1- to C_{12}-),

Desulfonema magnum (C_1- to C_{10}-) and *Desulfosarcina variabilis* (C_1- to C_{14}-). All of these bacteria occur in estuarine and marine environments and are described in detail by Widdel (1980). Several of the above genera are capable of the complete oxidation to CO_2 of many of the volatile fatty acids that occur as intermediates in the fermentative breakdown of organic matter in anoxic marine sediments. In addition to acetate, butyrate and propionate are often the predominant fatty acids in such sediments (Balba and Nedwell, 1982) and occur at appreciable levels in the surface 6 cm of Loch Eil E-70 sediment (Miller *et al*, 1979). Such compounds are oxidised to CO_2 although one propionate utilizing genera, *Desulfobulbus*, performs an incomplete oxidation of low molecular weight compounds (C_3-) to acetate and CO_2 (Widdel, 1980).

The presence of *Desulfobacter spp.* and *Desulfobulbus spp.* has recently been demonstrated in tidal sediments from the Ems-Dollard estuary (N.E. Netherlands) at populations of around 10^3 ml^{-1} in the surface 20 cm of sediment (Laanbroek and Pfennig, 1981). Concurrent with the reports of these novel genera of sulphate reducing bacteria has been an accumulation of evidence for their activities *in situ*. The importance of acetate oxidation *via* sulphate reduction has already been discussed and was suspected of occurring in a New Zealand intertidal sediment by Mountfort *et al* (1980); an observation that was confirmed in a subsequent paper (Mountfort and Asher, 1981). Recent studies have utilized ^{14}C-labelled short-chain fatty acids and gas chromatograph-gas proportional counting systems, together with $^{35}SO_4^{2-}$ and the inhibitor of sulphate reduction, molybdate (20mM MoO_4^{2-}). Fatty acid accumulations

under conditions of sulphate reduction inhibition have demonstrated the importance of acetate as an electron donor for sedimentary sulphate reduction, with Sørensen *et al* (1981) estimating that oxidation of the compound could account for as much as two thirds of the measured sulphate reduction activity. The contributions of butyrate and propionate were thought to be much less at between 5 to 10%, although Balba and Nedwell (1982) have suggested that sulphate reducing bacteria are the predominant organisms involved in the turnover of these compounds in a U.K. saltmarsh sediment.

At the time of writing relatively little information is available on the activities of these novel sulphate reducing bacteria in marine sediments. The absence of sporeforming organisms in acetate enrichment cultures described in the previous section suggested that members of these newly described genera may have been present in the sediments examined. Hence, the aims of the final section of this study were to investigate the distribution and significance of two new types of sulphate reducing bacteria (acetate and propionate oxidisers) in comparison to the classical lactate oxidising organisms. Two contrasting sediments were examined; E-70 in the polluted inner basin of Loch Eil and A-3, a low carbon, oxidised sediment on the Malin Shelf region of the British Isles continental shelf. It is thought that higher dissolved pools of low molecular weight compounds are present in sediments such as E-70 which contain larger amounts of organic carbon (see table i). As these compounds are electron donors for the three types of sulphate reducing bacteria, it was speculated as to whether there would be a difference in the activities of

the organisms between the two contrasting stations.

The study was conducted in three ways:

i. Acetate, lactate and propionate oxidising sulphate reducing bacteria were enumerated using modified culture techniques.

ii. The effect of acetate, lactate or propionate amendment on sedimentary sulphate reduction was measured using the previously described method with 0.1mM substrate.

iii. The rate of growth and sulphate reduction of a propionate oxidising organism on different substrates was determined and compared with that of a *D.desulfuricans* strain isolated from station E-70 in Loch Eil.

MATERIALS AND METHODS

ENUMERATION OF ACETATE, LACTATE AND PROPIONATE OXIDISING SULPHATE REDUCING BACTERIA

LOCH EIL E-70

The method used to enumerate these newly described strains of sulphate reducing bacteria was essentially that of Laanbroek and Pfennig (1981). The basal medium (solution 1) was autoclaved at 103.4 kPa for 30 minutes and cooled under an oxygen-free nitrogen gas stream (copper turnings at 350°C).

Solution 1	Distilled H_2O	970 ml
	Na_2SO_4	3.0 g
	NaCl	20.0 g
	KCl	0.3 g
	NH_4Cl	0.3 g
	$MgCl_2.6H_2O$	3.0 g
	KH_2PO_4	0.2 g
	$CaCl_2.2H_2O$	0.15 g

The following sterile additions were then made to the stirred basal medium under a stream of sterile nitrogen: 1 ml solution 2, 30 ml solution 3 and 3 ml solution 4.

Solution 2	Trace elements.	
	Distilled H_2O	993 ml
	HCl (25% v/v)	6.5 ml
	$FeCl_2.4H_2O$	1.5 g
	H_3BO_3	60 mg
	$MnCl_2.4H_2O$	100 mg

$CoCl_2.6H_2O$	120 mg
$ZnCl_2$	70 mg
$NiCl_2.6H_2O$	25 mg
$CuCl_2.2H_2O$	15 mg
$Na_2MoO_4.2H_2O$	25 mg

Solution 3: Sodium bicarbonate solution

Distilled H_2O	100 ml
$NaHCO_3$	8.5 g

- saturated with CO_2 and filtersterilized into a sterile gas-tight universal bottle.

Solution 4: Sodium sulphide solution

Distilled H_2O	100 ml
$Na_2S.9H_2O$	12 g

- autoclaved under nitrogen.

The resulting medium was adjusted to pH 7.2 (sterile 2M HCl or Na_2CO_3), transferred into an anaerobic chamber (Anaerobic System, 1024, Forma Scientific) and dispensed as 100 ml aliquots into sterile medical flat bottles. Acetate, lactate or propionate carbon sources were then added to each bottle to a final concentration of 20mM from sterile 2M stock solutions (Solution 5), together with 0.1 ml aliquots of four vitamin solutions (Solution 6).

Solution 5:

	Distilled H_2O	100 ml
i.	sodium acetate	16.4 g
or ii.	sodium lactate (70% w/v)	23.2 ml
or iii.	sodium propionate	19.2 g

Solution 6:	Distilled H_2O	100 ml
i.	Biotin	1 mg
or ii.	p-aminobenzoic acid	5 mg
or iii.	Vitamin B_{12}	5 mg
or iv.	Thiamin	10 mg

- filter sterilized.

Sediment taken at depths of 5 cm down the core sample was homogenized as described in the previous section and a 10^{-2} dilution made in basal medium (solutions 1 to 4) for each of the four sediment layers. These dilutions and the acetate, lactate and propionate media bottles were then sealed and removed from the anaerobic cabinet. The three media were warmed to 45°C in a water bath.

3 ml Aliquots of 3% w/v "Oxoid" agar no.1 containing 2% w/v NaCl and 0.3% w/v $MgCl_2.6H_2O$ were added to test tubes, dissolved by autoclaiving and held at 55°C. 6 ml aliquots of the acetate, lactate and propionate media were then mixed with 3 ml of this agar to yield sets of test tubes containing the three carbon sources, which were held at 45°C. 1 ml inocula from the 10^{-2} sediment dilutions were added to tubes containing the three substrates and decimal dilutions upto 10^{-6} made in these agar shake tubes. Prior to solidification and sealing with a 5 ml plug of agar, 0.01 ml aliquots of a sodium dithionite solution (solution 7) were added to each inoculated tube and mixed.

Solution 7:	Oxygen-free distilled H_2O	100 ml
	$Na_2S_2O_4$	3 g

- filtersterilized under nitrogen into a gas-tight bottle. Throughout all these manipulations anaerobiosis was maintained by means of the Hungate technique (Hungate, 1969).

The tubes were incubated in the dark at 30°C for four weeks before colony counts were performed.

