THE ROLE OF SULPHATE-REDUCING BACTERIA IN MERCURY-CONTAMINATED ESTUARINE SEDIMENTS: A CASE STUDY OF DURBAN BAY.

by

ELIZABETH ANNE SIMPSON

Dissertation submitted in compliance with
the requirements for the

MASTERS DEGREE IN TECHNOLOGY

in the

DEPARTMENT OF BIOTECHNOLOGY AND
THE DURBAN INSTITUTE OF TECHNOLOGY

SUPERVISOR: PROF G N TIVCHEV

DURBAN: APRIL 2003

DECLARATION

I declare that

THE ROLE OF SULPHATE-REDUCING BACTERIA IN MERCURY-CONTAMINATED ESTUARINE SEDIMENTS: A CASE STUDY OF DURBAN BAY is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE	**DATE**
	17 April 2003
(MRS E.A. SIMPSON)	

APPROVED FOR FINAL SUBMISSION	**DATE**
	17 April 2003

PROFESSOR G N TIVCHEV
PhD Bulgarian Academy of Science
MSc University of Sofia
(SUPERVISOR)

DEDICATION

Dedicated to the memory of my father who instilled in me the value of perseverance.
"If you start something, you finish it …"

ACKNOWLEDGEMENTS

I would like to extend my sincere thanks and appreciation to:

Mr Gary Parsons and Mr Basil Ngcobo who helped me collect samples under what were often adverse conditions.

Mr Ruben Mudaly for his assistance in much of the chemical analysis, and particularly for his determination and persistence in developing a method we could use for the measurement of methyl mercury concentrations.

Ms Khanyi Dlomo for her assistance in the processing of an endless stream of microbiology samples.

Mr Arjoon Singh for his graphics expertise.

The CSIR for allowing me the time and providing me with the equipment to undertake this project.

ABSTRACT

Stimulated by the findings of international researchers, that the sulfate-reducing microorganism *Desulfovibrio desulfuricans* could be incriminated in the process of mercury bio-methylation, it was decided to test this hypothesis on sediments from selected areas of Durban Bay where elevated levels of the bio-hazardous heavy metal had previously been detected. The Environmentek Division of the Council for Scientific and Industrial Research (Durban) is involved in an ongoing chemical assessment of heavy metal contamination (including levels of mercury) in the sediments of this estuary, but nothing is currently understood about the form in which mercury exists or the biological processes that could be determining its fate. The purpose of this project was to attempt to answer some of these questions.

The study involved attempting to isolate, identify and quantify microorganisms of the species *Desulfovibrio desulfuricans, Escherichia coli* and *Clostridium perfringens* in one hundred and eighty sediment samples taken from three designated sites in the bay. Each sample was additionally analysed for total and methyl mercury and sulfate content, as well as a number of physical parameters. Based on the outcome of the initial survey, it was envisaged that further laboratory experimentation would be conducted to determine whether or not isolates were responsible for the production of the highly toxic organic mercury and whether this process was occurring *in situ* in the sediments.

The findings of this project were contrary to what had been expected. Total mercury concentrations (apart from one instance) did not appear to be appreciably elevated in the areas under study. Similarly, the levels of methyl mercury were found to be either diminished or absent. Numbers of *D. desulfuricans* were low and not uniformly distributed throughout the sediments. *Cl. perfringens* was more in evidence, but counts were not perceptibly increased. Sulfate levels were consistently high, indicating significantly impaired rates of sulfate reduction. Difficulty experienced in sub-culturing isolates of *D. desulfuricans* meant that the laboratory experimentation part of the exercise could not be concluded.

The conclusion drawn from this study is that bio-methylation of mercury is not occurring to any great extent in the sediments taken from the three sites in Durban Bay. This finding is supported both by the chemical and microbiological results of the one hundred and eighty samples collected. International researchers have found that anaerobic aquatic sediments exposed to low-level inputs of inorganic mercury convert it to mono-methyl mercury. This process they have attributed predominantly to the sulfate-reducing microorganism, *Desulfovibrio desulfuricans*. From the results obtained in this study, however, it would appear that this process is not occurring in the sediments in Durban Bay.

TABLE OF CONTENTS

TABLE OF CONTENTS (contd.)

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1.

INTRODUCTION.

Industrial and municipal wastewater discharges are significant sources of river pollution, with a portion of the discharged dissolved constituent being adsorbed to the suspended particulate matter (Kern, Li & Westrich, 1998:1). When (as occurs in estuaries) there is a mergence of fresh and salt water, chemical and physical processes can occur that result in any suspended particulate matter (SPM) flocculating and precipitating out, consequently enriching the pollutant content in the aquatic sediments. Ultimately this could result in aquatic sediments becoming a reservoir for toxic trace metals. For a metal to be considered toxic it must be in a bio-available state, i.e., capable of being taken up into the organism. Its bio-availability and potential toxicity are dictated by the physio-chemical form in which the metal occurs (Wade, 1998). Hence the potential ecological and public health hazard posed by toxic metals is linked to the metal species.

Certain groups of microorganisms are capable of creating physical conditions that result in the formation of insoluble metal species, a state that reduces the bio-availability of the toxicant. Conversely, other microbiological species are capable of generating (many by way of their metabolic processes) solubilized metals, which result in an increase in their bio-availability (White, Sayer & Gadd, 1997:504). Thus microorganisms come to play an important role in the eventual environmental fate of toxic metals.

Mercury (Hg), the third element in Group IIB of the periodic table, has an average natural abundance in the earth's crust of 0.09ppm (*Standard Methods*, 1998:3-85). Apart from naturally occurring mercury, contamination of the environment is often exacerbated by anthropogenic pollution from industrial effluents, solid waste disposal and refining processes. The toxicity and bio-accumulative properties of mercury are well known, with inorganic, elemental and organic mercury having varying toxicological effects (Davis, Bloom & Que Hee, 1997:557). Like other heavy metals, the bio-availability of mercury is dependent upon its physio-chemical form. If it presents as the highly insoluble mercuric sulphide it is relatively non-bioavailable. However, in the organic form (as methyl mercury compounds) it becomes extremely toxic and bio-magnification-prone (Compeau and Bartha, 1985:498), affecting the brain and nervous system, and causing mental impairment (Davis, Bloom & Que Hee, 1997:557).

Of the numerous chemical and physical forms of the metal present in the environment, the species most relevant to this investigation are inorganic elemental mercury (Hg), its divalent form (Hg^{2+}) and organic methyl mercury (CH_3Hg). In this study all forms are measured together as the total mercury content of a sediment, with the organic form being measured as a separate parameter.

In anaerobic, undisturbed sediments containing significant amounts of sulphate, sulphate-reducing bacteria tend to produce sulphide, which results in the formation of highly insoluble mercuric sulphide (Archibald and Fowles, 1993). However, interconversion between inorganic and organic forms of mercury can be catalyzed by microbial activity in the sediments, with its subsequent release into the water column (Gadd, 1993:307). Although a number of facultative anaerobes belonging to the genera *Escherichia, Enterobacter, Staphylococcus*, *Streptococcus* and *Bacillus* have been incriminated in the methylation of mercury in sediments (Trevors, 1986:500), Compeau and Bartha (1985:498) unequivocally identified sulphate-reducing bacteria as the principal methylators of Hg^{2+} in anoxic sediments. It has subsequently been established that 95% of mercury bio-methylation is carried out by sulphate-reducing microorganisms, and that methyl mercury is synthesized predominantly by *Desulfovibrio desulfuricans* (Choi, Chase & Bartha, 1994:4072).

Desulfovibrio desulfuricans is a type species of the Genus *Desulfovibrio,* a group of Gram negative, spiral to vibroid-shaped cells. Capable of growing on simple defined media, members of this group are strict anaerobes that conduct dissimilatory sulphate reduction. During the process of dissimilatory sulphate reduction the sulphate ion acts as an oxidizing agent for the dissimilation of organic matter. Although a small amount of the reduced sulphur is assimilated into the organism, almost all of it is released into the environment as the sulphide ion in a form usually hydrolysed to free H_2S (Postgate, 1984:2). The importance of this organism to the project is two-fold. Firstly, its ability to produce sulphide results in the generation of an anion for the tentative production of insoluble mercuric sulphide in mercury-containing sediments. In addition (and in the presence of organic matter), it has also been incriminated in the enzyme-generated process that sees the formation of the highly bio-hazardous methyl mercury (Choi, Chase & Bartha, 1994, 4072-4077).

D. desulfuricans shares similar metabolic pathways with organisms of the Clostridium group, whose members are frequently found in estuarine sediments with high sulphate concentrations. *Clostridium perfringens* is a type species of the Genus *Clostridium,* a group of Gram positive, spore-bearing, rod-shaped, obligate anaerobes that are ubiquitous in the environment. Because *Cl. perfringens* is also a commensal inhabitant of the animal or human intestine, it continues to be regarded as a valuable choice where survival properties of the water quality indicator are paramount (Bisson and Cabelli, 1979:55). Although it does not carry out dissimilatory sulphate reduction (*Bergey's Manual of Systematic Bacteriology, Vol 2*, 1984:1141), its importance to this project lies in the fact that it is a strict anaerobe that reduces sulphites to sulphides. The significance of its obligatory anaerobic nature means that, if it is present in the sediments, prevailing conditions are sufficiently anaerobic to support the survival and propagation of other anaerobes such as Desulfovibrio sp. The fact that it is also capable of reducing sulphites to sulphides means that it, like *D. desulfuricans*, is capable of producing sulphide anions that could result in the production of insoluble metal sulphides.

These reactions are not necessarily limited to deep-water sediments either. If substantial amounts of organic matter are present, chemical and biochemical oxygen demand exhausts the dissolved oxygen just millimeters below the sediment-water interface (Compeau and Bartha, 1987:263). This creates not only ideal anaerobic conditions for the growth of SRB's, but also conditions conducive to mercury methylation (Archibald and Fowles, 1993).

Microbial methylation is not a one-way process and is balanced in nature by demethylation activities that can also be attributed to bacteria. Although the mechanism of microbial demethylation of methyl mercury has been well characterized and it is acknowledged that the types of bacteria that demethylate methyl mercury in sediments can be quite diverse (Pak and Bartha, 1998:1013), Ormeland, Cuthbertson and Winfrey (1991:134) identified a strain of *Escherichia coli* that demethylated methyl mercury compounds under fermentative anaerobic or aerobic growth conditions.

Escherichia coli is a type species of the Genus *Escherichia*, a group of Gram negative, facultatively anaerobic, straight rods. Like *Cl. perfringens* its presence in the environment can be indicative of pollution by animal or human faecal matter. However, its significance to this project is attributed to the fact that, as stated above, Ormeland, Cuthbertson and Winfrey (1991:134) identified a strain (DU 1040) capable of de-methylating methyl mercury compounds under fermentative anaerobic or aerobic conditions. Its presence in significant numbers might therefore be responsible, at least in part, for de-methylating processes that may be occurring in the sediments.

The chosen area for this study is Durban Bay. The Environmentek Division of the Council for Scientific and Industrial Research (Durban) is involved in an ongoing assessment focusing on the chemical contamination of sediments in this estuary, and the results have generally indicated elevated levels of heavy metals, including mercury (Archibald, 1997). Although the role of sulphate-reducing bacteria in the form and fate of mercury in estuarine sediments is a fairly well documented phenomenon and numerous studies have been conducted at various sites worldwide (Compeau and Bartha, 1985 and 1987; Ormeland, Cuthbertson & Winfrey, 1991), no such investigation has been undertaken in Durban Bay. In operations relating to the dredging and dumping of harbour sediments, the Durban Port authorities are governed by the London Dumping Convention and the sole indicator used at the present time to assess the level of contamination is the total metal concentration measured in the detritus. Nothing is currently understood about the form in which mercury exists or what biological processes could be determining its fate. It is hoped that this study will elucidate more clearly the prevailing situation.

CHAPTER 2.

LITERATURE REVIEW.

Begg (1978:246-247) describes Durban Bay as a 'land-locked' estuarine system surrounded by the City of Durban. With an area of some 1060ha this makes it the largest estuarine system within the Durban Metropolitan area. The head of the Bay is fed by the Amanzimnyama, Mbilo and Mhlatuzana Rivers, all of which are canalised in their lower reaches. These three rivers drain relatively small but heavily impacted catchments (Archibald, 2000:14). Their sediment loads are being deposited in the silt canal, the Congella turning basin and the Maydon channel due to a combination of river deposition and the dynamics of the harbour (Archibald and David, 2000:7). Begg (1978:247) has indicated that, in its pre-developed state, very little of the bay was 'deeper than 2m at high water on spring tide'. This, however, is not the case today. The physical qualities that have made Durban Bay a sheltered natural harbour have also led to the necessity for annual dredging operations to clear shipping channels of detritus and facilitate the free movement of large, modern, ocean-going bulk carriers.

As a contracting party to the London Convention of 1972 (the main provisions of which were incorporated into national legislation promulgated in 1982 through the Dumping at Sea Control Act 73), South Africa is governed by the guidelines and imperatives of the Convention in the disposal of its dredged spoil to the sea. Archibald (2000:53) has noted that this 'requires steps to be taken to avoid and prevent pollution of the sea from dumping of waste and other matter (e.g., dredge spoils) that is liable to create hazards to human health or harm living resources, or to damage amenities (e.g., ecological service) or to interfere with other legitimate uses (users) of the sea'.

In order to manage the risks linked to disposal to sea of dredged material, several ad hoc studies on toxic metals in the harbour sediments have been undertaken in recent years (Oliff et al, 1979; McClurg, Stanton & Combrink, 1990; Callow, 1994; Sibbald et al, 1994; Archibald, 1996; Wright, 1996). In 1997 the Port of Durban commissioned the CSIR (KwaZulu-Natal), as an independent accredited monitoring agency, to undertake a more formal annual sediment assessment programme to ascertain the pollution load impacting on certain areas of the harbour. The necessity for a monitoring programme of this nature has arisen because, as Archibald and David (2000:9) have pointed out, evidence has been presented elsewhere in the world to the effect that the disruption of sediments during dredging and transport of material to the marine disposal site carries with it the potential for heavy metal contamination.

The London Convention classifies mercury as an Annex I contaminant, and the *Guidelines for the Management of Dredge Spoil in South African Coastal Waters* (1996) indicate that the proposed limits for this toxic metal be set at an action level of 0.5-5.0μg/g dry weight sediment and a prohibition level of >5.0μg/g (Archibald & David, 2000:11). Between 1997 and 2000, Archibald and David reported that levels of mercury in sediments have variously exceeded the action limit in a number of sites throughout the harbour, but more notably in the upper reaches where ship repair activities are common practice and where the major river flows have the greatest impact (Archibald and David, 2000:24).

The method historically used to assess the mercury content of the sediments relies on a very aggressive process of total digestion of the sediment, followed by analysis of the resulting solution. Archibald and David (2000:25) have noted that this method of analysis reports on the total concentration of the metal, and that total concentration incorporates both bio-available and non-bioavailable fractions. Wade (1998) clearly distinguishes between bio-available and non-bioavailable forms of a metal. Bio-available forms he defines as those capable of being taken up by organisms and therefore, by this definition, being a form linked to potential toxicity. Despite the fact that, as Wade has pointed out, bio-available metals may become toxic only in concentrations above a certain threshold, bio-concentration in organisms does occur and bio-magnification manifests as a process inextricably linked to the ecosystem food chains.

In summary, therefore, the prevailing situation in Durban Bay is that South Africa, as a signatory to the London Convention, is bound by the guidelines and imperatives of an international protocol. At present, the procedure to determine and manage the potential environmental impact of the contaminated sediment relies on criteria involving analysis of total concentrations for various listed contaminants, including mercury. However, to be considered toxic the metal must be in a bio-available form, capable of entering the aquatic food chain. Current analytical procedures do not differentiate between the varying physio-chemical forms of the metal (although this can be achieved by predictive modelling), and hence do not distinguish between bio-available and non-bioavailable fractions. In fact, very little is presently understood about the biochemical processes occurring in the sediments of Durban Bay, and what influence they may be having on the determination of metal species. In addition, the subject of study relating to microbiological processes and their intervention in the form and fate of mercury in the sediments in Durban Harbour appears to be a topic as yet formally untackled by South African scientists.

Although the location of Durban Bay has generally been neglected as far as studies relating to the microbiological component of its sediments are concerned, much work has been done internationally during the past two decades on the bio-availability of methyl mercury in sediments and the processes via which this bio-hazardous state of the metal is produced. Choi, Chase and Bartha (1994:4072) note that: 'Anaerobic aquatic sediments exposed to low-level inputs of inorganic mercury convert it to mono-methyl mercury, a highly hazardous environmental pollutant prone to bio-magnification'. Ninety-five percent of mercury bio-methylation has been attributed to sulphate-reducing microorganisms, and in particular *Desulfovibrio desulfuricans* (Compeau and Bartha, 1985:498). Compeau and Bartha further note that, although some abiotic methylation by humic compounds does occur, this would account for only approximately one tenth of the methyl mercury formed. The rest should be ascribed to methylation by sediment microorganisms.