N.E. ATLANTIC A-3

The limitations of onboard ship facilities precluded the use of Laanbroek and Pfennig's (1981) method for the enumeration of acetate, lactate and propionate oxidising sulphate reducing bacteria. Hence, a simpler technique was employed based on the enumeration medium E of Postgate (1979). The medium was formulated as in the previous section with sulphate at a concentration of 30mM, 20mM acetate, lactate or propionate as electron donors and 1 ml litre^{-1} of the trace element solution 2. As the reducing agent sodium thioglycollate is inhibitory to some of the newly described sulphate reducers (Pfennig *et al*, 1981) it was omitted, with the ascorbate concentration of the medium being correspondingly increased to 0.2 g litre^{-1}.

9 ml aliquots of the three media were dispensed into test tubes, autoclaved in the usual manner and cooled and stored under an atmosphere of 95% H_2/5% CO_2 within an anaerobic jar. Just prior to use the tubes were melted and held at 45°C.

5 cm Sections of A-3 sediment were taken and homogenized as described previously. 10 ml inocula from each section were added to 90 ml of basal medium (no electron donor or agar) and 1 ml inocula from these 10^{-1} dilutions added to test tubes containing either of the three carbon sources. Decimal

dilutions upto 10^{-5} were again made in the agar shake tubes prior to agar solidification in a cold water bath, and plugging with 3 ml of medium. Colonies of sulphate reducing bacteria were counted after incubation in the dark at 30°C for upto 4 weeks.

EFFECT OF SUBSTRATE ADDITION ON SULPHATE REDUCTION RATES IN LOCH EIL E-70 AND N.E. ATLANTIC A-3 SEDIMENTS

Homogenized sediment from the 0-5 cm and 5-10 cm sediment layers of the two sampling stations was used. The method employed was as described in the previous section with the exception that each 50 ml reaction flask contained 10 ml 0.4M NaCl with either no substrate addition or 0.1mM sodium acetate, lactate of propionate. Sodium thioglycollate was again omitted due to its reported toxic effects on the novel sulphate reducing bacteria.

GROWTH AND SULPHATE REDUCTION OF *DESULFOBULBUS SP.* 3 pr 10 (DSM 2058) ON LACTATE, PROPIONATE AND PYRUVATE

Desulfobulbus sp. 3 pr 10 was obtained as an active culture from the Deutshe Sammlung von Mikroorganismen, Grisebachstrasse 8, D-3400 Göttingen, Germany. The strain (DSM 2058) was a marine *Desulfobulbus sp.* - a member of one of the newly described genera of sulphate reducing bacteria (Widdel, 1980). The organism was maintained by weekly subculture on a simplified version of the recommended marine *Desulfobulbus* medium (DSM medium 196) which had the following composition:

Na_2SO_4	2.8 g
KH_2PO_4	0.2 g
NH_4Cl	0.3 g
NaCl	20.0 g
$MgCl_2.6H_2O$	3.0 g
KCl	0.5 g
$CaCl_2.2H_2O$	0.15 g
sodium propionate	2.9 g
Trace element solution SL7	1.0 ml
Yeast extract	1.0 g
Sodium ascorbate	0.2 g
Resazurin (0.1% w/v aqueous soln.)	2 ml
Distilled H_2O	1,000 ml

Trace element solution SL7 contained in 1 litre of distilled H_2O: HCl (25% w/v) 10 ml, $FeCl_2.4H_2O$ 1.5 g (dissolved in the HCl, then diluted); $CoCl_2.6H_2O$ 190 mg; $MnCl_2.4H_2O$ 100 mg; $ZnCl_2$ 70 mg; H_3BO_3 62 mg; $Na_2MoO_4.2H_2O$ 36 mg; $NiCl_2.6H_2O$ 24 mg and $CuCl_2.2H_2O$ 17 mg.

The pH of the medium was adjusted to 7.5 using 2M NaOH and sterilized at 103.4 kPa for 20 minutes. The use of 0.02% w/v ascorbate as the reducing agent meant that the redox potential was only weakly poised - as indicated by a faint pink coloration of the resazurin (actually resorufin, formed irreversibly from resazurin at pH 7.5). However, the routine use of large inocula (10 to 20%) during subculture resulted in the carry-over of endogenous sulphide and further reduction of the culture

medium. This was indicated by the loss of the pink coloration of the medium. As resorufin becomes colourless at an Eh of -110mV (Costilow, 1981) it was assumed that the redox potential of an inoculated culture was probably below this value.

Growth experiments on strain 3 pr 10 were performed using the above medium at a sulphate concentration of 20mM and 30mM sodium lactate, propionate or pyruvate as electron donors. These three media were dispensed as 10 ml aliquots into reductase tubes, with reduction and sterilization being carried out as before. All the tubes were at an Eh$\leqslant$-110mV prior to inoculation. Ten tubes of each substrate were inoculated by injection of 1 ml of a 48 hour culture of 3 pr 10 grown on the relevant substrate and mixed. The absorbances of the cultures at 580 nm were read as described in the previous section and 2.5 ml 0.2M cadmium acetate were injected into one tube of each substrate. Any sulphide fixed was determined by iodine-thiosulphate titration as before. Growth at 30^{o}C was again followed by recording the mean absorbance and the culture sulphide concentration at noted time intervals until the stationary phase of growth was reached. The maximum specific growth rate (μm) and sulphate reduction rate during growth on each substrate were calculated as described in the previous section.

R E S U L T S

LOCH EIL E-70

COUNTS OF SULPHATE REDUCING BACTERIA

It can be seen from table 3.1 that in the surface 5 cm of sediment from station E-70 in the polluted inner basin of Loch Eil acetate and lactate utilizing organisms predominated at around 10^4 bacteria ml^{-1}. The number of propionate oxidisers was approximately half this number. The colonies formed in the acetate and propionate agar shakes were predominantly small and yellow-grey in coloration. Microscopic examination of several colonies from a high dilution of each substrate yielded short, non-motile rods when acetate was the growth substrate and lemon-shaped, non-motile cells with propionate.

At a depth of 5-10 cm down the sediment bacteria capable of using acetate as an electron donor for sulphate reduction predominated and were between five and ten times more numerous than lactate or propionate utilizing organisms. Further down the sediment at a depth of 10-15 cm the numbers of all types of sulphate reducing bacteria were similar, though below this depth no acetate oxidisers were recovered. 7.0×10^3 lactate utilizing organisms ml^{-1} were recorded at a depth of 15-20 cm in this reduced sediment.

SULPHATE REDUCTION RATE

The sulphate reducing activity in unamended surface 5 cm sediment from E-70 of 142.6 n mol SO_4^{2-} $ml^{-1}d^{-1}$ (table 3.2) was very similar to that recorded previously (see fig.2.3)

TABLE 3.1 COUNTS OF ACETATE, LACTATE AND PROPIONATE OXIDISING SULPHATE REDUCING BACTERIA IN LOCH EIL E-70 SEDIMENT

Sediment depth (cm)	Bacteria ml^{-1}		
	Acetate	Lactate	Propionate
0-5	1.1×10^4	1.4×10^4	7.0×10^3
5-10	2.0×10^4	4.0×10^3	2.0×10^3
10-15	2.0×10^4	1.9×10^4	1.4×10^4
15-20	-	7.0×10^3	n.d.

- not detected; n.d. not determined

TABLE 3.2 EFFECT OF SUBSTRATE ADDITION ON SULPHATE REDUCTION RATES IN LOCH EIL E-70 SEDIMENTS

Substrate addition	0-5 cm		5-10 cm	
	Sulphate reduction rate (nmol $SO_4^{2-}ml^{-1}d^{-1}$)	% Stimulation	Sulphate reduction rate (nmol $SO_4^{2-}ml^{-1}d^{-1}$)	% Stimulation
None	142.6	-	69.8	-
0.1mM Acetate	212.5	+49	121.9	+75
0.1mM Lactate	267.2	+87	111.3	+60
0.1mM Propionate	242.9	+70	116.7	+67

and was again high. The greatest stimulation of sulphate reduction rate in this surface sediment was with 0.1mM lactate which was over 80% greater than that in control samples. Although a stimulation in activity was recorded with both acetate and propionate additions, this was lower than that with lactate.