In 1997 Pak and Bartha used a strain of *Desulfovibrio desulfuricans* (ND-132), selected for its high Hg^{2+} methylating activity, to conduct laboratory experiments aimed at linking the methylation of inorganic mercury in anoxic aquatic sediments to the activities of certain sulfidogenic bacteria (Pak and Bartha, 1998:1013). Their selection of a strain specifically chosen for its high Hg^{2+} methylating activity suggests that not all isolates of *Desulfovibrio desulfuricans* necessarily have a high methylation potential. Compeau and Bartha (1985:501) also note that the 'methylating potential [of *D. desulfuricans*] is fully expressed only when sulphate is limiting and carbon sources are available that can also be utilized in the absence of sulphate'. They conclude that the 'mercury methylation can continue in the presence of high sulphate concentrations but not to the extent as when sulphate is at a lower concentration'. This they attribute to the fact that the hydrogen sulphide generated by sulphate respiration interferes with the methylation of Hg^{2+} by precipitating the latter as mercuric sulphide (Compeau and Bartha, 1985:501). The findings of these researchers go a long way to explaining some of the apparent anomalies that have occurred in the results of the samples taken in Durban Bay during the course of this investigation.

The process of bio-methylation has been variously documented (Trevors, 1986:500; Gadd, 1993:299; Choi, Chase & Bartha, 1994:4072-4077; White, Sayer & Gadd, 1997:504-512). Methyl mercury is formed principally through the microbial transfer of the methyl group (CH_3) from methylcobalamin to inorganic mercury. Trevors (1986:500) describes two different mechanisms responsible for this process. The first is 'an electrophilic attack by the metal on the Co-C bond of methylcobalamin' while the second 'involves a free radical transfer; a methyl-radical transfer of an ion pair between the metal and the portion of the methylcobalamin molecule that complexes to the central cobalt atom'. The methylation reaction occurs, as indicated below, in two phases:

$$Hg^{2+} + CH_3CoB_{12} \rightarrow CH_3Hg^{+} + CH_3CoB_{12} \rightarrow (CH_3)_2\,Hg$$

Mono-methyl mercury (CH_3Hg^{+}) is formed from Hg^{2+} and methylcobalamin, and then a second methyl group is transferred to the CH_3Hg^{+} to produce di-methyl mercury [$(CH_3)_2$ Hg] (Gadd, 1993:299).

Gadd (1993:299) has reported that the process of mercury methylation can be catalysed by many bacteria, with sulphate-reducing bacteria appearing to be the most important methylators of mercury and tin. Choi, Chase and Bartha (1994:4072-4077) identify enzymes in the acetyl-CoA pathway of *Desulfovibrio desulfuricans* metabolism that are responsible for the transference of the methyl group from CH_3-tetrahydrofolate via methylcobalamin as described above. They conclude that, although these pathways are not unique to *D. desulfuricans*, and that many sulphate-reducing bacteria utilize the acetyl-CoA pathway in their metabolism, the ability of *D. desulfuricans* to methylate mercury is most likely associated with the substrate-specificity of its enzymes.

Environmental factors that control the bioavailability of Hg^{2+} to methylating bacteria regulate the rate of methyl mercury synthesis. These factors include low pH, low alkalinity, low selenium concentration, and the presence of sulphate, dissolved organic carbon and decomposable organic matter (Barkay, Gillman & Turner, 1997:4267). The findings of these researchers support the hypothesis that the inhibitory effect of dissolved organic carbon (DOC) controls the availability of the Hg^{2+} substrate to bacteria. This inhibitory effect results from the competition between Hg^{2+} and H^+ for the negatively charged binding sites in DOC (Barkay, Gillman & Turner, 1997:4270). They also suggest that the availability of the Hg^{2+} for microbial transformations is reduced in estuarine and marine environments compared with that in freshwater systems. This latter conclusion supports the findings of Compeau and Bartha, who initially suggested that increased salinity resulted in a decrease in methyl mercury production (Compeau and Bartha, 1984:1206). In a later study, in which they revised their previous hypothesis, Compeau and Bartha (1987:263) concluded that slight increases in salinity (0.4% as opposed to 0.03%) resulted in a slightly higher rate of mercury methylation, but if the salinity was as high as 2.5% and the redox potential was low, then methylation activity was reduced by 38% - a confirmation of their initial findings. Based on their own results and the studies of Compeau and Bartha, Barkay, Gillman and Turner (1997:4272) concluded that reduced bio-availability of Hg^{2+} to methylating bacteria may be one reason why mono-methyl mercury accumulation is more common in freshwater than in estuarine and marine biota.

The redox potential (Eh) has also been cited as an important factor for consideration in the methylation of mercury. It has been reported that a negative redox potential appears to favour mercury methylation, and that the methylation of the Hg^{2+} molecule and the persistence of the methyl mercury formed are both favoured at –220mV as compared to +110mV (Compeau and Bartha, 1984:1206). This phenomenon of the redox effect, as Compeau and Bartha (1984:1206) suggest, is probably linked to the types of microbial metabolism they permit. *Desulfovibrio desulfuricans*, for example, requires for growth an environment where the redox potential has been reduced to below –150mV, although the organism is tolerant of short-term exposure to air making elaborate precautions to handle it anaerobically unnecessary (*Bergey's Manual of Systematic Bacteriology, Vol.1*, 1984:667).

If therefore, *as Bergey's Manual of Systematic Bacteriology, Vol.1* (1984:666) intimates, *Desulfovibrio desulfuricans* is to be found in 'anaerobic mud of fresh and brackish water and marine environments', one could deduce the possibility of it being present in the bottom muds of Durban Bay. Since it is apparent from the current literature survey that little has been done to address the extent of the microbial populations in the sediments in Durban Bay, the objective of this study is firstly to attempt to isolate sulphate-reducing species of bacteria from the aforesaid muds. Should *Desulfovibrio desulfuricans* be successfully isolated and identified, further attempts will be made (based on previous findings internationally) to link the organism to chemical processes that it might be stimulating in the mercury-containing sediments in Durban Bay.

CHAPTER 3

MATERIALS AND METHODS

1. STUDY DESIGN.

1.1 Study Area.

Three specific areas of Durban Bay, chosen for the very different types and degrees of catchment-impactment and physical properties they exhibit, were selected as the areas for study in this investigation. The three sites, A, B and C, are indicated on the aerial photograph (Fig. 3.1).

Site A is the silt canal in the upper reaches of the bay, which is fed by the canalised Amanzimnyama River. The Amanzimnyama River passes through industrial sites before opening into the silt canal, an area that is not regularly flushed by tidal movement. This has resulted in ongoing deposition of its silt load in the channel where, over the years, the sediments have become more and more polluted as the result of extraneous contaminants that settle out here. Various studies by the CSIR have indicated increased levels of heavy metals in the sediments, including elevated total mercury concentrations.

The deposition of detritus carried into this channel by the Amanzimnyama River, and the fact that it is a section of the bay not routinely dredged by the Port authorities, means that the depth of the water (even at its deepest point) is rarely more than a few meters. Although complete flushing of the area through regular tidal movement is limited, tidal fluctuations do result in exposure of its marginal regions (marked A1-A20 and A41-A60, Fig. 3.1) to atmospheric oxygen during cyclic periods of receding water levels. This sector of the bay is,

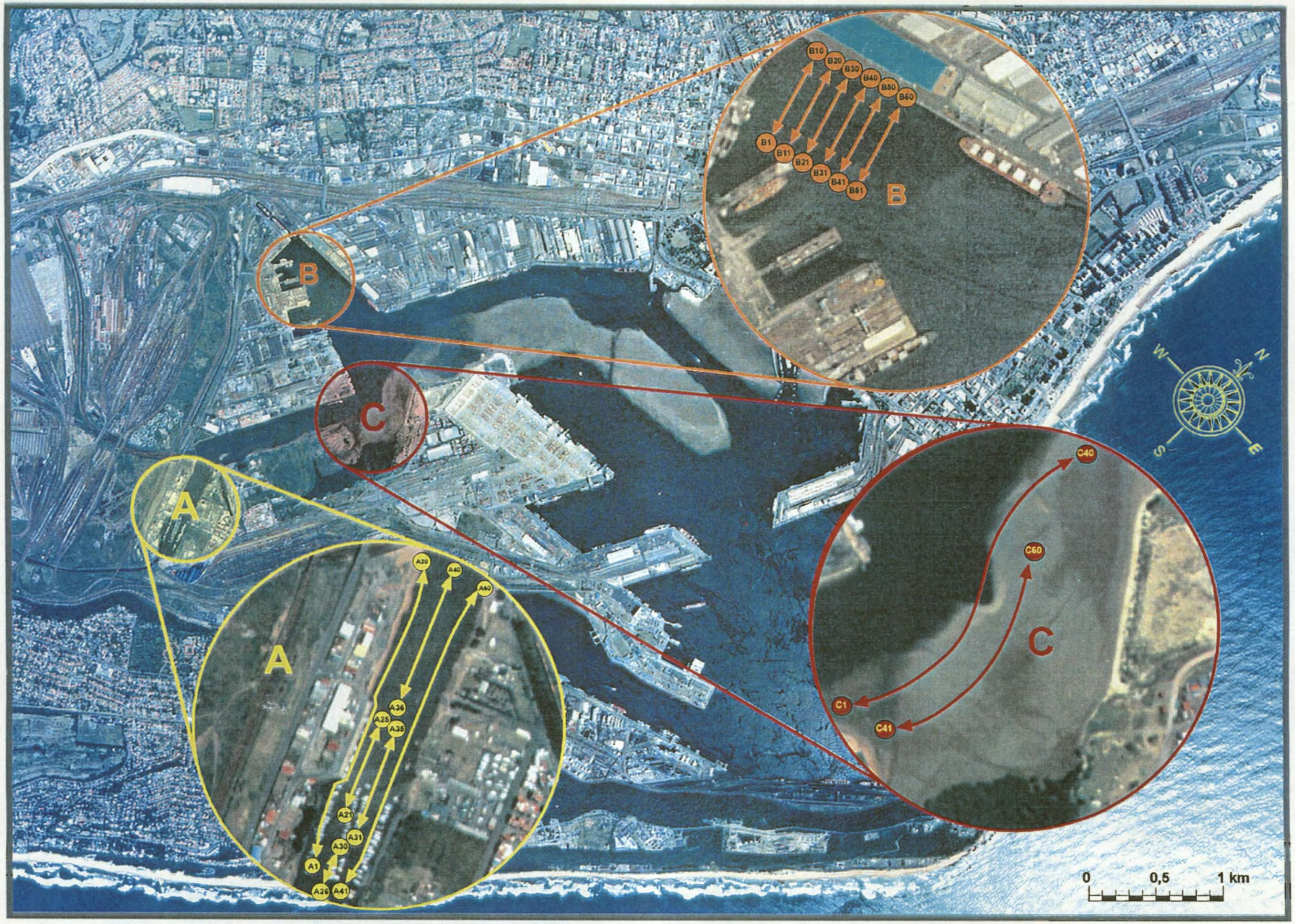


Fig. 3.1 Durban Bay : Study Sites A, B, and C.

therefore, one of relatively shallow water where certain sections of its sediments are regularly exposed to the air.

Slack tidal flushing means that the water here is relatively stagnant, unless impacted upon by sudden flood events originating in the Amanzimnyama River catchment. This, together with the chemical and biological oxygen demand created by contaminants, makes this area of the bay relatively anoxic and, therefore, creates conditions conducive to the proliferation of obligate anaerobes. For reasons indicated above, anoxic conditions are also more likely to occur in the central channel than along the marginal regions.

Finally, this area is one in which the production of hydrogen sulphide appears to be particularly prevalent. Whether this can be attributed to microbiological activity or chemical reaction is not fully understood but, at low tide, bubbles of hydrogen sulphide can be observed in abundance, rising from sediments just centimetres below the water surface.

Site B (Fig. 3.1) is the Congella turning basin situated in front of the dry dock at the head of the arterial channel. This is a deep-water area of the harbour that is regularly dredged by the Port authorities. Although not impacted upon by the inflow of industrial effluent, constant flushing out of the dry dock and the waste generated by the other ship-repair activities originating at the floating docks that surround the channel have resulted in elevated levels of heavy metals, including mercury, in the sediments.

Again, this area is not one that is adequately flushed during tidal movement. Consequently the sediments, that on average exist some fifteen meters below the water surface, have a tendency to be anaerobic in nature and are therefore likely to support the growth of obligate anaerobic microorganisms. Unlike Site A, however, no part of this channel is ever exposed to atmospheric oxygen, and its physical conditions therefore remain relatively constant.

Site C (Fig. 3.1) is located towards the head of the Maydon channel, in an area adjacent to the mangrove swamps. Positioned as it is at a bend in the water course, it accumulates deposits flushed down the canalised Mbilo and Mhlathuzana Rivers that spill into its upstream feeder-channel.

This area is regularly flushed by tidal movement and has large areas exposed to atmospheric oxygen at low tide. Even at high water, the sediments are only partially covered, thus creating conditions that would favour the growth of aerobic and microaerophilic microorganisms rather than obligate anaerobes. In addition, the particle size of the sediments here is larger than those at the other two sites. Since particle size can often be linked to concentration in heavy metals, it would be reasonable to suppose that, purely on physical conditions alone, this would be a site low in mercury (a fact supported by evidence from previous CSIR findings). Site C would therefore be used as a control site.

In summary, therefore, Site A is an unflushed, relatively shallow area where conditions are probably anaerobic (at least down the central channel), and where the total mercury concentration is variously elevated. Site B is an unflushed, deep-water area where conditions are undoubtedly continuously anaerobic, and where the total mercury concentrations are again variously increased. Site C is the control site, which is regularly flushed by tidal movement. It is probably also aerobic in nature due to exposure to atmospheric oxygen for considerable periods in a twenty-four hour cycle. Elevated total mercury concentrations at Site C are relatively non-existent.

1.2 Sampling.

1.2.1 Method of sampling

Sampling of the sediments taken at depth was effected through the use of a grab sampler. When the sampler was returned to the surface the sediment was scooped, as rapidly as possible to prevent aeration of the sample, into previously acid-washed and sterilized 250ml Schott bottles. The bottles were filled to the brim and the cap tightly applied to exclude as much oxygen as possible. Samples were returned to the laboratory to be processed within four hours of being taken.

Samples taken from Site C (Fig. 3.1) were collected at low tide using a scoop. The sediment was then scraped into a previously acid-washed and sterilized Schott bottle and processed as indicated above.

1.2.2 Sample Size

To satisfy the criteria for data capture using the MANOVA method (as detailed below) one hundred and eighty samples were collected from the three Sites A, B and C (sixty samples from each site) on a grid system as indicated in Fig. 3.1. Each sample consisted of 250ml of sediment in a Schott bottle for bacterial enumeration, total mercury and methyl mercury analysis, and 200ml of sediment in a plastic bucket with a fixed lid to analyse for pH, sulphate, particle size and organic matter content.

1.3 Statistical Analysis Method.

The three selected sites in Durban Bay each represent a different grouping of statistical variables. The statistical method used was the univariate multifactorial Analysis of Variance (MANOVA) method. In order to satisfy the criteria for data capture in this method, a balanced multifactorial experimental design was mapped out, as indicated above, which included sixty samples being taken from each site by the stratified random sampling technique. The number of samples was arrived at based on the assumption that there was one dependent variable (the bacterial count). It was anticipated that this dependent variable would interact with three types of bacteria (*D. desulfuricans*, *E. coli* and *Cl. perfringens*), two types of mercury (organic and inorganic) per type of bacterium, two concentration levels per type of mercury (zero concentration and 0.5µg/g dry weight of sediment) and fifteen observations per concentration level. The statistical package Statgraphics *Plus* was used for data entry and analysis.

2. METHODOLOGY.

2.1 Microbiology.

2.1.1 Desulfovibrio desulfuricans

All methods for the enumeration of *Desulfovibrio desulfuricans,* accessed in the literature, referred to that initially developed by Postgate (1984:30-51). Based on the findings of Iverson (1966:529), that Desulfovibrio will grow well on the agar surface of organic media supplemented with trace elements, it was decided to use a commercially prepared medium produced by Scharlau (Sulfate Reducing Agar Ref. 1-255), which employs the same formula as Postgate's Medium B but with the addition of 1.5% agar. Sediment samples were prepared as suggested by Postgate (1984:35) and the dilutions filtered through 47mm diameter 0.45µm sterile membrane filters. Various methods for positioning the membrane on the media surface were tried. These included placing the membrane face-up with and without an agar overlay, and placing the membrane face down on the agar. The most successful proved to be placing the membrane face down on the agar surface, and this method was then employed with all samples. Plates were incubated at room temperature for 21 days in an atmosphere of 9.5%

CO_2, 9.5% H_2 and 81% N_2 in a sealed I^2R inflatable glove chamber (Model IX-17-17). Sulphate reducing bacteria produced dark colonies surrounded by halos (resulting from the production of iron sulphurs), which fluoresced under UV due to the production of desulfoviridin. The results have been expressed as the number of presumptive *Desulfovibrio desulfuricans* colony-forming units per gram dry weight of sediment. Details of the method used for the enumeration of *Desulfovibrio desulfuricans* appear in Appendix A.