In the control 5-10 cm sediment samples the sulphate reduction rate of 69.8 n mol $SO_4^{2-}ml^{-1}d^{-1}$ was approximately twice that recorded previously (fig.2.3). In contrast to the higher sediments it was acetate supplementation which produced the greatest stimulation in activity with a lower level of rate elevation by 0.1mM propionate or lactate.

N.E. ATLANTIC A-3

COUNTS OF SULPHATE REDUCING BACTERIA

In the surface 5 cm of sediment from the Malin Shelf region of the British continental shelf organisms capable of oxidising lactate were the predominant sulphate reducers, being some ten times more numerous than either acetate or propionate utilizers (table 3.3). In the lower sediment section the numbers of all three types of sulphate reducing bacteria were similar at around 10^2 ml^{-1}.

SULPHATE REDUCTION RATE

The low sulphate reduction rate recorded of 2.2 n mol SO_4^{2-} $ml^{-1}d^{-1}$ recorded in unsupplemented 0-5 cm sediment (table 3.4)

TABLE 3.3 COUNTS OF ACETATE, LACTATE AND PROPIONATE OXIDISING SULPHATE REDUCING BACTERIA IN N.E. ATLANTIC A-3 SEDIMENT

Sediment depth (cm)	Bacteria ml^{-1}		
	Acetate	Lactate	Propionate
0-5	$3.0x10^2$	$5.0x10^3$	$3.0x10^2$
5-10	$3.0x10^2$	$4.0x10^2$	$2.0x10^2$

TABLE 3.4 EFFECT OF SUBSTRATE ADDITION ON SULPHATE REDUCTION RATES IN N.E.ATLANTIC A-3 SEDIMENT

Substrate addition	0-5 cm		5-10 cm	
	Sulphate reduction rate (nmol $SO_4^{2-}ml^{-1}d^{-1}$)	% Stimulation	Sulphate reduction rate (nmol $SO_4^{2-}ml^{-1}d^{-1}$)	% Stimulation
None	2.2	-	0.4	-
0.1mM Acetate	2.6	+17	0.8	+81
0.1mM Lactate	3.1	+41	2.8	+573
0.1mM Propionate	2.9	+33	0.5	+10

was similar to that obtained on an earlier cruise (table 2.1). As with E-70 surface sediment, the addition of 0.1mM lactate gave the greatest increase in sulphate reducing activity (+41%), with correspondingly less stimulation by propionate or acetate. The very low activity below this depth was massively stimulated by the addition of lactate to over 500% of that with no substrate amendment. Supplementation with acetate produced more stimulation than in the surface sediment (+81%), whilst the addition of propionate had a less marked effect.

GROWTH AND SULPHATE REDUCTION OF *DESULFOBULBUS SP.* 3 pr 10 (DSM 2058) ON LACTATE, PROPIONATE OR PYRUVATE

LACTATE

Growth of strain 3 pr 10 on lactate as electron donor occurred exponentially and was mirrored by sulphide production (fig.3.1). Both these parameters levelled off after approximately 60 hours incubation at 30°C. The maximum specific growth rate (μm) was recorded at the start of logarithmic growth, whilst the maximum rate of sulphate reduction (sulphide production) occurred in contrast towards the end of this period (33 to 46 hours). In each instance the values recorded were the highest obtained in this study (table 3.5).

PROPIONATE

Very slow growth was observed on propionate and it was unclear as to whether this was exponential or linear (fig.3.2). In contrast, the use of this compound as electron donor for

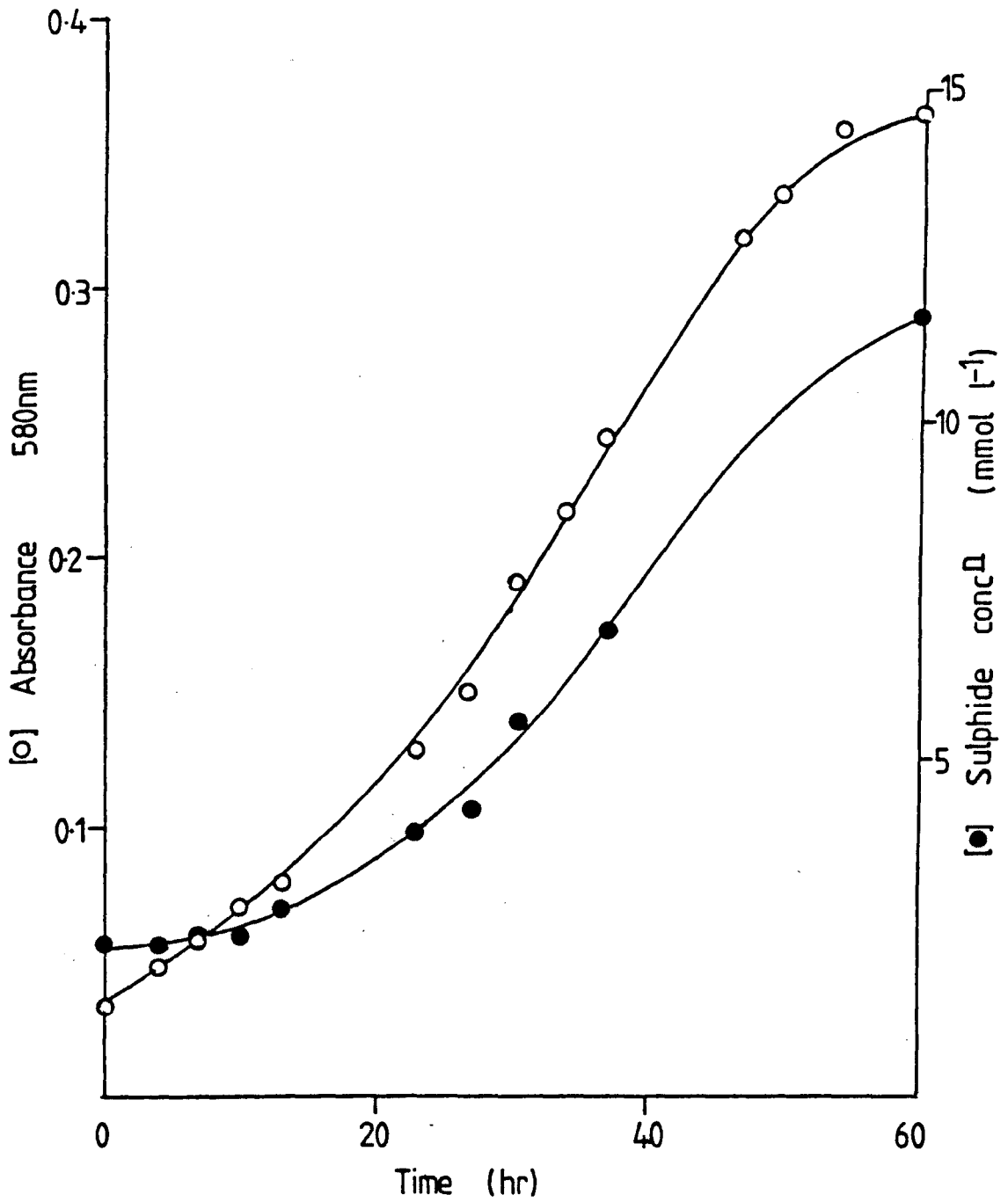

Fig.3.1 Growth and sulphide production of *Desulfobulbus sp.* 3 pr 10 growing on 30mM lactate.