2.1.2 Clostridiun perfringens

The method used for the enumeration of *Clostridium perfringens* was that initially developed and documented by Bisson and Cabelli (1979:55-66) and later modified by Armon and Payment (1988:78-79). This mCP method is a membrane filtration method based on the use of a tryptose yeast extract medium containing sucrose, to which is added D-cyclocerine and polymixin B sulphate. These ingredients, together with an incubation temperature of 45^0C in an atmosphere of 9.5% CO_2, 9.5% H_2 and 81% N_2, act to inhibit background growth while promoting differentiated colonies of *Cl. perfringens*. The results have been expressed as the number of presumptive *Clostridium perfringens* colony-forming units per gram dry weight of sediment. Details of this method appear in Appendix A.

2.1.3 Escherichia coli

The method used for the enumeration of *Escherichia coli* is based on the faecal coliform membrane procedure detailed in *Standard Methods for the Examination of Water and Wastewater* (1998:9-63), using commercially prepared Difco m-FC agar. A known weight of the sediment sample, reconstituted in 2.5% saline, was passed through a membrane filter. The filter was placed on the surface of the m-FC agar and the petri dish incubated at 44.5^0C for 24hrs. Faecal coliforms appear as blue colonies and all other colonies are grey. Suspected

Escherichia coli colonies were verified by their ability to produce indol in tryptone water and gas in brilliant green lactose bile (2%) broth. The results have been expressed as the number of presumptive *Escherichia coli* colony-forming units per gram dry weight of sediment. A detailed account of this method appears in Appendix A.

2.2 Physical Chemistry.

2.2.1 pH

A slurry was made using 10g sediment and 100ml deionised water. This was allowed to stand for 30 min and the pH was then read on a WTW MultiLab 540 instrument equipped with a WTW SenTix 97T KCl filled combination pH electrode. pH measurements were done within 24hrs of sample collection.

2.2.2 Organic Matter Content

The organic matter content was determined as weight loss on ignition (550^0C, overnight) of dried (100^0C overnight) sediment sample.

2.2.3 Particle Size

Particle size determinations were done by the University of Natal Geoscience Unit using previously dried samples and a series of sieves with decreasing mesh-grid sizes.

2.2.4 Redox, Salinity and Temperature

These measurements were done in the field using a SenTix ORP electrode coupled to a portable SENSION 2 ISE meter to measure redox potential. A mercury-in-glass thermometer was used to measure temperature. At each of Sites A,B, and C five points were selected. At each point a grab sample of sediment was taken and the redox potential and temperature of the sample measured as soon as the sampler surfaced. At each point a bottom-water sample, taken at the water/sediment interface, was collected and the salinity measured in the laboratory using a MultiLab 540 salinity/pH meter.

2.3 Organic / Inorganic Chemistry.

2.3.1 Sulphate

Since sulphates are generally soluble compounds, it was presumed that they would reach equilibrium in the water phase of the sediment and so sulphate determinations were carried out on the pore-water extracted by centrifugation (3000rpm for 10 min). Measurement of the sulphate concentration employed the automated methylthymol blue method, as described in *Standard Methods for the Examination of Water and* Wastewater (1998:4-179), using a Technicon AutoAnalyser II instrument.

2.3.2 Total Mercury

The sediment samples were pre-treated with concentrated (55%) nitric acid by microwave digestion in the pressure vessels of a CEM Microwave Digester (Model MDS 2000). The total mercury content of the digested sample was measured by cold-vapour atomic absorption spectrometry on a Varian SpectrAA-30 atomic absorption spectrophotometer. The results were expressed as μ/g dry weight of sample, to three significant figures. The minimum detection limit for mercury on this instrument is 0.002μ/g.

2.3.3 Methyl mercury

The instrument used for the measurement of organic mercury was a Varian Saturn Star Gas Chromatograph equipped with a Varian Saturn Star 2000 MS detector (Model 3400 CXGC). The organic mercury content of the sediment was extracted using a method developed by the

CSIR to complement the use of its GC/MS instrument. This procedure involved treating the homogenised, air-dried sediment sample with hydrochloric acid (0.01M solution). After filtering the extract through a Whatman 40 filter paper, the filtrate was buffered with sodium acetate–acetic acid buffer (pH5) and then treated with dithiozone (1% solution). This was followed by the addition of sodium tetraphenyl borate (1% solution in acetone) and dichloromethane, and the subsequent extraction of the dichloromethane portion with sodium sulphate. The extract was concentrated using a rotary evaporator and 1µL of the concentrate was injected onto the DB5 30mm x 250µm x 0.25µm capillary column of the Gas Chromatograph. The instrument parameters were as follows: Injector temperature – 250^0C, splitless; carrier gas – helium at 5mL/min; oven programme – 2min at 80^0C, then ramp at 10^0C/min to 180^0C and hold for 5min, then ramp at 10^0C/min to 280^0C and hold for 3min. The methyl mercury detected was quantified on the MS detector with a scan range of 40-400amu. At the time of sample measurements detection limits for the instrument had not been established, but the lowest quantifiable standard used was 0.5ng and anything lower than this was considered as insignificant.

3. EXPERIMENTAL PROCEDURE.

3.1 Field Survey

The objective of the initial survey was to attempt to isolate, identify and quantify microorganisms of the species *D. desulfuricans, E. coli* and *Cl. perfringens* in the one hundred and eighty samples taken from the three Sites A, B and C (Plate 3.1). For each of the samples both the total and the methyl mercury content were measured. It was hoped that these results would indicate the concentration and distribution of the aforementioned microorganisms relative to areas where elevated levels of total mercury existed, and whether the process of mercury methylation might also be occurring *in situ*. It was also envisaged that isolates of *Desulfovibrio desulfuricans* could be purified and propagated for use in laboratory experiments to be carried out in the second part of the exercise.

3.2 Laboratory Experimentation

The second part of the exercise, the laboratory trials, was dependent on the findings in the field survey. If substantial numbers of *Desulfovibrio* were encountered at sites where the process of mercury methylation appeared to be occurring, it was envisaged that larger samples would be collected from these identified sites and treated as follows:

- Two parallel portions of each sample would be incubated anaerobically for five days, undisturbed. On Day 0, to one of these portions, would be added 20nM $NaMoO_4$, which is an inhibitor of SRB (sulphate-reducing bacteria) activity. The concentration of methyl mercury in each portion would be determined on Day 0 and Day 5. This experiment would determine whether or not mercury methylation was occurring in the samples taken, and whether it was due to the presence of SRB's.
- Two further portions of each sample would be sterilized by tyndallisation (intermittent exposure to moist heat at 100^0C for 20-45min on each of 3 successive days). They would then be spiked with a known concentration of mercuric chloride and inoculated with the purified isolates obtained during the initial field survey. To one portion would be added 20nM $NaMoO_4$ to suppress the SRB activity. Both portions would be incubated anaerobically for five days, undisturbed. Total and methyl mercury estimations would be conducted on each sample on Day 0 and Day 5. Results from this experiment would determine whether or not the isolates obtained in the field survey were capable of generating methyl mercury under controlled laboratory conditions.

CHAPTER 4.

RESULTS

Tabulated summaries of data gathered from the three sites (A, B and C) are contained in Appendix B. All quantitative data relating to a particular sample have been normalised by computing each result obtained with its dry weight factor. The computed results are those appearing in Appendix B. The results will be dealt with firstly on the basis of general observations at each site, followed by a statistical analysis that will attempt to highlight statistical correlation and co-dependence between factors.

1. SITE A – Amanzimnyama River Area.

The pH results for samples taken from the sixty sites in this series show an insignificant variation between sampling points. The results range between pH 7.56 and pH 8.95, with the majority of samples having a pH between 8.0 and 8.5. With a few exceptions, the levels of sulphate appear to be generally constant throughout the sampling area, and within the limits expected in marine sediments. The percentage organic matter at Site A, however, shows considerable variation between sampling points, with levels ranging between 1% and 22%.

The total mercury content of these sediments was generally low, varying between zero at point A54 and 0.076µg/g at point A26. No sample showed a result equal to or above the lower limit for the action level (0.5 – 5.0µg/g dry weight sediment) as defined in the *Guidelines for the Management of Dredged Spoil in South African Coastal Waters* (1996). In only 10% of the samples was the total mercury content shown to be above 0.05µg/g. Two of these results were from samples taken at the head of the silt canal where the Amanzimnyama River enters the bay and where one could reasonably expect the greatest deposition of contaminated silt loads. Apart from that there appears to be no discernible pattern in distribution related to samples that showed mercury levels in excess of 0.05µ/g.

In only eleven of the sixty samples (16%) was any methyl mercury detected, and all but two of these produced results below 0.0005µg/g (the lowest quantifiable standard used). Methyl mercury was not detected in any of the remaining forty-nine samples. In samples A22 and A33 methyl mercury levels of 0.0012µg/g and 0.00276µg/g respectively were recorded. The significantly quantifiable amount of organic mercury in these two samples cannot be reconciled with any evidently increased levels in the corresponding total mercury results. Likewise, these detectable amounts of methyl mercury do not correspond with an increase in numbers of *D. desulfuricans* in samples taken at A22 (where the *D. desulfuricans* count was 0 cfu/g DW) and A33 (where the *D. desulfuricans* count was 6 cfu/g DW). The percentage organic content at these two points was, however, recorded in the upper half of the range, i.e., 22% and 12% at A22 and A33 respectively.

Microbiologically all samples produced a relatively healthy growth of *Cl. perfringens*. Numbers of *E. coli* at all sampling points were insignificant, while the counts for *D. desulfuricans* showed that only in 20% of samples was this organism isolated. In the samples from which *D. desulfuricans* was isolated only four showed a count of greater than or equal to 10^3 cfu/g of sediment, and none of these corresponded with increase levels of total or methyl mercury. Attempts to sub-culture apparent *D. desulfuricans* colonies proved unsuccessful, and identification was therefore made on the morphology in Gram stain, their ability to reduce sulphates (and hence cause blackening of the medium on which they were isolated), and weak fluorescence under ultra violet light due to the production of desulfoviridin.

2. SITE B – Dry Dock Area.

As with Site A, this site again shows consistency of results for both pH and sulphate measurements. In the sixty samples taken from Site B, the pH ranges between pH 7.32 and pH 9.0, with the majority falling between pH 8.0 and pH 8.5. The levels of sulphate at all sampling points appear to remain fairly constant and concentrations here are a reflection of those found at Site A. The percentage organic matter is, on the whole, much lower by comparison with Site A, the results falling between 2% and 10%.

The total mercury concentrations at Site B were, however, generally higher than those found at Site A. In 66% of the samples taken at Site B the total mercury concentration exceeded 0.05µg/g dry weight of sediment (compared with only 10% of samples at Site A), although at only one point (B18 where a value of 0.535µg/g was recorded) was it greater than the lower limit for the action level as defined in the *Guidelines for the Management of Dredged Spoil in South African Coastal Waters* (1996). In 10% of the samples the total mercury concentration exceeded 0.1µg/g. A trace of methyl mercury was only detected in five out of sixty samples (8%), but of these none showed concentrations above that of the lowest quantifiable standard used (0.0005µg/g).

Counts of *Escherichia coli* at Site B were either very low or completely absent, and tended to only feature significantly in sediments where storm water drains fed into their immediate environments. While counts of *Cl. perfringens* were obtained in 60% of the samples, these counts were considerably lower than those found at Site A. Numbers of *D. desulfuricans* appeared to be more widely dispersed at Site B although the counts themselves were relatively low, except at B45, B49 and B50 where they were equal to or exceeded 10^3 cfu/g sediment. Visually there again appears to be a lack of correlation between numbers of *D. desulfuricans* and the total and methyl mercury content of the sediments.

3. SITE C – Mangrove Mud Flats Area.

Site C was essentially included as a control site, since the physical conditions here differ markedly from those at the other two sites. Differences in physical characteristics are paralleled by similarly divergent results in samples taken from this site. The pH ranges between pH 7.8 and pH 9.98, with 61% of samples having a pH value above 8.5. These results show a considerable increase in pH over similar results at Sites A and B (Fig. 4.1). At Site C the percentage organic matter varies between 0.6% and 2.0% (Fig. 4.2), which is considerably lower than that at Site A (1% to 22%) and Site B (2% to 10%). As with its two predecessors, the sulphate results at Site C are markedly high, and do not notably differ from those recorded at Sites A and B.

The overall total mercury content of the samples taken at Site C was low, ranging between 0.004μg/g in sample C5 to 0.073μg/g in sample C56. No sample showed a result equal to or above the lower limit for the action level (0.5 – 5.0μg/g dry weight of sediment) as defined by the *Guidelines for the Management of Dredged Spoil in South African Coastal Waters* (1996). In only 5% of samples did the total mercury content exceed 0.05μg/g, as compared with 10% of samples at Site A and 66% of samples at Site B (Fig. 4.3). No methyl mercury was detected in any of the sixty samples taken at Site C (Fig. 4.4).