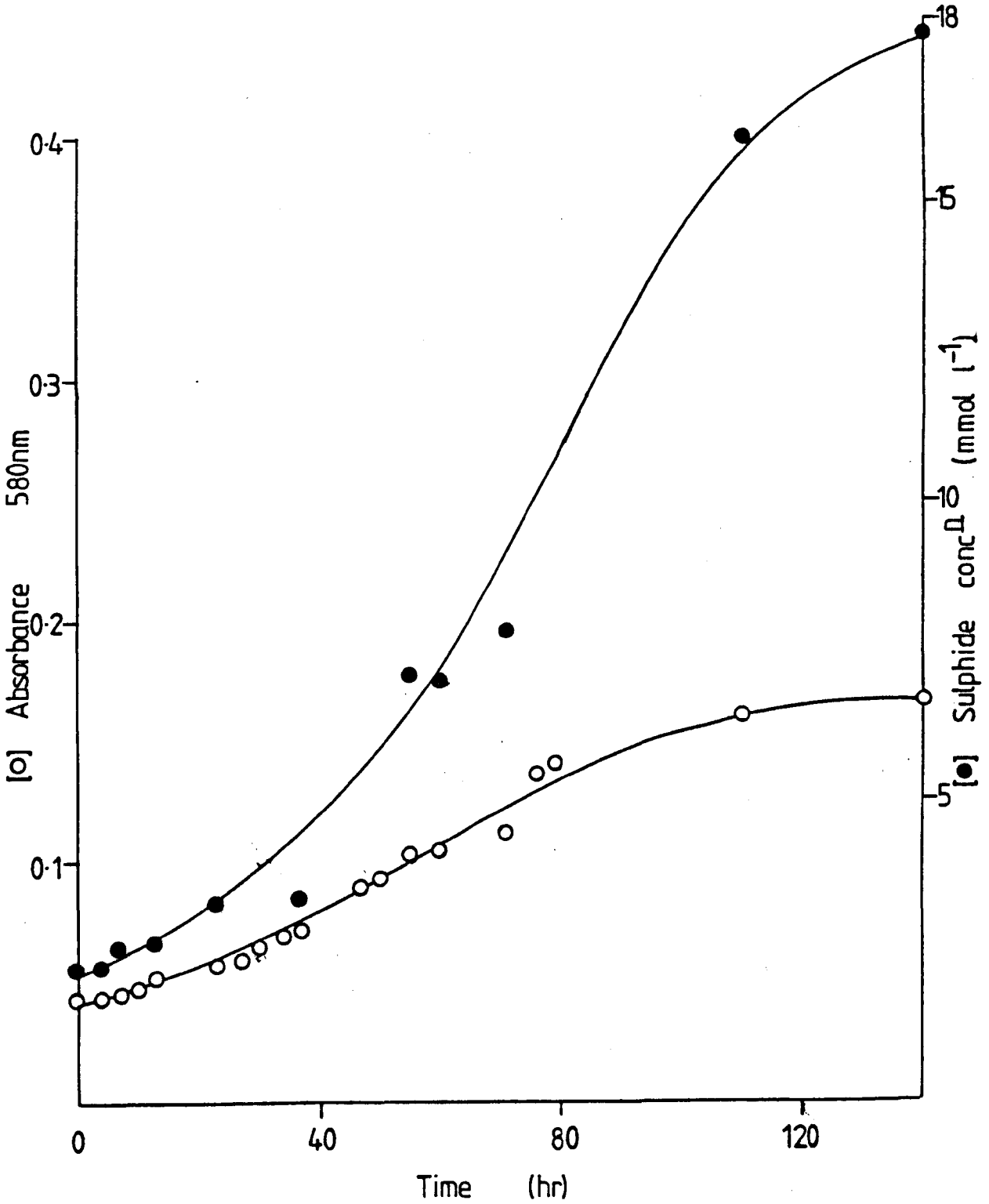

Fig.3.2 Growth and sulphide production of *Desulfobulbus sp.* 3 pr 10 growing on 30mM propionate.

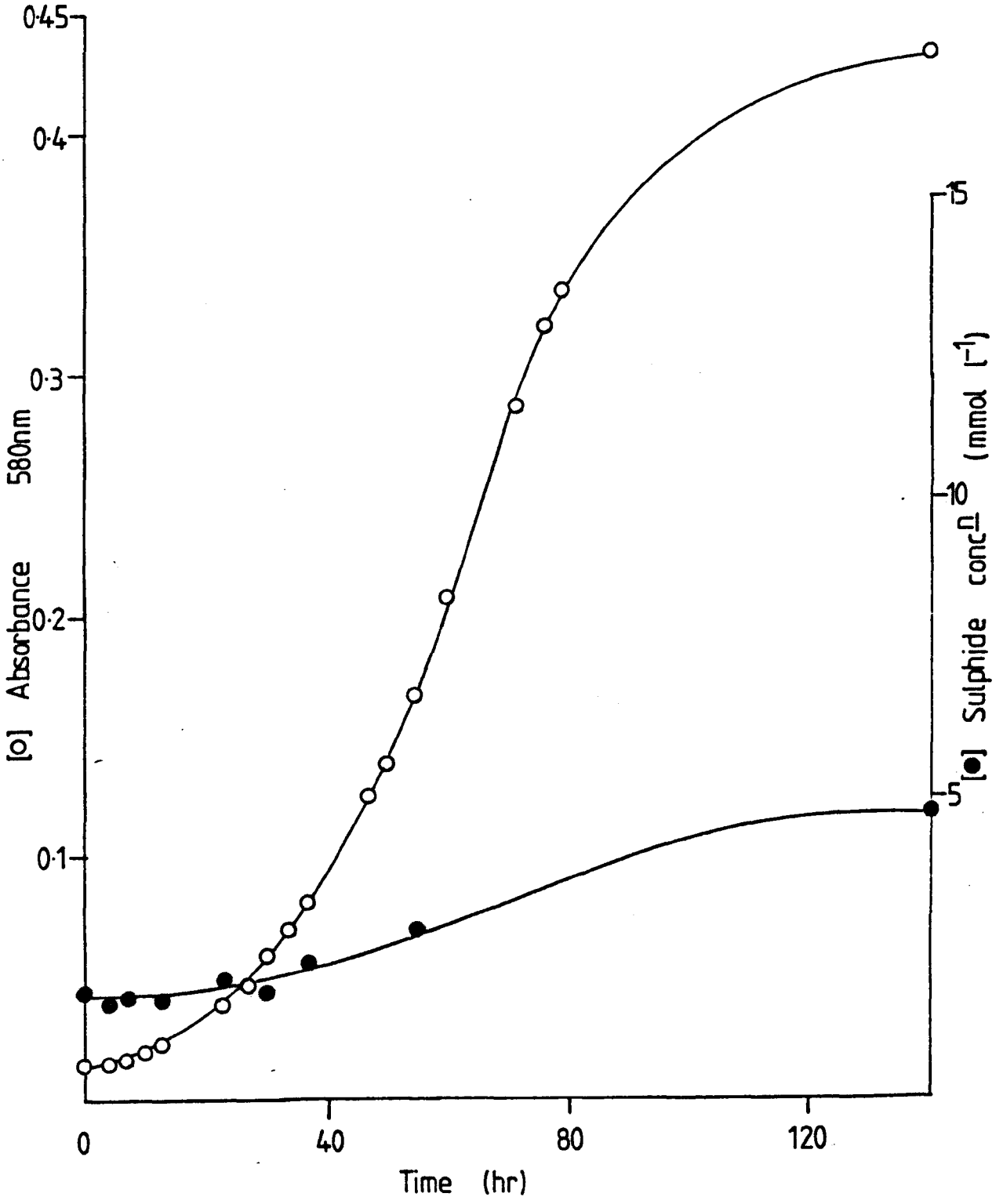

Fig.3.3 Growth and sulphide production of *Desulfobulbus sp.* 3 pr 10 growing on 30mM pyruvate.