Fig. 4.1. Site Comparison: pH

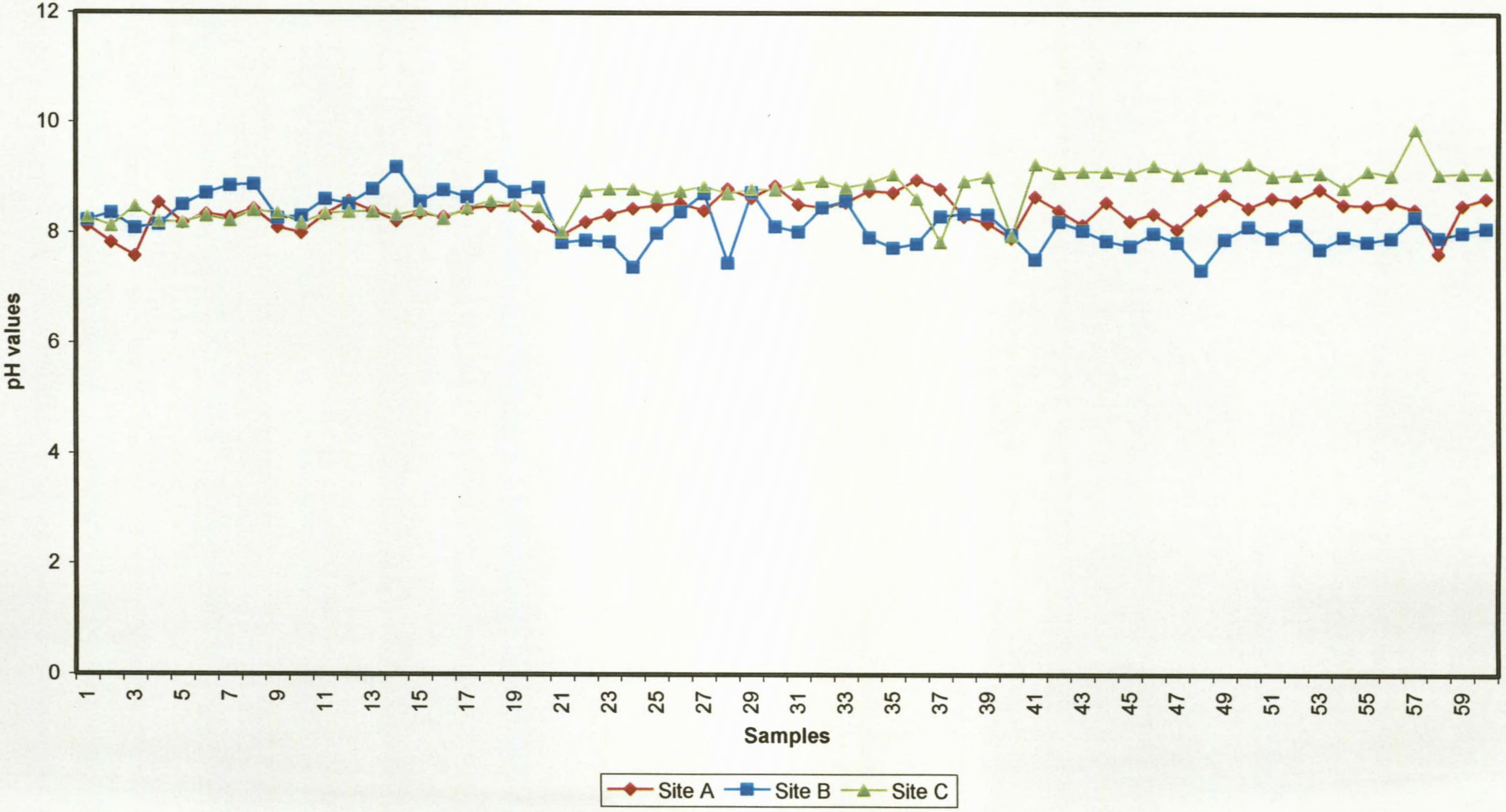


Fig. 4.2. Site Comparison: Organic Matter Content

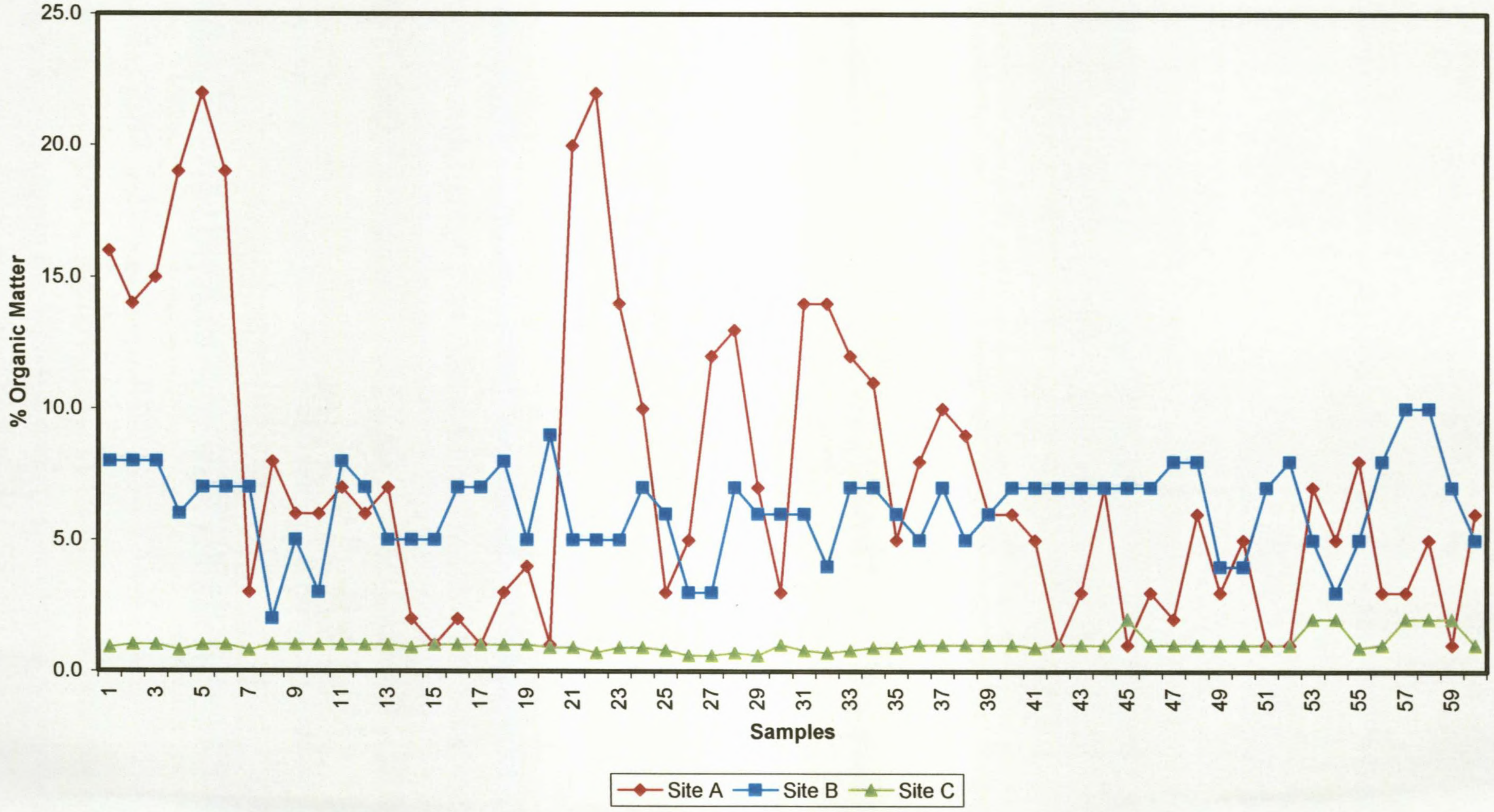


Fig. 4.3. Site Comparison: Total Mercury

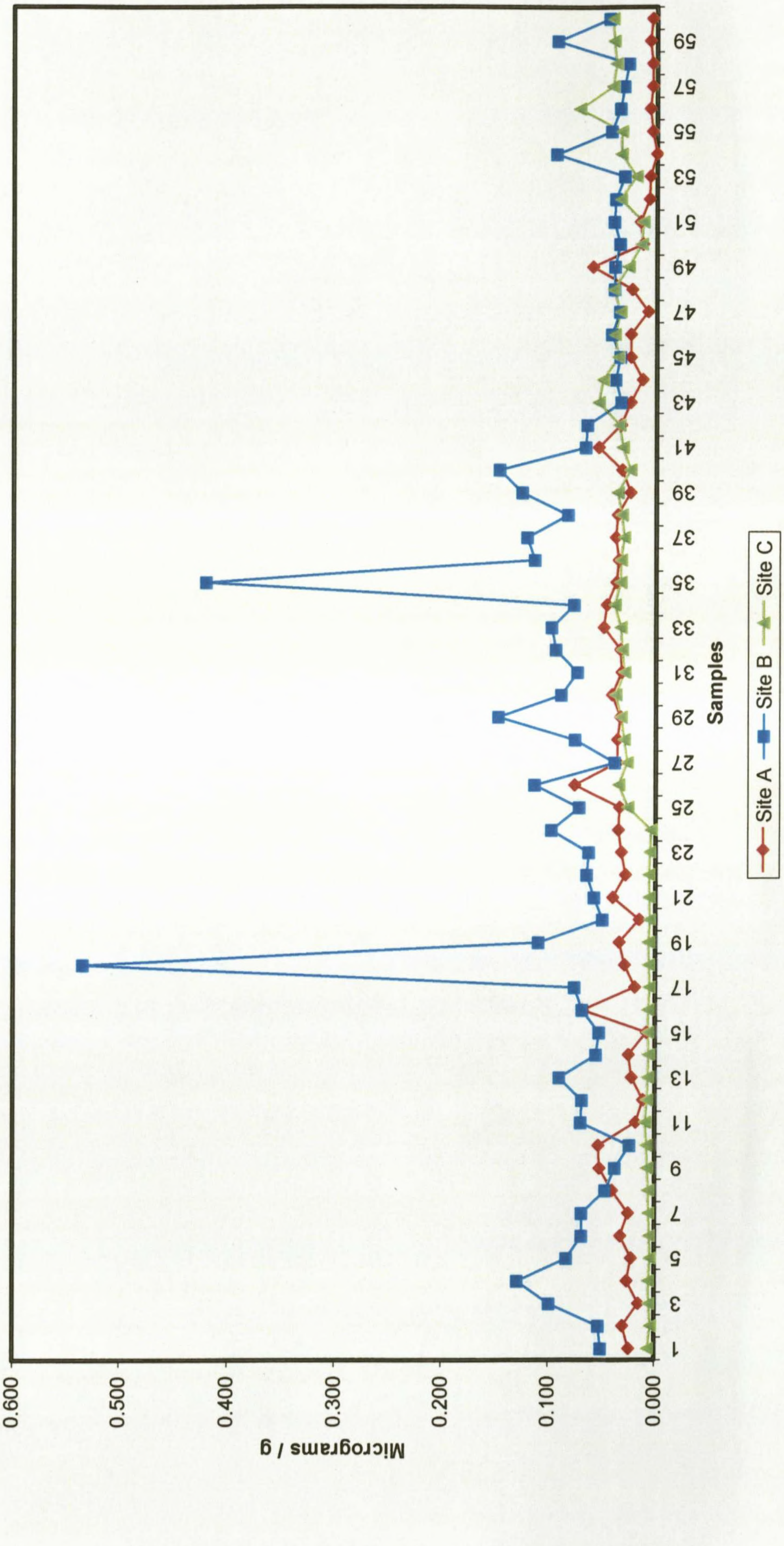


Fig. 4.4 Site Comparison: Methyl Mercury

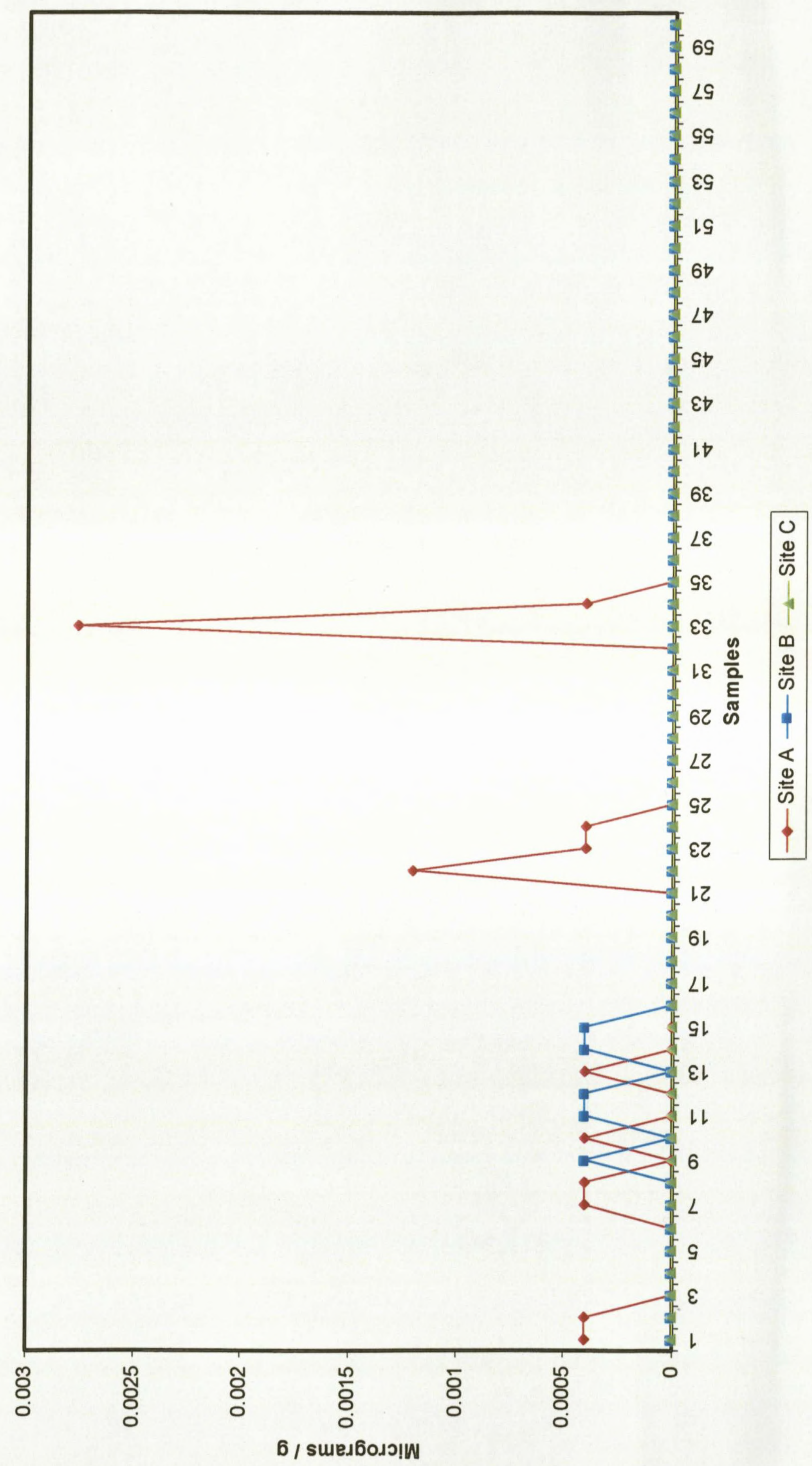


Fig. 4.5. Site Comparison: D. desulfuricans

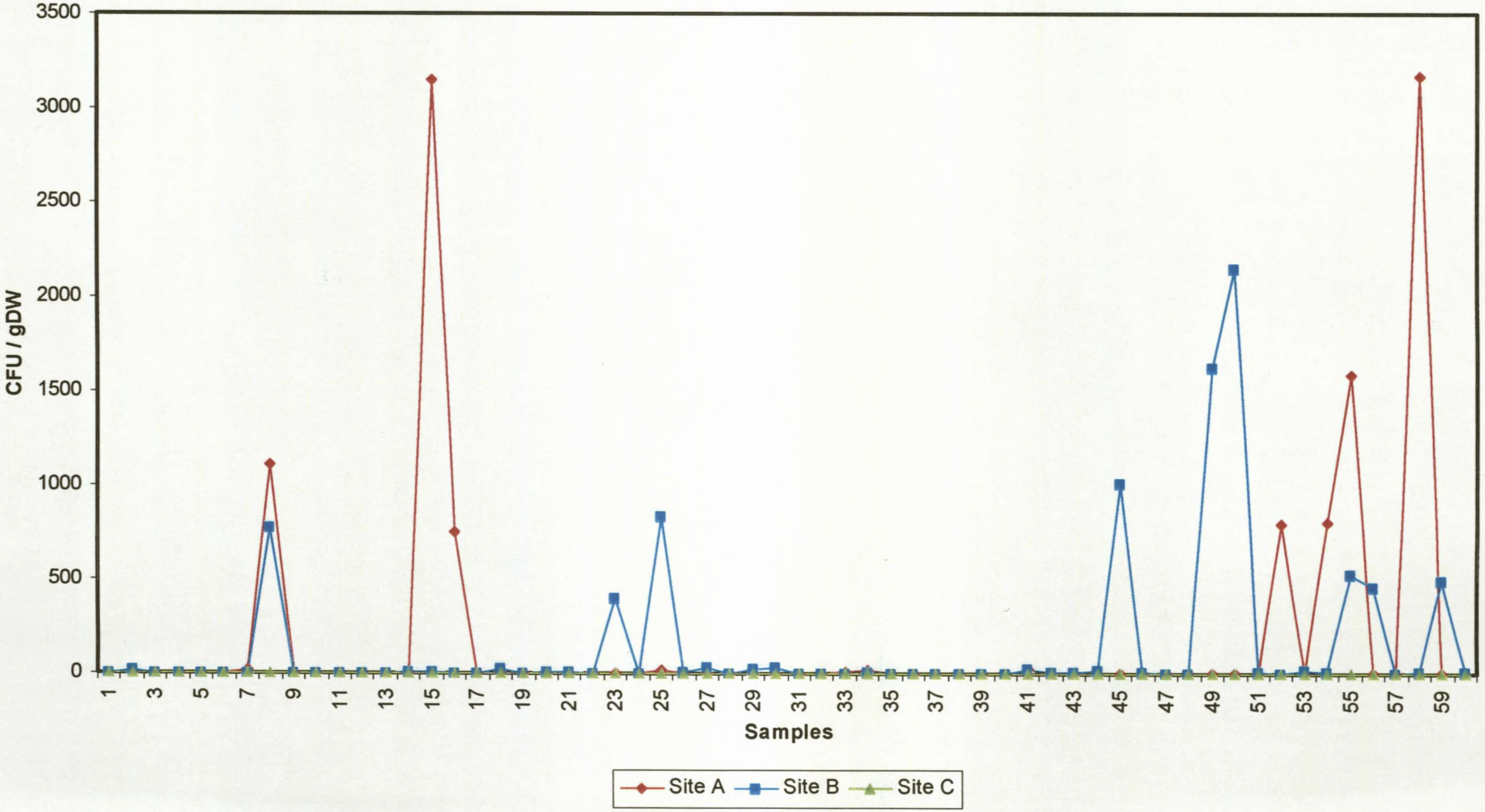


Fig. 4.6 Site Comparison: Cl. perfringens

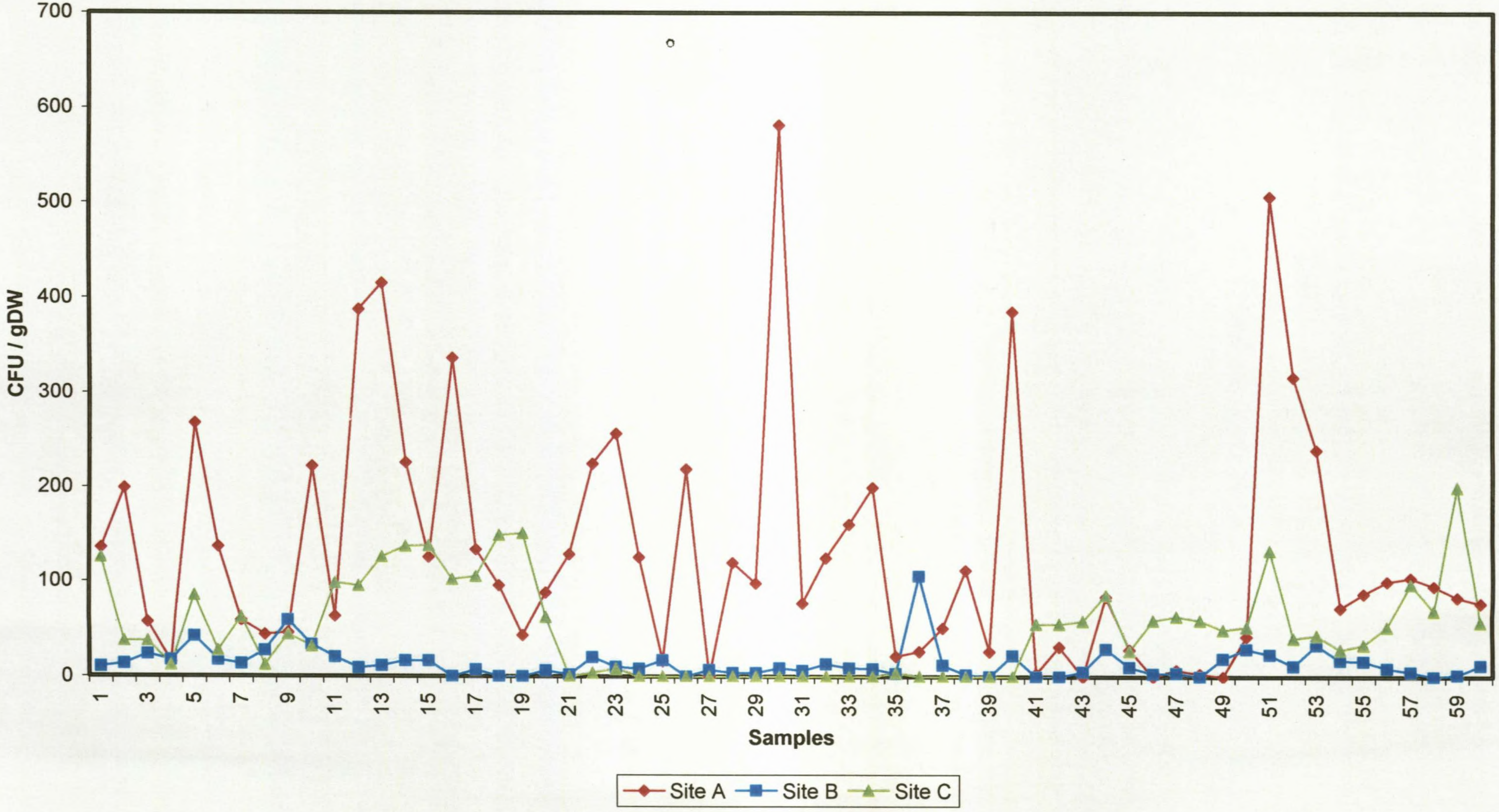
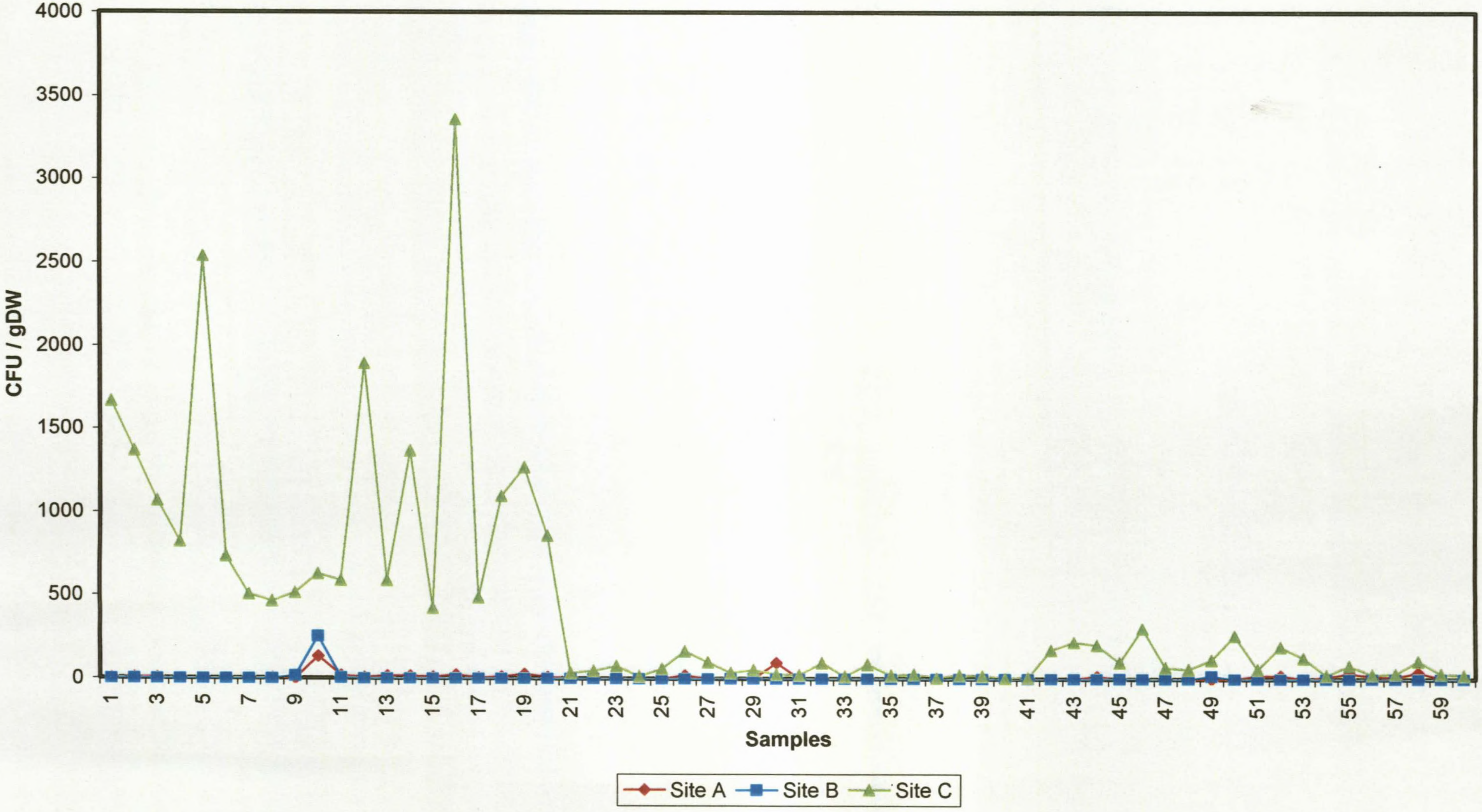


Fig. 4.7. Site Comparison: Escherichia coli



No micro-organisms of the group *Desulfovibrio desulfuricans* were isolated from any of the samples taken at Site C, as compared with moderate numbers detected at Sites A and B (Fig. 4.5). Numbers of *Clostridium perfringens* (particularly between sampling points C1 to C20 and C40 to C60), while more in evidence here than at Site B, did not mirror the notably high counts recorded at Site A (Fig. 4.6). Numbers of *Escherichia coli* were variable throughout the area, but were generally numerically more in evidence (particularly between sampling points C1 and C19) and more ubiquitous than at Sites A and B (Fig. 4.7).

4. STATISTICAL ANALYSIS.

Using the package Microsoft Excel, the results were initially subjected to a correlation analysis with a preliminary view to establishing the relationships between variables. Results from the correlation analysis procedure were then used to provide input for further statistical factor analysis. The Multifactor ANOVA component of the package Statgraphics *Plus* was used to test the effects of two or more factors on one key variable.

4.1 Site A.

Table 4.1 shows the results of the correlation analysis for Site A. For the purpose of this study the figures of interest in the generated matrix of correlation coefficients are those that appear in the lower left quadrant. Each of these computed correlation coefficients provides a normalised and scale-free measure of the linear association between variables, e.g., between *D. desulfuricans* and methyl mercury (where the correlation coefficient is -0.64, indicating a negative correlation that sees variables varying in opposite directions) and between

Table 4.1. Site A: Correlation

	Total Hg	Methyl Hg	%TC	pH	SO4	Faecal coliforms/g DW	E.coli/g DW	Cl. perfringens/g DW	D.desulf/g DW
Total Hg	1								
Methyl Hg	0.212592031	1							
% Organic Matter Content	0.154144259	0.291710773	1						
pH	0.109664012	-0.03034454	-0.175615003	1					
SO4	0.307308754	0.082393932	0.085967967	-0.068028479	1				
Faecal coliforms/g DW	0.109738001	-0.016468963	-0.204393994	-0.079095818	0.09345547	1			
E.coli/g DW	0.183604045	0.082975918	-0.043085818	-0.181136282	0.075864202	0.74565158	1		
Cl. perfringens/g DW	0.055645932	0.119690321	0.027758828	0.048906943	0.245198307	0.408458652	0.098016338	1	
D.desulf/g DW	-0.271525067	-0.064862585	-0.174544968	-0.205579094	0.148192232	0.169468578	-0.03316912	-0.016508878	1

Table 4.2. Site B: Correlation

	Total Hg	Methyl Hg	*%TC*	*pH*	*SO4*	*Faecal coliforms/g DW*	*E.coli/g DW*	*Cl. perfringens/g DW*	*D.desulf/g DW*
Total Hg	1								
Methyl Hg	-0.093405783	1							
%Organic Matter Content	0.052433148	-0.041705843	1						
pH	0.164647372	0.306926194	-0.113796893	1					
SO4	0.179021995	-0.078568834	0.023338985	0.154750689	1				
Faecal coliforms/g DW	-0.105064669	-0.002918953	-0.263281332	0.0266531	-0.327073449	1			
E.coli/g DW	-0.071450963	0.43178777	-0.095185529	0.018887826	-0.31945027	0.051277077	1		
Cl. perfringens/g DW	-0.125010949	0.186260027	-0.272983457	-0.065273021	-0.154858243	0.178780076	0.356392259	1	
D.desulf/g DW	-0.153966023	-0.107049686	-0.280385466	-0.131402391	0.100507466	-0.012885857	-0.047415305	0.104449465	1

Table 4.3. Site C: Correlation

	Total Hg	Methyl Hg	%TC	pH	SO4	Faecal coliforms/g DW	E.coli/g DW	Cl. perfringens/g DW	D.desulf/g DW
Total Hg	1								
Methyl Hg	0	1							
% Organic Matter Content	0.212574303	0	1						
pH	0.678901927	0	0.32966052	1					
SO4	0.457942913	0	-0.171332265	0.268938341	1				
Faecal coliforms/g DW	-0.551346032	0	-0.094851557	-0.510482521	-0.577120056	1			
E.coli/g DW	-0.396515657	0	-0.058660433	-0.396604369	-0.441016767	0.894573801	1		
Cl. perfringens/g DW	-0.213035012	0	0.308396197	-0.007133998	-0.646618608	0.443703472	0.424307922	1	
D.desulf/g DW	0	0	0	0	0	0	0	0	1

Table 4.4. Sites A + B: Correlation

	Total Hg	**Methyl Hg**	***%TC***	***pH***	***SO4***	***Faecal coliforms/g DW***	***E.coli/g DW***	***Cl. perfringens/g DW***	***D.desulf/g DW***
Site									
Total Hg	1								
Methyl Hg	-0.051808357	1							
% Organic Matter Content	-0.017689036	0.281561201	1						
pH	0.01614282	0.090160139	-0.080492815	1					
SO4	0.290275212	-0.011587956	0.012962328	-0.071283612	1				
Faecal coliforms/g DW	-0.101068239	0.003448862	-0.157977154	0.015511478	-0.126314677	1			
E.coli/g DW	-0.013031835	0.100446758	-0.02871016	-0.070084966	0.009905007	0.418702651	1		
Cl. perfringens/g DW	-0.237438358	0.185583824	0.08759606	0.160099945	-0.069090351	0.247104063	0.137651634	1	
D.desulf/g DW	-0.133466273	-0.060140613	-0.176310209	-0.137862249	0.105375017	0.076923343	-0.024749752	0.019269793	1

D. desulfuricans and sulphate (where the correlation coefficient is 0.148, indicating a positive correlation that sees variables varying in same direction). From the results shown in Table 4.1 it would appear that no random variable shares a strong relationship with any other, i.e., in the matrix no two variables produce a correlation coefficient that shows a close affinity with either –1 or +1. The strongest correlation exists between *Cl. perfringens* and sulphate with a correlation coefficient of 0.245, indicating a weak relationship between an increasing/decreasing count for *Cl. perfringens* and an increasing/decreasing value for sulphate. The weakest linear relationship, i.e., that closest to zero correlation, exists between the methyl mercury content and the faecal coliform count (correlation coefficient of –0.016). This suggests that these two variables (methyl mercury and faecal coliforms) are very close to being statistically independent.