TABLE 3.5 MAXIMUM SPECIFIC GROWTH RATE μm, INCREASE IN A_{580nm} AND MAXIMUM SULPHATE REDUCTION RATE OF *DESULFOBULBUS SP.* 3 pr. 10 GROWING ON 30mM LACTATE, PROPIONATE OR PYRUVATE

Substrate	μm (hr^{-1})	Increase in $Absorbance_{580nm}$	Maximum sulphate reduction rate (μmol SO_4^{2-} $ml^{-1}d^{-1}$)
Lactate	0.18	0.33	6.72
Propionate	(0.02)*	0.12	5.04
Pyruvate	0.16	0.42	0.96

* μm value assuming that growth was exponential

dissimilatory sulphate reduction yielded the highest amount of sulphide produced in this study (15.5 mmol l^{-1}) and a maximum rate of sulphide production at the end of growth (68 to 78 hours) which was three quarters of that with lactate (table 3.5).

PYRUVATE

Exponential growth occurred on pyruvate (fig.3.3) and the maximum specific growth rate at the beginning of logarithmic growth were similar to that with lactate as electron donor (table 3.5). The maximum growth of strain 3 pr 10, as indicated by the change in absorbance at 580 nm, was recorded with this substrate. Sulphide production during 140 hours incubation was low with a correspondingly low maximum sulphate reduction rate.

D I S C U S S I O N

The data presented in table 3.1 indicated that the newly isolated acetate and propionate oxidising sulphate reducing bacteria occurred at similar levels to the "classical" lactate utilizers in Loch Eil surface sediments. This illustrated the diversity of the sulphate reducing flora in Loch Eil, which could be capable of oxidising through to CO_2 almost half of the organic matter degraded by the sediment *per annum*. The number of bacteria recovered was slightly higher than that reported for the Ems-Dollard estuary (Laanbroek and Pfennig, 1981) and this was probably a reflection on the lower organic carbon content of the estuarine sediment (see Schröder and van Es, 1980). The population of lactate oxidising organisms at the four sediment depths examined was generally a factor of ten greater than that recorded previously using the rich Postgate medium (fig.2.3). Approximately 80% of the species of sulphate reducing bacteria described in Widdel's thesis (1980) are capable of using lactate as an electron donor for sulphate reduction. Although these species contain many members of the genus *Desulfovibrio*, there are also members of the genera *Desulfobulbus*, *Desulfococcus*, *Desulfonema* and *Desulfosarcina* represented. As these organisms often require sulphide as the medium reductant and vitamin and trace element additions for growth, it was feasible that these organisms would not have been recovered in a thioglycollate reduced medium such as Postgate's medium B (Postgate, 1979).

From morphological examination it would appear that the asporogenous, short rods present in the acetate shake tubes

were either *Desulfobacter* or *Desulfosarcina spp.* The characteristic lemon-shaped appearance of many of the cells growing on propionate as sole electron donor suggested that they were members of the genus *Desulfobulbus* (Widdel, 1980) and these organisms bore a close morphological similarity to *Desulfobulbus sp.* 3pr10 (see below). In contrast to the previous study on Loch Eil there was no rapid decrease in the numbers of sulphate reducing bacteria on increasing sediment depth. The predominance of acetate oxidising sulphate reducers at a depth of 5-10 cm was possibly a reflection of fatty acid concentration as discussed below.

The stimulation of sulphate reduction rate in the surface 5 cm of E-70 sediment by added substrates was as follows:

Lactate > Propionate > Acetate

This observation was consistent with the decreasing energy changes for the reduction of sulphate by the three substrates (Wake *et al*, 1977). A similar preference in electron donors by sulphate reducing bacteria has been reported by Smith and Klug (1981) who found that in an eutrophic lake sediment molybdate decreased the mineralization rate of lactate by 58%, propionate by 52% and acetate by 14%. The importance of lactate as an electron donor for sedimentary sulphate reduction in both freshwater and marine sediments is well known (*e.g.* Cappenberg, 1975; Oremland and Silverman, 1979) and was demonstrated to be the preferred substrate of a representative *D.desulfuricans* strain isolated from the surface 5 cm of E-70 sediment. As the majority of sulphate reducing bacteria are

capable of utilizing lactate as an electron donor (Widdel, 1980) it was still thought that lactate was an important substrate for sedimentary sulphate reduction.

Below the sediment surface however, acetate became the most important electron donor for sulphate reduction, as indicated by the predominance of acetate oxidising organisms and by the increased stimulation of sulphate reduction:

Acetate > Propionate > Lactate

The greatest concentration of degradable organic matter was found at the surface of E-70 sediment and decreased rapidly with depth as it was metabolized by sedimentary bacteria (Duff, 1981). The electron donors utilized by the three groups of sulphate reducing bacteria are produced by the fermentative breakdown of this material and would tend to occur at maximum concentrations in the surface 5 cm of sediment (Nedwell, 1982). It would appear that lactate and propionate were utilized preferentially in this zone due to thermodynamic considerations and as a consequence would be less abundant below this depth. Less biodegradable matter would also be present below this depth and it has been postulated that when the supply of available carbon is limited fermentative bacteria can obtain a slightly greater energy yield by re-oxidising their reduced co-enzymes *via* H_2-transfer as opposed to fermentative re-oxidation (Nedwell, 1982). This process results in the production of proportionately more acetate than reduced fermentative products such as lactate, propionate or succinate. The nutritionally limited *Desulfovibrio spp.* are

capable of acting as the H_2-acceptor as discussed in the previous section and under such conditions could utilize hydrogen as an electron donor, as opposed to their preferred substrate lactate. Such a situation would favour sulphate reducing bacteria capable of using acetate as an electron donor and would account for the observed stimulation of activity in the 5-10 cm zone of E-70 sediment by this organic acid. In addition the oxidation of lactate and propionate is often incomplete and results in acetate formation due to the reactions:

$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + S^{2-} + 2H_2O$$

$$4CH_3CH_2COO^- + 3SO_4^{2-} \rightarrow 4CH_3COO^- + 4CO_2 + 3S^{2-} + 4H_2O$$

The requirement for sulphate reducing bacteria to oxidise this acetate through to CO_2 has already been discussed and from the data obtained in this study it would appear that in Loch Eil this process occurs in the deeper sediment where the supply of more favourable substrates is limited.

In the previous section it was shown that the specific rate of sulphate reduction per colony of sulphate reducing bacteria recovered from Loch Eil E-70 surface sediment (table 2.20) was some five thousand times higher than that of a representative *D.desulfuricans* isolate growing under good cultural conditions. As the measured sulphate reduction rate was not thought to be this much in error, it was concluded that the numbers of sulphate reducing bacteria had been underestimated by this amount. A similar observation has been made by Jørgensen (1977b) who considered this to be due to naturally occurring sulphate reducing bacteria which were

physiologically different to *Desulfovibrio spp.*– being incapable of growth on conventional lactate based media (Jørgensen, 1978c). The necessity of modified cultural conditions for the newly described acetate and propionate oxidising sulphate reducing bacteria has already been discussed, together with the possibility of many lactate oxidising bacteria going undetected. The measured sulphate reduction rate of 142.6 n mol SO_4^{2-} $ml^{-1}d^{-1}$ for the surface 5 cm of E-70 sediment was very similar to that recorded previously (table 2.18). This figure gives a specific rate of metabolism (sulphate reduction) for the enumerated acetate, lactate and propionate oxidising bacteria of $4.5x10^{-12}$mol SO_4^{2-} $bacterium^{-1}$ d^{-1}. Although the recovery of these new types of sulphate reducing bacteria had reduced this rate to a thirtieth of that when the bacteria were enumerated with Postgate's medium B (table 2.20), it was still a 100-fold higher than that of *D.desulfuricans* isolate 19 growing on lactate as electron donor. As the maximum sulphate reduction rate of these novel bacteria appeared less than that of isolate 19 (table 3.5) it would seem that the difference in specific rates of metabolism of sulphate reducing bacteria *in situ* and *in vitro* was due to an underestimation of cell numbers and not to an elevated specific rate of metabolism of any novel organism. As this underestimation was not now thought to be due to a deficient medium, it was assumed that the failure to recover sedimentary sulphate reducing bacteria was due to bacterial absorption and insufficient homogenization as discussed in the previous section. In fact, this extreme underestimation of true cell numbers occurs frequently in viable counts of heterotrophic bacteria (Jørgensen, 1978c).

N.E. ATLANTIC A-3

In the surface 5 cm of this oxic, low carbon sediment bacteria capable of reducing sulphate at the expense of lactate predominated and occurred in numbers comparable with those previously reported for coastal sediments (table 2.19). Below this sediment depth however, acetate, lactate and propionate oxidising bacteria were present in similar numbers, which would indicate that earlier studies on such sediments may have underestimated the sulphate reducing flora by up to 50%.

The stimulation of sulphate reduction rate by substrate supplementation of the surface 5 cm of sediment was as follows:

Lactate > Propionate > Acetate

This pattern of stimulation was identical to that of the more organically rich Loch Eil sediment and was again consistent with the relative energy changes for the appropriate reactions involving sulphate. Although low molecular weight compounds are undetectable in A-3 porewater (S.Malcolm, pers.comm.), the substrate preference of the sulphate reducing flora was identical to that present in the surface sediment of E-70 which contains several biologically important organic acids (Miller *et al*, 1979). This observation would suggest that lactate is still a good substrate for ecological studies on sulphate reducing bacteria, although its use with a modified medium such as that described by Pfennig *et al* (1981) would seem necessary.

The stimulation of sulphate reduction in A-3 surface sediment by the addition of 0.1mM substrates was less than that

in the more organically rich sea-loch sediment. This would suggest that some factor other than electron donor availability was limiting sulphate reduction. The Eh at a depth of 4 cm in A-3 sediment was typically over +300mV and as dissimilatory sulphate reduction requires much lower redox potentials it would appear that sulphate reduction was limited by the oxidised conditions. It was thought that sulphate reduction occurred in A-3 surface sediment within reduced microniches. As these anaerobic microenvironments often occur within small particles of organic matter, such as faecal particles (Jørgensen, 1977a), it was conceivable that the low levels of such organic matter in A-3 sediment (%C, 0.5) limited microniche formation.

Below the surface 5 cm of sediment lactate remained the most important electron donor, with sulphate reduction rate stimulation as follows:

Lactate >> Acetate > Propionate

This was in contrast to the sea-loch sediment and suggested that lactate was not normally depleted in the surface sediment. The massive stimulation of sulphate reducing activity again emphasised the importance of lactate as an electron donor in marine sediments and indicated that the restraint on activity in this lower sediment appeared to be electron donor availability.

The difficulties in interpreting the results of carbon source amendment on sedimentary sulphate reduction rate have already been mentioned. The addition of 0.1mM substrate to the sediment obviously resulted in unnaturally high levels of the organic acid and stimulation by a compound derived from the substrate could not be discounted. Nonetheless, the

changing importance of lactate and acetate with increasing depth in E-70 sediment has been reflected in recent measurements on the concentration of these organic acids in the sediment porewater (S.Malcolm, unpub. data). The use of recently described radiotracer techniques (see Balba and Nedwell, 1982) would appear necessary for the further understanding of the role of these low molecular weight organic acids in the anaerobic oxidation of sedimentary detritus by the sulphate reducing bacteria.

GROWTH AND SULPHATE REDUCTION OF *DESULFOBULBUS SP.* 3 pr 10

From the above considerations it would appear that the newly described acetate and propionate oxidising sulphate reducing bacteria are active participants in anaerobic mineralization in Loch Eil sediment. Little information is currently available on their rates of growth and sulphate reduction in pure culture, although doubling times have been published for the acetate oxidising organism *Desulfobacter postgatei* (Widdel and Pfennig, 1981).

Desulfobulbus sp. 3 pr 10 was a marine sulphate reducing bacterium isolated on propionate as the sole electron donor and carbon source. It was assumed to have similar nutritional characteristics to the marine propionate oxidising organisms isolated by Laanbroek and Pfennig (1981) - namely, utilization of ethanol, hydrogen, lactate, propionate and pyruvate as growth substrates. The organism seemed to have a morphological similarity to several of the E-70 propionate oxidising sulphate reducers and it was thought that many of these bacteria were *Desulfobulbus spp.* Hence, a comparison was made of the rates

of growth and sulphate reduction of strain 3 pr 10 with those of *D.desulfuricans* isolate 19 (table 2.14). Two common (lactate and pyruvate) and one unique (propionate) substrates were employed at a concentration of 30mM.

As with isolate 19, maximum rates of growth and sulphate reduction were recorded during growth on lactate as the sole electron donor (table 3.5). The values for the maximum specific growth rate of the two organisms were similar and yielded a doubling time for 3 pr 10 of 4 hours. This figure was much smaller than those reported for the acetate oxidiser *Desulfobacter postgatei* - 20 hours in uncontrolled batch culture and 15 hours at constant pH and low H_2S concentration in fermenter cultures (Widdel and Pfennig, 1981). Low levels of slow (linear?) growth occurred with propionate as electron donor, although the highest levels of sulphate were reduced. This resulted in a maximum sulphate reduction rate at the end of growth which was around 75% of that with lactate. This illustrated that high sulphate reducing activity need not necessarily involve the production of a high biomass and was a reflection on the stoichiometry of the reduction of 3 moles of sulphate by 4 moles of propionate (Sørensen *et al*, 1981). Although rapid growth and the highest cell density of this study were recorded during the growth of strain 3 pr 10 on pyruvate, the rate and yield of sulphate reduction were low. This again was consistent with the stoichiometry for the incomplete oxidation of pyruvate (4 moles) to acetate and CO_2 by the reduction of one mole of sulphate (see below).

With each of the three substrates growth tended to cease

with the cessation of sulphate reduction. However, the amounts of sulphate reduced on lactate (9.2 m mol l^{-1}), propionate (15.5 m mol l^{-1}) and pyruvate (3.0 m mol l^{-1}) were much less than the theoretical maxima of 15.0, 22.5 and 7.5 m mol l^{-1} assuming ratios of 2 mol lactate: 1 mol SO_4^{2-}: 4 mol propionate : 3 mol SO_4^{2-} and 4 mol pyruvate: 1 mol. SO_4^{2-}. This suggested that unlike isolate 19, the cessation of growth was not due to electron donor depletion. From studies on *Desulfobacter postgatei* (Widdel and Pfennig, 1981) it would appear that sulphide toxicity or medium pH alteration could result in growth limitation and any further studies on this organism would necessitate the use of a fermenter with Eh and pH control (see Brown *et al*, 1973). As has already been mentioned the maximum rates of sulphate reduction of *Desulfobulbus sp* 3 pr 10 were on the whole less than those of *D.desulfuricans* isolate 19 (tables 2.14 and 3.5). Although this may have been due to cultural considerations, it seems unlikely that massive sulphate reduction by this novel organism was a cause of the high specific rate of metabolism calculated for Loch Eil E-70 sulphate reducing bacteria *in situ* when compared with that of pure cultures *in vitro*.