Despite the low levels of correlation that appeared to exist between variables at Site A, a multifactor analysis of variance was performed for various dependent variables chosen from this set. Using first methyl mercury as the key dependent variable and *D desulfuricans* and the organic matter content as possible factors that might affect its production, it was established that the former gave a p-Value of 0.0000 while the latter produced a p-Value of 0.2601. This indicated the *D. desulfuricans* factor, with its p-Value of less than 0.05, has a statistically significant effect on methyl mercury at the 95.0% confidence level. *Cl. perfringens* was similarly shown to have a statistically significant effect on methyl mercury with a p-Value of 0.0017. *E. coli* and faecal coliforms were found to be statistically insignificant as far as methyl mercury was concerned, with p-Values of 0.7256 and 1.0000 respectively. Supplanting the various microorganisms as the key dependent variables, it was then established that sulphate had a statistically significant effect on only *D. desulfuricans*, with a p-Value of 0.0000. No other combination of factors and dependent variables produced results that suggested statistically significant effects at the 95% confidence level.

4.2 Site B.

Table 4.2 shows the results of the correlation analysis for Site B. An analysis of the correlation coefficients in the lower left hand quadrant of Table 4.2 again reveals that no variable exhibits a particularly strong relationship with any other. The strongest statistical relationship appears to be that between *E. coli* and methyl mercury (with a correlation coefficient of 0.431). The weakest statistical relationship exists between faecal coliforms and methyl mercury (with a correlation coefficient of –0.002).

Factor analysis, with methyl mercury as the key dependent variable, indicated that *Cl. perfringens*, faecal coliforms and *E. coli* all had statistically significant effects on methyl mercury at the 95% confidence level, with p-Values of 0.0276, 0.0002 and 0.0006 respectively. Substituting the various microorganisms as the key dependent variables, it was found that only with *D. desulfuricans* and sulphate as the factor was a relationship of statistical dependence established (p-Value 0.0000).

4.3 Site C.

Table 4.3 shows the results of the correlation analysis for Site C. Because no values were obtained for either methyl mercury or *D. desulfuricans* at Site C, these have been transposed as zero correlation with other variables in the matrix table. Ignoring these coefficients of zero correlation, the next weakest linear relationship between variables is that between pH and *Cl.*

perfringens, with a correlation coefficient of –0.007. The strongest linear relationship appears to be that between sulphate and faecal coliforms, with a correlation coefficient of 0.577. At this site there were again no variables showing strong relationship with other variables, i.e., none attained a correlation coefficient close to either –1 or +1.

Analysis of variance for results at Site C was limited because of the zero values obtained with all sixty samples for the parameters of methyl mercury and *D. desulfuricans*. The only p-Value less than 0.05, for this set of results, was the 0.0282 obtained for the organic matter content when the key dependent variable was *Cl. perfringens*. Site C appears statistically to be a very unproductive site.

4.4 Sites A and B Combined.

In addition to that performed on the three sites individually, a statistical analysis was also carried out on the combined data from Sites A and B. This was done because Sites A and B bear a general similarity to one another both in a lot of their physical characteristics and in some of the results they produced. The results of this correlation analysis are presented in Table 4.4.

As with the correlation analysis for the individual sites, the procedure applied to the combined data from Sites A and B did not produce proof of any particularly strong linear relationships between the random variables. The strongest correlation coefficient (0.185) was that between methyl mercury and *Cl. perfringens*, while the weakest was that between sulphate and *E. coli* (correlation coefficient 0.009). These results did not reinforce any previous findings from the individual site analyses.

Multifactor analysis of variance revealed that *Cl. perfringens* had a statistically significant effect on methyl mercury in the combined data set, with a p-Value of 0.0000. Sulphate was shown to have a statistically significant effect on both *Cl. perfringens* and *D. desulfuricans* with p-Values of 0.0001 and 0.0000 respectively, while the organic matter content appeared to statistically impact on methyl mercury production with a p-Value of 0.0017.

CHAPTER 5.

DISCUSSION

Stimulated by the findings of international researchers, that the sulphate-reducing microorganism *Desulfovibrio desulfuricans* could be incriminated in the process of mercury bio-methylation, it was decided to test this hypothesis on the sediments in Durban Bay where previous South African researchers had variously found increased levels of total mercury. The results of this investigation have been presented in the previous chapter. Generally these findings proved contrary to what had been expected.

Of the one hundred and eighty samples taken from the various sites in Durban Bay, only one (B18) showed a level of total mercury (0.535µg/g dry weight sediment) above the lower limit for the action level (0.5 – 5.0µg/g dry weight of sediment) as defined in the *Guidelines for the Management of Dredged Spoil in South African Coastal Waters* (1996). This finding is consistent with previous CSIR observations. Since 1977 the CSIR has, on behalf of the Port authorities in Durban Harbour, been involved in an annual sediment assessment programme. Although the sampling sites for the CSIR/Portnet assessment programme remain unchanged, the results obtained from each survey have been found to vary considerably from year to year. Whereas the CSIR/Portnet programme identifies perhaps only six widely distributed points for sampling in each area, this survey has focused on sixty sampling points concentrated on a rigid grid system within a fairly limited area. Nevertheless, results from this survey bear strong similarity to previous findings by the CSIR, i.e., that elevated mercury levels vary site-by-site and year-by-year, probably due to sediment deposition and shifting dynamics in the harbour.

Site B, on the whole, appears to be more contaminated in respect of its total mercury content than do either of Sites A or C. This is understandable since it is in the Congella Turning Basin in front of the dry dock, an area consistent with ship maintenance and repair activities that could be responsible for actively introducing mercuric compounds into the immediate environment. Despite this potential for pollution and the apparently elevated levels of total mercury in the sediments from this site, only one sample violated the lower limit of the action level. It would therefore appear that contamination with mercury does not present a generalised problem in the three areas (Site A, B and C) under study.

In only two of the one hundred and eighty samples taken (A22 with a result of 0.0012μg/g and A33 with a result of 0.00276μg/g) was there detected any presence of methyl mercury above the lowest quantifiable standard used, i.e., 0.0005μg/g. This lower limit for detection shows a degree of extreme sensitivity in the method. Compeau and Bartha (1985:498) reported operating within a lower detection limit of 3ng/g (or 0.003μg/g) of dry sediment. A lower limit for detection of 0.5ng/g or 0.0005μg/g is therefore almost one hundred times more sensitive than that used by Compeau and Bartha, and still methyl mercury was not being detected in the majority of samples taken during this study. In a number of samples minute amounts of methyl mercury were detected on the trace of the chromatograph, but these could not be quantified on the MS detector because they fell below the lowest quantifiable standard of 0.0005μg/g. These results were subsequently recorded as 0.0004μg/g for data manipulation purposes.

It would therefore appear that the methylation of mercury is not occurring to any great extent in the areas under study in Durban Bay. The method being used for its detection is sufficiently sensitive, but concentrations of organic mercury do not necessarily appear to be pervasive in the sediments collected. This apparent absence of organic mercury production could be attributed to a number of factors.

Compeau and Bartha (1985:498 and 1984:1204) identify a neutral pH value of 6.8, found in salt marsh sediments, as being essentially conducive to the methylation of mercury. The pH of sediments taken in Durban Bay varied between pH 7.32 and pH 9.98, with the majority exceeding pH 8.0. Based on the findings of Compeau and Bartha it would therefore appear that the predominantly high pH values experienced in Durban Bay sediments would preclude the formation of methyl mercury. This, however, was not necessarily the case, since the pH was pH 8.18 at A22 and pH 8.54 at A33 where methyl mercury results of 0.0012µg/g and 0.00276µg/g respectively were recorded. In all other samples accredited with methyl mercury values of 0.0004µg/g, the recorded pH values were either close to or exceeded pH 8.0. Thus it would appear that suppositions by Compeau and Bartha (1985:498) and later by Barkay, Gillman and Turner (1997: 4267), that the enhanced accumulation rates of methyl mercury can be correlated with low pH, does not necessarily appear to be the case in Durban Bay.

Barkay, Gillman and Turner (1997:4267) do, however, cite high levels of decomposable organic matter as being one of the environmental parameters necessary for organic mercury production. This was found to be true in the case of samples taken in Durban Bay. A relatively high percentage organic content was recorded in samples A22 and A33, and appreciably large amounts were also recorded in other samples showing detectable levels of methyl mercury. The argument might also be used to explain the apparent absence of organic mercury since, in the majority of samples taken, the organic matter percentage was extremely low.

By comparing the results obtained at control Site C with those obtained at Sites A and B, it is possible to discern how the different parameters interact. The overall increase in pH at Site C over that at Sites A and B is a phenomenon that might be accounted for by the corresponding drop in organic matter content (which is acidic by nature) in Site C sediments. Similarly, the fact that no microorganisms of the group *D. desulfuricans* were isolated from any samples taken at Site C (compared with moderate numbers detected at Sites A and B) is consistent with the findings of low organic matter content, increased pH and absence of methyl mercury in Site C samples.

Compeau and Bartha (1987:262) also conclude that, while slight increases in salinity (from 0.03% to 0.4%) favoured a higher rate of mercury methylation, salinity as high as 2.4% (3.5% is the salinity normally found in marine environments) results in a reduction by 38% of methylation activity. The sediments in this study, being marine sediments, would have contained a high percentage of sodium chloride (probably close to 3.5%), which could have therefore limited the production of methyl mercury.

Compeau and Bartha (1987:263) also suggest that, apart from the precipitation of mercury as the highly insoluble mercuric chloride compound, an explanation for the decline in the formation of methyl mercury could be related to a microbial population in high salinity sediments that is more efficient at demethylating methyl mercury than producing the opposite reaction. The apparently low numbers of *Desulfovibrio desulfuricans* isolated here might be consistent with the argument that the process of demethylation is outstripping the methylation process in the sediments. While no attempt was made in this study to isolate organisms responsible for the demethylation process, counts for *E. coli* at the various sites were established. A report by Ormeland, Cuthbertson and Winfrey (1990:134) identified a strain of *Escherichia coli* (DU 1040) that had the ability to demethylate methyl mercury. No attempt was, however, made to type isolates obtained from samples in this survey. Although the numerical preponderance of *E. coli* was found to be quite widely distributed, it was felt that they were not being isolated in sufficient numbers to be able to attribute their presence as the cause of mercury demethylation.

Results of statistical analysis do not suggest the incrimination of *E. coli* in the demethylation process either. From none of the sites (A, B or C) was there a strongly negative correlation coefficient between methyl mercury and *E. coli*, a result that would have indicated that the numbers of *E. coli* were going up as the concentration of methyl mercury was going down, or vice versa. The positive correlation coefficient between *E. coli* and methyl mercury at Site B is possibly suggestive of another factor, not clearly apparent, that is influencing the relationship here, rather than supposing that increasing numbers of *E. coli* can be directly linked to increasing levels of methyl mercury.

The results obtained in a number of the statistical analyses are logical. One would expect, for example, the analysis to show that *D. desulfuricans* has a significant effect on methyl mercury production because this is a proven scientific fact recorded in the literature. Although statistical analysis suggests that this is also happening in the samples under study, it would be equally correct to suppose that the phenomenon could be due to an unknown factor not included in the analysis – rain makes the grass grow and rain makes the mealies grow, but if only the results of grass growth and mealie growth are included in the analysis the result would be skewed to the extent that it would appear as if grass was dependent on mealies for its growth, or vice versa.

Assessing the correctness of statistical analysis is often problematical, other than to take the logical approach. It is not logical, for example, to conclude that *E. coli* and sulphate have a strong relationship with one another when their correlation coefficient result is 0.577 (as has occurred with results in Site C) because *E. coli* does not reduce sulphates. This result should be interpreted as interference by a third unidentified factor. However, it is logical to accept statistically significant correlation coefficients computed between other factors and variables, e.g., between sulphates, *D. desulfuricans* and *Cl. perfringens*, because the two latter are known to reduce sulphates and sulphites respectively. Similarly, a p-Value of 0.0000 between methyl mercury and organic matter in the combined A and B site data is a logically correct result since, as Barkay, Gillman and Turner (1997:4267) have indicated, high levels of decomposable organic matter affect the accumulation rate of methyl mercury.

Finally it should be noted that, because attempts to sub-culture colonies of *Desulfovibrio desulfuricans* proved unsuccessful, pure isolates of the organism could not be obtained. As a result of this the laboratory experimentation part of this exercise (3.2 of Chapter 3) could not be concluded. Execution of the proposals in 3.2 of Chapter 3 were further complicated by the difficulty in relocating the exact position at each site were numbers of *D. desulfuricans* were found. Distance between grid points at the site often did not vary by more than a few meters and yet results differed substantially between points. The likelihood of being able to relocate the exact point at which the previous sample had been taken was thus extremely unlikely. Therefore, it could not be guaranteed that the same conditions could be met when obtaining a second sample as had been found in the first sample.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The conclusion drawn from this study is that the bio-methylation process for mercury is not occurring to any great extent in the sediments taken at Sites A, B and C in Durban Bay. This finding is supported by both the chemical and microbiological results. No total mercury results (apart from that at B18) appear to be appreciably elevated. This fact is supported by correspondingly low to absent levels of methyl mercury. Numbers of *D. desulfuricans* (cited in the literature as the principle methylators of mercury) appear to be low and not uniformly distributed throughout the sediments. Sulphate levels are consistently high, indicating significantly impaired rates of sulphate reduction. Lack of sulphate-reducing bacteria (*D. desulfuricans* and *Cl. perfringens)* might thus also be contributing to the high levels of sulphate in the sediments.

Based on the outcome of this project the author makes the following recommendations, which is suggested should be observed if any part of the research is to be repeated:

- Samples should be collected in the form of sediment cores (and preferably by a diver) so that they remain as close to the *in situ* state as possible. This would negate the consequence of possible oxygen contamination, which might have a detrimental effect on the numbers of obligate anaerobes. The taking of large sediment cores (that are sealed while held at the sediment/water interface) would allow an accurate measurement of redox potential in the core itself once it had been returned to the laboratory. Such a procedure would give a far more informed indication of whether or not anaerobic conditions to support the survival of *D. desulfuricans* were being met in the sediments under study.

- Media for the isolation of *D. desulfuricans* should be checked for its growth potential and reliability using a known strain of *D. desulfuricans* obtained from a reputable supplier. This would establish that the numbers of organisms being isolated were an accurate reflection of their numbers in the sediments.
- The possible presence and numbers of the organisms capable of demethylating methyl mercury in the sediments should be established. This would clarify whether or not bio-methylation of mercury is actively being counterbalanced by a demethylating process, so that outwardly it appears that little change is taking place.
- The particle size of all samples taken should be analysed, since the particle size is often related to the total mercury content. Small particle size means greater surface area for the adsorption of heavy metals. Hence concentrations of metals in sediments with a small particle size would necessarily be greater than in those with a large particle size. In order, therefore, to deduce whether or not the increased concentration of mercury was due to bacterial processes in the sediments, a knowledge of sediment particle size would provide invaluable information.

REFERENCES.

Archibald, C.G.M. 1996. *Environmental Management of the Port: A Water Quality Information System for the Port of Durban.* Progress Report of the 1997/97 monitoring programme of Durban Bay. CSIR Report JEA. Durban: CSIR.

Archibald, C.G.M. 1997. *Port of Durban: Water Quality Monitoring Programme for Durban Bay.* CSIR Report Watqm96.rep/19970715/cgma/s205. Durban: CSIR.

Archibald, C.M.G. 2000. *Port of Durban: Water Quality Aspects of Durban Bay.* Progress Report of the 2000/2001 monitoring programme of Durban Bay. CSIR Report Dbayrep 2000A/2001-03-18/S370. Durban: CSIR.

Archibald, C.G.M and David, R. 2000. *Port of Durban: Sediment Assessment Programme.* Annual Report . CSIR Report Podsed2krep/cgma/2000-09-24. Durban: CSIR.

Archibald, C.G.M. and Fowles, B.F. 1993. Water Quality and Quantity Trends. *In*: G. Begg *Initial Assessment Concerning the Potential Environmental Impact of the Shongweni Dam Rehabilitation Proposals.* Contract Report. Durban: CSIR.

Armon, R. and Payment, P. 1988. A Modified m-CP Medium for Enumerating *Clostridium perfringens* from Water Samples. *Canadian Journal of Microbiology,* **34**:78-79.

Barkay, T., Gillman, M. and Turner, R.R. 1997. Effects of Dissolved Organic Carbon and Salinity on the Bioavailability of Mercury. *Applied and Environmental Microbiology,* **63**(11): 4267-4271.

Begg, G.W. 1978. The Estuaries of Natal. *Natal Town and Regional Planning Comission Report*, Vol. 41. Durban: Printing Centre.

Bergey's Manual of Systematic Bacteriology, 1984. Vol.1. Baltimore: Williams & Williams.

Bergey's Manual of Systematic Bacteriology, 1984. Vol.2. Baltimore: Williams & Williams.

Bisson, J.W. and Cabelli, V.J. 1979. Membrane Filter Enumeration Method for *Clostridium perfringens*. *Applied and Environmental Microbiology,* **37**(1): 55-66.

Callow, S. 1994. A Review of Findings: Aspects of Heavy Metal Concentration in Natal Estuarine Sediments. M.Sc. thesis, University of Natal, Durban.

Choi, S-C, Chase, T. and Bartha, R. 1994. Metabolic Pathways Leading to Mercury Methylation in *Desulfivibrio desulfuricans* LS. *Applied and Environmental Microbiology*, **60**(11): 4072-4077.

Compeau, G.C. and Bartha, R. 1984. Methylation and Demethylation of Mercury Under Controlled Redox, pH and Salinity Conditions. *Applied and Environmental Microbiology*, **48**(6): 1203-1207.

Compeau, G.C and Bartha, R. 1985. Sulfate-Reducing Bacteria: Principal Methylators of Mercury in Anoxic Estuarine Sediment. *Applied and Environmental Microbiology*, **50**(2): 498-502.

Compeau, G.C. and Bartha, R. 1987. Effect of Salinity on Mercury-Methylating Activity of Sulfate-Reducing Bacteria in Estuarine Sediments. *Applied and Environmental Microbiology*, **53**(2): 261-265.

Davis, A., Bloom, N.S. and Que Hee, S.S. 1997. The Environmental Geochemistry and Bioaccessibility of Mercury in Soils and Sediments: A Review. *Risk Analysis*, **17**(5): 557-569.

Gadd, G.M. 1993. Microbial formation and transformation of organometallic and organometalloid compounds. *FEMS Microbiology Reviews*, **11**: 297-316.

Iverson, W.P. 1966. Growth of *Desulfovibrio* on the Surface of Agar Media. *Applied Microbiology*, **14**(4): 529-534.

Kern, U., Li, C-C. and Westrich, B. 1998. Assessment of sediment contamination from pollutant discharges in surface waters. *Wat. Sci. Tech.*, **37**(6-7): 1-8.

McClurg, T.P., Stanton, R.C. and Combrink, E.A. 1990. *Report on a Survey of Benthic Macrofauna and Chemistry in Sediments Near the Durban Yacht Mole.* CSIR, Durban (unpublished report).

Oliff, W.D., Connell, A.D., McClurg, T.P., Gardner, B.D., Stanton, R.C., Engelbrecht, E.M and Ugifalusi, M.J. 1979. *National Marine Pollution Surveys: East Coast Section. Estuarine Surveys: Durban Bay Complex.* Annual Report, NIWR, Durban.

Oremland, R.S., Culbertson, C.W. and Winfrey, M.R. 1991. Methymercury Decomposition in Sediments and Bacterial Cultures: Involvement of Methanogens and Sulfate-Reducers in Oxidative Demethylation. *Applied and Environmental Microbiology,* **57**(1): 130-137.

Pak, K-R. and Bartha, R. 1998. Mercury Methylation and Demethylation in Anoxic Lake Sediments by Strictly Anaerobic Bacteria. *Applied and Environmental Microbiology,* **64**(3): 1013-1017.

Postgate, J.R. 1984. *The Sulphate-Reducing Bacteria.* Cambridge: Cambridge University Press.

Sibbald, R.R., Connell, A.D., Muller, M.S. and Calder, M.M. 1994. *Wilson's Wharf Study: A Chemical Survey.* CSIR Report: Durban (unpublished).

South Africa. Department of Environmental Affairs and Tourism. 1996. *Guidelines for the Management of Dredged Spoil in South African Coastal Waters.* Cape Town: Government Printer.

Standard Methods for the Examination of Water and Wastewater. 20th ed. 1998. Baltimore: United Book Press, Inc.

Trevors, J.T. 1986. Mercury methylation by bacteria. *J. Basic Microbiol.*, **26**(8): 499-504.

Wade, P. 1998. Appendix II: Bioavailable Versus Total Concentrations of Metals. *In* Archibald, C.G.M & Parsons, G. *Sediment Assessment of a Proposed Floating Dock Site for Elgin Brown & Hamer (Pty.) Ltd. - Heavy Metal Survey.* Contract Report. Durban: CSIR.

White, C., Sayer, J.A. and Gadd, G.M. 1997. Microbial Solubilization and Immobilization of Toxic Metals: Key Biochemical Processes for Treatment of Contamination. *FEMS Microbiology Reviews,* **20**: 503-516.

Wright, C.I. 1996. *A Study of Heavy Metal Concentrations Within the Congella Basin, Durban Harbour.* Report No. 1996-003. Council for Geoscience Geological Survey (Marine Geoscience Unit, Durban).

APPENDIX A.

1. METHOD FOR THE DETECTION OF FAECAL COLIFORMS AND *Escherichia coli.*

1.1 PRINCIPLE

Faecal coliform bacteria can be used as indicators in testing water quality. They are aerobic or facultative anaerobic Gram-negative, non-spore-forming, rod-shaped bacteria that produce blue colonies within 18 - 24 hours of incubation at 44.5±0.5°C on m-FC agar.

The faecal coliform membrane filtration procedure uses an enrichment lactose medium and incubation temperature of 44.5±0.5°C for selectivity. This method has an established 93% accuracy in differentiating between coliforms found in the faeces of warm-blooded animals and those from other environmental sources.