GENERAL DISCUSSION

This study was concerned with the use of nitrate or sulphate as terminal electron acceptors for the anaerobic oxidation of organic matter within the surface layers of marine sediments. In contrast to many other studies a wide variety of sediments were examined which ranged in character from cellulose polluted, reduced fjordic through to organically poor, oxic abyssal. The technique used to investigate nitrate reduction activity within the sediments could only give an indication of the potential for dissimilatory nitrate reduction due to the elevated NO_3^- levels employed. This was in contrast to the true measure of sulphate respiration *via* the use of the radiotracer $^{35}SO_4^{2-}$. However, some comparisons of the importance of the two processes can be made.

All the sediments examined had the potential for nitrate respiration and appeared to be electron acceptor limited due to the low nitrate concentrations that occur in the marine environment. When nitrate was present in excess ($\geqslant$2mM) activity tended to be rate limited by the availability of organic matter (electron donor levels) and not by the population of nitrate reducing bacteria. In contrast, sulphate reduction was never electron acceptor limited due to the abundance of sulphate in the sediments examined. Hence it was concluded that sulphate respiration was the predominant means of anaerobic mineralization. Sulphate reducing bacteria numbers did not reflect the differences in sulphate reduction rate between different stations but rather indicated the potential for sulphate reducing activity. The respiration rate appeared to be dependent on the organic input into the sediment and

more specifically,on the levels of available carbon (electron donors). Activity in sediment from the N.E. Atlantic continental slope and abyssal sampling stations was also affected by the highly oxic nature of the sediments and by the high pressures and subsequent decompression on sediment recovery.

The effect of organic carbon supply on sulphate reduction rate was demonstrated by the specific rate of metabolism of sulphate reducing bacteria in the sediments tending to increase with increasing organic content. Although the organic carbon content often reflects that material which is unavailable for sulphate reduction, it was thought that such data were a reflection of organic input and carbon availability. Station E-70 in Loch Eil which had a high organic content also had a very high level of available carbon (Duff, 1981) and yielded the highest specific rate of metabolism. Sulphate reducing bacteria in the surface 10 cm of this sediment were shown to be capable of degrading around 50% of the total carbon mineralized per year. This sediment was subjected to high levels of cellulose pollution although no net accumulation of cellulose had been recorded in the sediment. It was thought that input and decomposition were balanced in this sediment (Pearson, 1982) and the data obtained in this study would suggest that sulphate reducing activity and not bacterial numbers had increased with increased organic input. It was unclear as to what was limiting sulphate reduction in this sediment, although recent data suggests that the confinement of cellulose degradation to the aerobic, surface sediments may be a factor (S.Malcolm, pers.comm.).

The importance of sulphate reducing bacteria in the mineralization of organic matter in Loch Eil was paradoxical when compared with their limited range of electron donors in pure culture. All the lactate-oxidising isolates obtained from the surface sediment were *D.desulfuricans* strains and characteristically incompletely oxidised a relatively small number of simple organic compounds to acetate and CO_2. This range was expanded somewhat by mutualism in enrichment cultures and by mixotrophy in pure culture, where amino acids could act as sources of cell carbon. The use of newly described cultural techniques enabled the presence of novel sulphate reducing bacteria to be demonstrated in comparable numbers to the more conventional lactate oxidising organisms in both E-70 sediment and in samples from the continental shelf. This indicated that both sediments possessed a varied sulphate reducing microflora and had the potential for a complete oxidation of organic matter through to CO_2 by dissimilatory sulphate reduction.

Substrate supplementation of E-70 and A-3 sediment, although a somewhat limited technique, demonstrated that in the surface 5 cm of sediment lactate was preferentially utilized as an electron donor for sulphate reduction. This was consistant with thermodynamic considerations and illustrated the preference of sulphate reducing bacteria for lactate in both organically rich and poor marine sediments. Below 5 cm depth in the Loch Eil sediment the utilization of lactate and propionate in the surface layers resulted in acetate being the preferred substrate, with a corresponding predominance of acetate oxidising sulphate reducing bacteria. Contrasting sediment from the British continental shelf however, still

showed a preference for lactate below a depth of 5 cm and this was thought to be a reflection on the lower sulphate reducing activity in the surface sediment. Pure culture work on a representative *D.desulfuricans* strain from Loch Eil demonstrated that lactate yielded a specific rate of sulphate reduction which was higher than other published values or with malate or pyruvate as electron donors. Similarly the maximum rate of sulphate reduction of a type strain of *Desulfobulbus* was recorded during growth on lactate as electron donor.

The specific rate of sulphate reduction of a representative sulphate reducing bacterium from Loch Eil under good cultural conditions was found to be considerably less than that of bacteria *in situ*. It was demonstrated that this was not due to novel groups of organisms being incapable of growth on the enumeration medium as was previously thought. Similarly, it was not due to elevated metabolic rates of pure cultures of novel sulphate reducing bacteria. The difference in the calculated rates was thought to be possibly caused by insufficient homogenization of sediment samples during the enumeration procedure.

Pure cultures of Loch Eil sulphate reducing bacteria were shown to be capable of utilizing amino acids as sources of reducing power and nitrogen. Ammonia was also utilized as a nitrogen source for growth, as was nitrate which was in disagreement with some published reports. The high levels of interstitial NH_4^+ that occur in Loch Eil (and other marine sediments) suggested that this compound was used as the nitrogen source of sulphate reducing bacteria *in situ*. This ammonia

can originate by ammonification or by the dissimilatory reduction of nitrate. The low levels of nitrite in all the sediments examined indicated that NO_2^- was not the end product of nitrate respiration. Net ammonia production by the dissimilatory reduction of nitrate was demonstrated in both Loch Eil and continental shelf sediments. In the other Atlantic sediments and in samples from the Lynn of Lorne ammonia assimilation by sedimentary organisms caused a decrease in NH_4^+ levels on incubation. However, these sediments could reduce NO_3^- to NO_2^- and contained bacteria which were capable of reducing this NO_2^- to NH_4^+.

The counts of nitrate reducing bacteria recovered from the sediments decreased with increasing water depth as was the case with the sulphate reducers. Similarly, no nitrate reducers were recovered from the abyssal Atlantic plain and this was thought to be due to pressure considerations as the sediment had the potential for dissimilatory nitrate reduction. The nitrate reducing flora from all the sediments was predominantly composed of fermentative bacteria of the *Aeromonas/Vibrio* genera. This indicated that the N.E.Atlantic and sea-loch sediments possessed a similar nitrate reducing flora to that found in estuarine and North Sea sediments (see Herbert, 1982). The majority of the isolates were capable of reducing nitrate through to ammonia which if repeated within the sediment would provide a "short circuit" in the biological nitrogen cycle and result in the conservation of ammonia within the sediment.

Ammonia can also arise in marine sediments by the process

of heterotrophic nitrogen fixation which is generally considered to be performed predominantly by sulphate reducing bacteria. All the inshore sediments examined could fix nitrogen, with activity tending to increase with increasing organic content. However, despite nitrogen fixation requiring considerable amounts of energy, there was no correlation between acetylene reduction rate and sulphate reduction rate. It was thought that the high interstitial NH_4^+ concentrations in the organically rich sediments examined inhibited the sulphate reducing bacterial nitrogenase. As ammonia was also a more energetically favourable nitrogen source for sulphate reducing bacteria, it appeared that the increased energy yield for the organisms in organically rich sediments, did not necessarily enable a matched increase in nitrogen fixing activity due to the correspondingly higher ammonia levels from organic breakdown. Unfortunately, molybdate inhibition experiments (*e.g.* Nedwell and Abdul Aziz, 1980) were not performed and obviously such experiments would give a clearer indication of the role played by the sulphate reducers in heterotrophic nitrogen fixation in inshore marine sediments. In conclusion, it would appear that the main source of ammonia in inshore marine sediments is ammonification, although a limited input could originate from dissimilatory nitrate reduction (depending on the availability of nitrate) and heterotrophic nitrogen fixation.