1.2 EQUIPMENT AND CONSUMABLES

Vacuum pump with suction flasks

Autoclave

Fridge at 1-8°C

Waterbath set at 44.5±0.5°C

Automatic pipettes (P1000 and P10ml)

Sterile saline solution (2.5% - made using commercially available sodium chloride and distilled or deionised water)

Sterile test tubes

Test tube rack

Forceps

65mm sterile disposable petri dishes

Sterile 1 ml and 10 ml pipette tips

0,45 μm sterile membrane filters (47 mm diameter)

Measuring cylinders

1.3 MEDIA AND REAGENTS

m-FC Agar – Produced commercially by Difco (Ref. 0677-01-1)

Formula	**g/L**
Bacto Lactose	12,5 g
Bacto Tryptose	10,0 g
Proteose Peptone No. 3 Difco	5,0 g
Sodium chloride	5,0 g
Bacto Yeast extract	3,0 g
Bacto Bile salts No. 3	1,5 g
Aniline blue	0,1 g
Bacyo Agar	13,0 g

Suspend 52g of powder in 1L of distilled / deionised water. The addition of rosalic acid is not mandatory. Heat to near boiling, promptly remove from heat and cool to below 50°C. Pour media into 65 mm petri dishes. Do not autoclave.

Store finished medium in the dark at 1-8°C.

Use within 2 weeks of preparation.

Lauryl Tryptose Broth – Produced commercially by Oxoid (Ref. CM451)

Formula	**g/L**
Tryptose	20,0 g
Lactose	5,0 g
Sodium chloride	5,0 g
Di-potassium hydrogen phosphate	2,75 g
Potassium dihydrogen phosphate	2,75 g
Sodium lauryl sulphate	0,1 g

Suspend 36.5g of powder in 1L of distilled / deionised water. Before sterilization, dispense 4ml volumes of media into test tubes containing fermentation tubes (Durham). Sterilize by autoclaving at 121°C for 15 minutes. Store prepared media in the dark at 1- 8°C.

Brilliant Green Bile (2%) Broth – Produced commercially by Oxoid (Ref.CM31)

Formula	**g/L**
Peptone	10,0 g
Lactose	10,0 g
Ox-bile (purified)	20,0 g
Brilliant green	0,0133 g

Suspend 40g of powder in 1L of distilled / deonized water. Before sterilization, dispense 4ml volumes of media into test tubes containing fermentation tubes (Durham). Sterilize by autoclaving at 121°C for 15 minutes. Store prepared media in the dark at 1- 8°C.

Tryptone Water – Produced commercially by Oxoid (Ref. CM87)

Formula	**g/L**
Tryptone	10,0 g
Sodium chloride (NaCl)	8,5 g

Dissolve 15g of powder in 1L of distilled / deionised water ans distribute in 4ml volumes in test tubes. Sterilize by autoclaving at 121°C for 15 minutes.

Kovac's reagent – prepared as follows:

4-Dimethylaminobenzaldehyde GR	10.0 g
Isoamyl alcohol GR	150 ml
Hydrochloric acid (concentrated HCl) RG	50 ml

Dissolve the aldehyde in the alcohol by gentle heating on a heating mantle.

NB: work in a fume hood to prevent inhalation of alcohol fumes.

Cool and carefully add the acid to the solution.

Mix properly.

Allow to stand for 24 hours prior to use.

Note: the reagent should be light yellow to light brown in colour; some samples of amyl alcohol are unsatisfactory, and give a dark colour with the aldehyde.

Store reagent in an amber bottle below 10°C.

After the 24 hour period and prior to use, quality control must be performed as follows on the reagent:

Inoculate two tubes of tryptone water, one tube with the *Escherichia coli* reference culture as positive control and another tube with the *Enterobacter cloacae* reference culture as negative control. Incubate the inoculated tubes in a water bath at 44.5±0.5°C for 18-24 hours. Include a blank uninnoculated tube. Read results according to Section 3.6. If results are incorrect, discard reagent appropriately and re-prepare.

Complete appropriate label with the necessary information and place it on the bottle containing the freshly prepared Kovaks reagent.

MUG Media – Nutrient agar containing 4-methylumbelliferyl-B-D glucuronide

Nutrient Agar – Produced commercially by Oxoid (Ref.CM3)

Formula	**g/L**
Lab-Lemco powder	1.0 g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g

4-methylumbelliferyl-B-D glucuronide is purchased as a powder from Sigma or as a freeze dried supplement from Oxoid (Ref.BR71).

Suspend 28g of powder in 1L of distilled / deionised water. Bring to the boil to dissolve completely. Decant 100 ml volumes into 200 ml medical flat bottles. Sterilize by autoclaving at 121°C for 15 minutes. Place in 50°C waterbath until use or cool and store in the dark at 1-8°C for up to 3 months.

To 100 ml of molten sterile nutrient agar add 1 ml of a solution containing 10 mg/ml, 4-methylumbelliferyl-B-D glucuronide and thoroughly mix. Pour media into 65 mm diameter petri dishes and store in the dark at 1- 8°C for up to 2 weeks.

1.4 COLLECTION OF SAMPLES

Collect the sample in a sterile Schott bottle, filling the vessel to its rim with sediment. Fill as quickly as possible and screw the lid on tightly to exclude as much air as possible. Store in a cool bag and transport to the laboratory for processing as quickly as possible.

1.5 FAECAL COLIFORM TEST PROCEDURE - MEMBRANE FILTER TECHNIQUE (*Standard Methods*, 1995:9-6; *Standard Methods*, 1998:92220).

1.5.1 Sample Analysis

1. Remove prepared m-FC plates from the fridge and allow them to warm to room temperature. Make sure that the plates are dry and that there is no condensation on the insides of the lids.
2. Disregarding the surface layer of sediment in the Schott bottle, weigh 10g of sediment into a 100ml Erlenmeyer flask. Add 50ml of sterile 2.5% saline. Shake for 15 min. Stand for 10 min. Decant supernatant. Use 1ml and 0.1ml volumes of supernatant for filtration.
3. Using sterile forceps, place a sterile membrane filter (grid side up) over porous plate of filtering receptacle.
4. Wet filter with a small volume of sterile saline solution. Filter the 1ml and 0.1ml volumes of sample through separate filter papers under partial vacuum. When less

than a 10ml volume is to be filtered, add approximately 10ml sterile saline solution to the funnel before filtration, then filter the entire dilution.

5. With filter still in place, rinse the interior surface of the funnel 3 times with 10ml of sterile saline solution.
6. Remove the membrane filter with sterile forceps and place on the m-FC agar plate, avoiding entrapment of air bubbles.
7. Incubate the plates, inverted in a stainless steel canister, in a waterbath at 44.5±0.5°C for 18-24 hours.

1.5.2 Sterility Controls

1. To monitor sterility of the media, incubate an m-FC plate inverted at 44.5±0.5°C for 18 - 24 hours.
2. To monitor sterility of the saline solution, filter 100ml saline solution and incubate inverted at 44.5±0.5°C for 18 - 24 hours.
3. Examine the media and saline controls for contamination. Discard all sample batches where sterility controls were contaminated and repeat samples and sterility controls using another batch of saline and/or media.

1.5.3 Positive And Negative Controls

Include positive and negative controls using a certified reference organism on a monthly basis or with each request for analysis if the time lapse between each request is greater than a month.

Positive control: *Escherichia coli*

Negative control: *Streptococcus faecalis*

Inoculate an m-FC plate with the above. Record their growth on the relevant positive and negative control form (DBN 5:3).

1.5.4 Faecal Coliform Verification

If colonies appear doubtful, test the colonies for lactose fermentation to verify faecal coliforms.

1. Transfer growth of 10 typical colonies from the m-FC plate to lauryl tryptose and brilliant green bile (2%) broth tubes containing Durham tubes.
2. Incubate at 44.5±0.5°C and examine for gas production after 24 and 48 hours.
3. Gas formed in lauryl tryptose broth and confirmed in brilliant green lactose bile broth within 48 hours verifies the colony as a faecal coliform.

1.6 COUNTING AND CALCULATION OF RESULTS

1. Count all colonies exhibiting various shades of blue. The final count is calculated as the number of colony-forming units per gram of sediment. This is then multiplied by the dry weight factor (the expression of the dry weight of the sediment divided by the wet weight of the sediment) to ensure uniformity in all counts per gram of sediment.
2. The results are expressed as the number of presumptive *Escherichia coli* colony-forming units per gram dry weight of sediment.
3. Record results of sterility controls on the faecal coliform worksheet for each batch of

samples.

1.7 *Escherichia coli* I CONFIRMATION

One of two methods may be used to confirm the presence of *E.coli* I:

1.7.1 Conventional method:

1. Subculture a confirmed faecal coliform colony into a test tube containing tryptone water and one containing brilliant green lactose bile (2%) broth and a durham tube.
2. Incubate in a water bath at 44.5±0.5°C for 24 hours.
3. After incubation, test for the formation of indole by adding 0,3-0,5 ml of Kovacs reagent to each tube of inoculated tryptone water. Shake gently and allow to stand for 10 minutes. The development of a red colour indicates the production of indole.
4. The presence of indole in tryptone water and growth and gas in brilliant green lactose bile (2%) broth confirm the presence of *E.coli* I.
5. The confirmed *E.coli* 1 count is calculated as a proportion of the presumptive *E.coli* (faecal coliform) count from those tubes that give a positive reaction for both indol and brilliant green lactose bile as indicated in 4 above, e.g., If the presumptive *E.coli* (faecal coliform) count is 100 faecal coliforms / 100ml. and 5 of the 10 sets of confirmatory tubes are both indol and BGB positive, then the number of confirmed *E.coli* will be 50 *Escherichia coli* / 100ml.

1.7.2 Rapid MUG method:

Membranes with counts >50 may not easily be counted by the MUG method. In such instances the conventional technique for confirming *E.coli* is recommended. Where membranes have counts of less than 50 colonies, the rapid MUG method described below may be used as a confirmatory test.

1. Mark, by stabbing through the filter paper, the colonies that do not present as faecal coliforms on the m-FC plate, i.e., the white or grey colonies.
2. Aseptically transfer the membrane filter to a plate containing the MUG media, followed by incubation for 3 hours at 35±0.5°C.
3. The blue colonies will lose their colour. Count the unmarked colonies that fluoresce when viewed under long wavelength UV light.

1.8 REFERENCES

Clark, D.L., Milner, B.B., Stewart, M.H., Wolfe, R.L. and Olson, B.H., 1990. Comparative study of commercial 4-methylumbelliferyl-B-D-glucuronide preparations with the standard methods faecal coliform test for the detection of *Escherichia coli* in water samples. *Appl. Environ. Microbiol.* **57**(5): 1528-1534.

Mates, A and Schaffer, M., 1988. Quantitative determination of *Escherichia coli* and faecal coliforms in seawater. *Microbios.* **53**: 161-165.

South African Bureau of Standards. 1990. *Bacteriological Quality of Water. SABS Method 221*, 2nd revision. Pretoria: South African Bureau of Standards.

Standard Methods for the Examination of Water and Wastewater. 19th ed, 1995, Baltimore:United Book Press, Inc.

Standard Methods for the Examination of Water and Wastewater. 20th ed, 1998, Baltimore:United Book Press, Inc.

2. METHOD FOR THE DETERMINATION OF *Clostridium perfringens.*

2.1 PRINCIPLE

The spores of sulphite-reducing anaerobes (clostridia) are wide-spread in the environment. They are present in human and animal faecal matter, in waste water and in soil. Unlike *Escherichia coli* and other coliform organisms, the spores survive in water for long periods as they are more resistant than vegetative forms to the action of chemical and physical factors. They may thus give an indication of remote or intermittent pollution (Agg, Stanfield & Gould, 1978). They may even be resistant to chlorination at levels which are normally used for the treatment of water, and they are thus useful for control purposes (International Standard: ISO 6461/2, 1986).

The use of *Clostridium perfringens* as a water quality indicator has been a subject of considerable controversy. At least part of the controversy may have been due to the measurement of a heterogenous group of organisms, "sulphite-reducing, spore-forming anaerobes" on one hand or the use of a rather imprecise method (Bisson and Cabelli, 1979).

C. perfringens is more widely spread in nature than any other pathogenic microorganism. It produces a number of soluble substances that cause a variety of toxic effects in vitro or in vivo or both. *C. perfringens* has been divided onto 5 types (A, B, C, D E) on the basis of major lethal toxins (Cruikshank, 1965). Type A strains are found as part of the microflora of both soil and intestinal tracts, while types B, C, D and E are obligate parasites of animals and are occasionally found in man.

Sources yielding *C.perfringens* include soil and marine sediments worldwide, clothing, raw milk, cheese, semi-preserved meat products and venison. It has been isolated from the intestinal contents of virtually every animal investigated.

2.2 EQUIPMENT AND CONSUMABLES

Autoclave

Membrane filtration apparatus

0.45µm sterile membrane filters

Incubator - set at 37± 1 ^{0}C

Incubator - set at 44.5± 0.5 ^{0}C

65mm petri dishes

Automatic pipettes (P10ml and P1000)

Sterile 10 and 1ml pipette tips

Inflatable glove chamber (Model X-17-17)

Gas mixture: 9.5% CO_2, 9.5% H_2 and 81% N^2

Anaerobic catalyst (BR42)

2.3 COLLECTION OF SAMPLES

Collect the sample in a sterile Schott bottle, filling the vessel to its rim with sediment. Fill as quickly as possible and screw the lid on tightly to exclude as much air as possible. Store in a cool bag and transport to the laboratory for processing as quickly as possible.

2.4 CULTURE MEDIA

2.4.1 m-CP Agar (no commercially available product)

The original method obtained from Bisson and Cabelli (1979) with modifications taken from Armon and Payment (1988)

Formula	**g/90ml**
Tryptose	3.0
Yeast Extract	2.0
Sucrose	0.5
L-cysteine hydrochloride	0.1
$MgSO_4.7H_20$	0.01
Bromocresol purple	0.004
Agar	1.5

Supplements

- 40mg of D-Cycloserine (Sigma) and 2.5mg of polymyxin B sulphate (Sigma) as the dry ingredients

- Indoxyl β-D-glucoside (Sigma), 6.0mg in 8.0ml of sterile distilled water

- 2.0ml of a filter-sterilized 0.5% solution of phenolphthalein diphosphate (Sigma)
- 0.2ml of a filter-sterilized 4.5% solution of $FeCl_3.6H_2O$.

Dissolve the ingredients of the medium and adjust the pH to 7.6 (imperative!). After autoclaving at 121 ^{0}C for 15 minutes, cool the medium to 50 ^{0}C and add the supplements. Dispense the medium in 5ml quantities into sterile petri dishes. Store the poured plates in an anaerobic jar until use.

2.4.2 Gelatin medium

Formula	**g/100ml**
Tryptone	1.5
Yeast extract	1.0
Gelatin	1.5
Agar	1.5

Dissolve the ingredients and autoclave at 121 for 15 minutes. Dispense in 3.0ml volumes into sterile petri dishes.

2.4.3 Frazier's reagent

$HgCl_2$	5.0g
Concentrated HCl	20ml
Distilled water	100ml

Mix $HgCl_2$ with water, add the acid, and shake well until solution is complete.

2.5 TEST PROCEDURE

1. Disregarding the surface layer of sediment in the Schott bottle, weigh 10g of sediment into a 100ml Erlenmeyer flask. Add 50ml of sterile 2.5% saline. Shake for 15 min. Stand for 10 min. Decant supernatant. Use 1ml and 0.1ml volumes of supernatant for filtration.

2. Filter the 1ml and 0.1ml volumes of sample through separate 0.45μm, grided, sterile membranes. Volumes smaller than 10ml should be mixed with 10ml sterile saline.

3. After filtration, remove the membrane with sterile forceps and place it face upwards on the agar. Make sure the agar surface is dry before attaching the membrane.

4. Invert plates and place them in an inflatable glove chamber (Model X-17-17). Put into the bag an anaerobic catalyst (BR42). With a vacuum pump, extract as much air as

possible and replace it with a mixture of 9.5% CO_2. 9.5% H_2 and 81% N_2. Incubate at 44 ± 1°C for 24 hrs.

5. Typical *C.perfringens* are 1.0 to 3.0mm in diameter, convex with entire edges, somewhat opaque, slightly butyrous in consistency and a pale yellow colour. Mark the yellow colonies. Expose the plates to a solution of concentrated ammonium hydroxide for 20 to 30 seconds. Count the red or dark pink colonies and record them as presumptive *C.perfringens* (acid phosphatase positive).

6. After performing the acid phosphatase test, transfer the filter with sterile forceps from the m-CP agar to a plate containing the gelatin medium for a primary confirmation test. After 1-2 hours incubation at 37°C, flood the plates with 2ml Frazier's reagent. The reagent reacts with the gelatin in the medium to produce opacity. Where gelatin has been hydrolysed the medium remains clear. Count the gelatinase-positive colonies and express as a percentage of the presumptive phosphatase positive colonies.

7. The final count is calculated as the number of colony-forming units per gram of sediment. This is then multiplied by the dry weight factor (the expression of the dry weight of the sediment divided by the wet weight of the sediment) to ensure uniformity in all counts per gram of sediment.