Two interesting observations to come from the study of nitrate and sulphate reduction at station E-70 in Loch Eil were the presence of large numbers of budding yeasts in the

surface sediment and the failure of the *D.desulfuricans* isolates to grow on a widely used medium for sulphate reducing bacteria. The yeasts appeared to be inshore forms as defined by van Uden and Fell (1968) and two isolates were capable of utilizing nitrate as an alternative electron acceptor to oxygen. They did not appear to originate from the pulp mill effluent and their role in organic mineralization was unclear. It was thought that the organisms were growing *in situ* on hydrolysis products of cellulose (*e.g.* glucose) produced by cellulolytic bacteria. The failure of the Loch Eil sulphate reducing bacteria isolates to grow in Postgate's medium C was unexpected. It appeared that the citrate in this medium bound magnesium much more tightly than the natural binding sites within the cell. It was speculated that this was due to a low stability constant for Mg^{2+} of the binding sites of the inorganic pyrophosphatase-which resulted in its inhibition and a prevention of the initial activation step of dissimilatory sulphate reduction. If these isolates were not atypical of naturally occurring organisms then it would indicate that the use of chelated media for enrichment and isolation should be avoided.

This observation serves to illustrate the problems involved in the cultivation of sulphate reducing bacteria, with the medium employed often being a compromise between convenience and the need to recover the predominant nutritional groups present in the sediment. Comprehensive surveys of the various types of sulphate reducing bacteria can be made using the basal medium of Pfennig *et al* (1981) with numerous special

additions. However, the logistic constraints of most field work prevents the use of this sophisticated cultural system. The data presented in this thesis has suggested that the "traditional" electron donor lactate is preferentially utilized by sulphate reducing bacteria in the active upper horizons of both polluted and organically poor marine sediments. As around 80% of all sulphate reducing bacteria currently described are capable of utilizing lactate as a growth substrate (Widdel, 1980) it would appear that this compound is still ironically the closest to a "universal" substrate for sulphate reducing bacteria. Its use in a simple medium without sodium thioglycollate or chelated iron could enable a close approximation to be made of the total sulphate reducing flora of marine sediments under field work conditions.

A P P E N D I X

INTRODUCTION

The data presented in this appendix were obtained by co-workers in the S.M.B.A. bio-geochemical cycling group during the period of this work (November 1978 until August 1981). The group consisted of the following members:

N. Battersby, C. Brown - Department of Biological Sciences, University of Dundee and latterly, Department of Brewing and Biological Sciences, Heriot-Watt University.

F. Drake, R. Parkes - Sediment Microbiology, S.M.B.A.

S. Malcolm - Biogeochemistry, S.M.B.A.

S. Stanley - Organic degradation, S.M.B.A.

MATERIALS AND METHODS

EXTRACTION OF PORE WATERS

Sediment pore water was extracted using a "Teflon" diaphragm-type squeezer as described by Robbins and Gustinis (1976) under a nitrogen pressure of 138 kPa. Squeezing was carried out as soon as possible on landfall and was performed within the nitrogen filled glove bag. Pore water was extruded into pre-cleaned polyethylene vials for later analysis.

AMMONIA DETERMINATION

Ammonia was measured on a 1 or 5 ml sample by the colorimetric phenol-hypochlorite method of Solórazo (1969).

NITRATE DETERMINATION

Nitrate was determined by reduction to nitrite on passage through a copper/cadmium column with the resulting nitrite being assayed as described by Montgomery and Dymock (1961).

SULPHATE DETERMINATION

Sulphate was measured on a 4 or 5 ml sample of porewater from which the sulphide had been precipitated as zinc or cadmium sulphide. Analysis was by $BaSO_4$ precipitation gravimetrically (American Public Health Association, 1971) or by complexometric titration (Howarth, 1978).

TABLE 4.1 INORGANIC NITROGEN ANALYSES FOR LOCH EIL E-24 AND E-70 SEDIMENTS

	E-24		E-70	
Depth (cm)	NH_4^+ (μM)	NO_3^- (μM)	NH_4^+ (μM)	NO_3^- (μM)
OSW	0	0.8	878	3.4
0-2	6.8	21.8	271	15.8
2-4	1.1	14.0	889	9.5
4-6	28.9	2.8	871	0.7
6-8	42.5	6.0	1004	0.3
8-10	44.6	2.3	900	0.4
13-15	157.1	1.1	1007	0.3
18-20	276.4	7.9	900	0.3
23-25	331.8	25.7	1192	0.6
28-30	384.3	2.2	1057	2.3
33-35	451.8	18.2	1421	1.3
38-40	490.0	0.9	1232	0.3
43-45	602.5	28.5	1600	0.3
48-50	699.6	9.5	1496	0.2

OSW, overlying sea water

TABLE 4.2 SULPHATE ANALYSES FOR THE N.E. ATLANTIC SEDIMENTS A-1, A-2 AND A-3

Depth (cm)	A-1 SO_4^{2-} (mM)	A-2 SO_4^{2-} (mM)	A-3 SO_4^{2-} (mM)
OSW	27.2	26.3	26.7
0-2	27.1	26.4	25.4
2-4	27.1	26.3	25.4
4-6	27.2	26.3	24.7
6-8	27.0	26.2	24.6
8-10	27.3	26.3	23.9
10-12	27.1	26.2	23.2

TABLE 4.3 SULPHATE ANALYSES FOR LYNN OF LORNE LY-1 SEDIMENT

Depth (cm)	SO_4^{2-} (mM)
0-5	24.6
5-10	23.2
10-15	21.4

TABLE 4.4 SULPHATE ANALYSES FOR LOCH EIL E-24 AND E-70 SEDIMENTS

Depth (cm)	E-24 SO_4^{2-} (mM)	E-70 (9/79) SO_4^{2-} (mM)	E-70 (7/81) SO_4^{2-} (mM)
OSW	28.1	28.1	
0-2	25.8	21.6	(0-5 cm)
2-4	23.8	20.5	14.6
4-6	22.7	17.8	
6-8	22.0	17.8	(5-10 cm)
8-10	23.8	21.4	14.8
13-15	24.1	19.2	
18-20	22.8	19.3	
23-25	23.0	19.8	
28-30	22.1	18.6	
33-35	21.8	14.4	
38-40	20.6	18.6	
43-45	22.1	14.4	
48-50	20.3	11.7	
53-55	19.3	10.7	
58-60	-	10.5	

- not determined

TABLE 4.5 SULPHATE ANALYSES FOR LOCH CRERAN CR-1 AND CR-2 SEDIMENTS

Depth (cm)	CR-1 SO_4^{2-} (mM)	CR-2 SO_4^{2-} (mM)
OSW	29.0	28.0
0-1	23.5	28.0
1-2	25.0	30.5
2-3	24.5	28.7
3-4	24.5	27.0
4-5	27.0	29.7
5-6	24.0	28.0
6-7	25.0	26.0
7-8	21.0	27.0
8-9	25.0	30.0
9-10	24.0	28.7
13-15	22.0	27.7
18-20	22.5	28.0
23-25	22.0	27.5
28-30	24.0	26.0
33-35	25.0	27.5
38-40	24.0	-
43-45	24.0	-

TABLE 4.6 SULPHATE ANALYSES FOR SULLOM VOE D-4 AND COLLA FIRTH CF-1 SEDIMENTS

Depth (cm)	D-4 SO_4^{2-} (mM)	CF-1 SO_4^{2-} (mM)
0-5	29.5	29.7
5-10	16.5	24.0
10-15	14.6	-
15-20	7.5	-
20-25	6.8	-
25-30	3.8	-
30-35	4.8	-
35-40	3.5	-
40-45	2.5	-
45-50	2.3	-

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