2.6 EXPRESSION OF RESULTS

The results are expressed as the number of presumptive *Clostridium* perfringens colony-forming units per gram dry weight of sediment.

2.7 REFERENCES

Agg, A R, Stanfield, G, Gould, D J. 1978. Chapter 2: Indicators of pollution. *In*: *Investigations of Sewage Discharges to some British Coastal Waters*. Water Research Centre, Technical Report TR67.

Armon, R and Payment, P. 1988. A modified m-CP medium for enumerating *Clostridium perfringens* from water samples. *Can. J. Microbiol.*, **34**: 78-79.

Bisson, J W and Cabelli, V J. 1979. Membrane Filter Enumeration for *Clostridium perfringens*. *Appl. Environ.Microbiol.*, **37**(1): 55-66.

Cruikshank, R. 1965. *Medical Microbiology*. Edinburgh and London: E & S Livingstone Limited.

International Standard: ISO 6461/2 - 1986 (E) - Water Quality: Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration.

3. METHOD FOR THE DETERMINATION OF *Desulfovibrio desulfuricans.*

3.1 PRINCIPLE

Desulfovibrio desulfuricans are Gram negative spiral to vibroid-shaped motile cells. They are strictly anaerobic, inhabiting anoxic fresh water and marine sediments, oil fields and other industrial water systems and the intestines of animals. Members of this species are capable of reducing sulphate (and also often sulphite or thiosulphate) to hydrogen sulphide. It is this latter property that is used to distinguish their characteristic growth on simple defined media used for their cultivation (Iverson, 1966; *Standard Methods*, 1998). According to Postgate (1984:9) the genus *Desulfovibrio* are the best known because its members are usually relatively easy to isolate and purify.

3.2 EQUIPMENT AND CONSUMABLES

Autoclave
Membrane filtration apparatus
0.45μm sterile membrane filters
65mm petri dishes
Automatic pipettes (P10ml and P1000)
Sterile 10 and 1ml pipette tips
Inflatable glove chamber (Model X-17-17)
Gas mixture: 9.5% CO_2, 9.5% H_2 and 81% N^2

Anaerobic catalyst (BR42)

3.3 COLLECTION OF SAMPLES

Collect the sample in a sterile Schott bottle, filling the vessel to its rim with sediment. Fill as quickly as possible and screw the lid on tightly to exclude as much air as possible. Store in a cool bag and transport to the laboratory for processing as quickly as possible.

3.4 CULTURE MEDIA

Sulfate Reducing Agar - produced commercially by Scharlau (Ref.1-255)

Formula	g/L
Yeast extract	1,0
Ascorbic acid	0,1
Monopotassium phosphate	0,5
Ammoniun chloride	1,0
Calcium sulfate	1,0
Magnesium sulfate	2,0
Sodium thioglycolate	0,1
Ferrous sulfate	0,5
Sodium lactate	3,5
Agar	15,0

Suspend 25g of powder in 1L of distilled / deionised water and let it soak. Bring to the boil and distribute into containers. Sterilize by autoclaving at 121^0C for 15 min. The mediun should be clear and without precipitate. Cool media to 56^0C and pour a layer into 65mm petri dishes. Allow to harden. The medium should be prepared on the day of use to prevent excess absorption of oxygen.

3.5 TEST PROCEDURE

1. Disregarding the surface layer of sediment in the Schott bottle, weigh 1g of sediment into a sterile test tube. Add 9ml of 2.5% saline (this makes a 10^{-1} dilution). Mix well and stand for about 5 min to allow the heavy particles to settle. From supernatant make dilutions 10^{-2} through 10^{-5} in sterile 2.5% saline.
   NB. Most sediments dealt with in these assays were composed of very fine particulate material. It was found that if the supernatant from well-suspended sediments was used for the dilutions, then the resulting suspended debris deposited on the filter paper after filtration tended to obscure colonies. For this reason the tube containing the initial dilution was allowed to stand for 5 min before the supernatant was removed.

2. Filter the 1ml and 0.1ml volumes of each of the 10^{-1}, 10^{-3} and 10^{-5} through separate 0.45μm, grided, sterile membranes. Volumes smaller than 10ml should be mixed with 10ml sterile saline. Where the sediment particle size was larger and

therefore suspended debris did not obscure colonies growing on the surface of the membrane (i.e. at Site C), 1ml and 5ml volumes were filtered through the membrane.

3. After filtration, remove the membrane with sterile forceps and place it face downwards on the agar. Make sure the agar surface is dry before attaching the membrane.

4. Invert plates and place them in an inflatable glove chamber (Model X-17-17). Put into the bag an anaerobic catalyst (BR42). With a vacuum pump, extract as much air as possible and replace it with a mixture of 9.5% CO_2. 9.5% H_2 and 81% N_2. Incubate at room temperature (21^0C to 24^0C) for up to three weeks.

5. Sulphate reducing bacteria produce dark colonies with halos due to the production of iron sulphurs. Growth usually starts between the second and the seventh day, but may occur anywhere between two and twenty-one days.

6. Count those colonies with a black halo. Place the plate under a UV lamp and observe for fluorescence due to the production of desulfoviridin. Gram a number of the colonies to ensure characteristic morphology.

7. The final count is calculated as the number of colony-forming units per gram of sediment. This is then multiplied by the dry weight factor (the expression of the

dry weight of the sediment divided by the wet weight of the sediment) to ensure uniformity in all counts per gram of sediment.

3.6 EXPRESSION OF RESULTS

The results are expressed as the number of presumptive *Desulfovibrio desulfuricans* colony-forming units per gram dry weight of sediment.

3.7 REFERENCES

Iverson, W.P. 1966. Growth of *Desulfovibrio* on the Surface of Agar Media. *Applied Microbiology,* **14**(4):529-534.

Postgate, J.R. 1984. *The Sulphate-Reducing Bacteria*. Cambridge: Cambridge University Press.

Standard Methods for the Examination of Water and Wastewater. 1998. 20th ed. Baltimore: United Book Press, Inc.

1. SITE A - AMANZIMNYAMA RIVER AREA

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
A1	0.024	0.0004	16.0	8.11	2040.5	2	0	136	0
A2	0.030	0.0004	14.0	7.18	1613.3	7	0	199	0
A3	0.015	0	15.0	7.56	2170.8	4	0	57	0
A4	0.026	0	19.0	8.53	2394.3	0	0	15	0
A5	0.022	0	22.0	8.17	2334.0	0	0	268	0
A6	0.032	0	19.0	8.32	3487.6	0	0	137	0
A7	0.025	0.0004	3.0	8.27	3983.3	0	0	59	15
A8	0.040	0.0004	8.0	8.41	2828.6	3	0	44	1108
A9	0.052	0	6.0	8.08	3824.5	0	0	46	0
A10	0.053	0.0004	6.0	7.98	2676.8	133	53	222	0
A11	0.018	0	7.0	8.32	2935.0	17	4	63	0
A12	0.011	0	6.0	8.56	669.8	6	0	388	0
A13	0.022	0.0004	7.0	8.38	2493.0	15	0	416	0
A14	0.025	0	2.0	8.20	2176.5	15	0	226	0
A15	0.008	0	1.0	8.34	3989.1	8	0	126	3149
A16	0.068	0	2.0	8.27	3195.5	22	0	337	750
A17	0.019	0	1.0	8.41	3345.2	4	0	134	0
A18	0.029	0	3.0	8.47	1711.7	12	0	96	0
A19	0.034	0	4.0	8.47	2336.1	27	0	43	0
A20	0.015	0	1.0	8.10	529.7	8	0	88	0
A21	0.040	0	20.0	7.94	2084.6	3	0	129	0
A22	0.028	0.0012	22.0	8.18	1533.6	0	0	225	0
A23	0.032	0.0004	14.0	8.31	2609.1	1	0	257	0
A24	0.035	0.0004	10.0	8.43	2169.3	0	0	126	0
A25	0.034	0	3.0	8.48	1625.2	0	0	16	13
A26	0.076	0	5.0	8.52	4706.9	19	0	219	0
A27	0.036	0	12.0	8.39	1658.8	0	0	93	0
A28	0.036	0	13.0	8.79	2404.6	0	0	120	0
A29	0.033	0	7.0	8.62	1473.4	0	0	98	0
A30	0.041	0	3.0	8.84	2123.8	96	0	582	0

1. SITE A - AMANZIMNYAMA RIVER AREA

(contd.)

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
A31	0.032	0	14.0	8.51	1169.6	0	0	77	0
A32	0.033	0	14.0	8.45	2214.4	0	0	125	0
A33	0.049	0.0028	12.0	8.54	2533.0	0	0	161	6
A34	0.046	0.0004	11.0	8.75	2647.1	0	0	200	13
A35	0.037	0	5.0	8.72	2571.0	0	0	21	0
A36	0.035	0	8.0	8.95	3598.7	2	0	26	0
A37	0.038	0	10.0	8.79	2340.8	0	0	51	0
A38	0.036	0	9.0	8.29	3931.1	0	0	112	0
A39	0.024	0	6.0	8.17	3154.0	0	0	26	0
A40	0.032	0	6.0	7.90	3476.1	0	0	386	0
A41	0.054	0	5.0	8.65	185.3	0	0	3	26
A42	0.033	0	1.0	8.40	2676.8	0	0	31	0
A43	0.024	0	3.0	8.14	863.1	0	0	0	0
A44	0.013	0	7.0	8.55	100.5	16	0	84	0
A45	0.024	0	1.0	8.21	1034.0	0	0	28	0
A46	0.024	0	3.0	8.34	355.4	0	0	0	0
A47	0.008	0	2.0	8.06	612.4	0	0	7	0
A48	0.023	0	6.0	8.42	516.9	0	0	3	0
A49	0.060	0	3.0	8.69	191.3	0	0	0	0
A50	0.013	0	5.0	8.45	478.5	0	0	42	0
A51	0.015	0	1.0	8.63	3083.6	19	0	507	0
A52	0.007	0	1.0	8.58	2100.3	20	0	316	791
A53	0.006	0	7.0	8.79	1198.9	0	0	239	0
A54	0.000	0	5.0	8.51	1241.2	12	0	72	798
A55	0.004	0	8.0	8.49	1969.8	32	0	87	1585
A56	0.006	0	3.0	8.55	1347.2	16	0	100	0
A57	0.004	0	3.0	8.42	3083.6	12	0	104	0
A58	0.004	0	5.0	7.62	1913.1	40	0	95	3170
A59	0.006	0	1.0	8.49	956.9	4	0	83	0
A60	0.004	0	6.0	8.62	1678.3	4	0	77	0

2. SITE B - DRY DOCK AREA

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
B1	0.051	0	8.0	8.21	3808.4	0	0	10	4
B2	0.053	0	8.0	8.34	2739.1	2	0	13	13
B3	0.099	0	8.0	8.07	3238.7	0	0	23	0
B4	0.129	0	6.0	8.13	3718.8	0	0	17	0
B5	0.083	0	7.0	8.49	2698.6	0	0	42	0
B6	0.069	0	7.0	8.70	3454.3	0	0	17	4
B7	0.069	0	7.0	8.84	2961.6	0	0	13	0
B8	0.047	0	2.0	8.86	3808.4	0	0	27	778
B9	0.038	0.0004	5.0	8.25	1199.5	18	3	59	0
B10	0.024	0	3.0	8.29	1261.1	255	0	33	0
B11	0.070	0.0004	8.0	8.59	3030.9	6	0	20	0
B12	0.069	0.0004	7.0	8.51	2686.1	0	0	9	0
B13	0.090	0	5.0	8.77	3069.4	0	0	11	4
B14	0.056	0.0004	5.0	9.17	3573.2	0	0	16	6
B15	0.053	0.0004	5.0	8.55	3069.4	0	0	16	12
B16	0.069	0	7.0	8.76	3339.9	0	0	0	0
B17	0.076	0	7.0	8.63	3030.9	0	0	7	4
B18	0.535	0	8.0	9.00	3339.9	0	0	0	26
B19	0.110	0	5.0	8.72	3729.5	0	0	0	0
B20	0.050	0	9.0	8.80	3223.8	0	0	6	6
B21	0.058	0	5.0	7.81	3808.4	0	0	2	10
B22	0.065	0	5.0	7.85	2739.1	0	0	20	0
B23	0.063	0	5.0	7.82	3238.7	0	0	10	397
B24	0.098	0	7.0	7.36	3718.8	0	0	8	0
B25	0.072	0	6.0	7.98	2698.6	0	0	17	836
B26	0.114	0	3.0	8.37	3454.3	0	0	0	6
B27	0.039	0	3.0	8.70	2861.6	0	0	7	30
B28	0.076	0	7.0	7.44	3808.4	2	0	4	0
B29	0.148	0	6.0	8.72	1199.5	2	0	4	25
B30	0.089	0	6.0	8.10	1261.1	0	0	9	31

2. SITE B - DRY DOCK AREA

(contd.)

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
B31	0.074	0	6.0	8.01	3030.9	0	0	6	0
B32	0.094	0	4.0	8.45	2686.1	0	0	13	0
B33	0.098	0	7.0	8.57	3069.4	0	0	9	0
B34	0.077	0	7.0	7.91	3573.2	0	0	8	12
B35	0.421	0	6.0	7.72	3069.4	0	0	3	0
B36	0.114	0	5.0	7.79	3339.9	0	0	106	0
B37	0.121	0	7.0	8.29	3030.9	0	0	12	0
B38	0.083	0	5.0	8.34	3339.9	0	0	2	4
B39	0.126	0	6.0	8.33	3729.5	0	0	0	0
B40	0.147	0	7.0	7.96	3223.8	0	0	22	0
B41	0.067	0	7.0	7.51	2469.4	0	0	0	21
B42	0.065	0	7.0	8.20	2074.3	0	0	0	10
B43	0.034	0	7.0	8.04	2247.1	0	0	5	9
B44	0.039	0	7.0	7.85	1876.7	0	0	29	20
B45	0.035	0	7.0	7.76	2716.3	3	0	10	1011
B46	0.042	0	7.0	7.99	2247.1	0	0	3	5
B47	0.035	0	8.0	7.82	2321.2	0	0	4	0
B48	0.040	0	8.0	7.32	2247.1	0	0	0	0
B49	0.040	0	4.0	7.88	3160.8	19	0	9	1626
B50	0.035	0	4.0	8.11	3531.2	0	0	29	2154
B51	0.040	0	7.0	7.91	2347.7	0	0	23	9
B52	0.039	0	8.0	8.14	2785.1	0	0	11	4
B53	0.031	0	5.0	7.70	1802.2	0	0	33	14
B54	0.094	0	3.0	7.92	2760.6	0	0	17	11
B55	0.043	0	5.0	7.84	2492.5	0	0	16	523
B56	0.035	0	8.0	7.90	1802.2	0	0	9	459
B57	0.031	0	10.0	8.29	3996.3	0	0	5	0
B58	0.027	0	10.0	7.91	2858.9	0	0	0	5
B59	0.093	0	7.0	8.00	3156.6	0	0	2	489
B60	0.045	0	5.0	8.08	2834.2	0	0	12	10

3. SITE C – MANGROVE MUD FLATS AREA

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
C1	0.006	0	0.9	8.27	1619.3	1666	333	125	0
C2	0.004	0	1.0	8.12	1619.3	1370	548	37	0
C3	0.005	0	1.0	8.47	1441.8	1069	0	37	0
C4	0.006	0	0.8	8.19	1730.2	820	164	12	0
C5	0.004	0	1.0	8.18	1530.5	2540	1016	85	0
C6	0.005	0	1.0	8.30	1530.5	733	147	27	0
C7	0.006	0	0.8	8.21	1419.6	505	0	62	0
C8	0.006	0	1.0	8.40	1708.0	464	93	12	0
C9	0.007	0	1.0	8.35	1552.7	515	0	44	0
C10	0.008	0	1.0	8.17	1153.5	627	125	31	0
C11	0.009	0	1.0	8.34	1513.8	588	235	98	0
C12	0.008	0	1.0	8.37	1698.3	1894	1105	95	0
C13	0.007	0	1.0	8.38	1242.3	589	235	126	0
C14	0.006	0	0.9	8.31	1490.9	1369	822	137	0
C15	0.007	0	1.0	8.40	1675.1	422	253	138	0
C16	0.007	0	1.0	8.25	1468.1	3359	1343	102	0
C17	0.005	0	1.0	8.44	1354.7	485	194	105	0
C18	0.006	0	1.0	8.57	1377.3	1097	0	149	0
C19	0.007	0	1.0	8.49	1698.3	1271	508	151	0
C20	0.006	0	0.9	8.46	2123.8	857	0	62	0
C21	0.006	0	0.9	8.00	3066.9	36	14	0	0
C22	0.006	0	0.7	8.75	2371.2	48	19	4	0
C23	0.005	0	0.9	8.79	2965.9	76	46	8	0
C24	0.004	0	0.7	8.79	3092.2	16	4	0	0
C25	0.026	0	0.8	8.66	3079.9	62	25	0	0
C26	0.035	0	0.6	8.75	2989.3	166	0	0	0
C27	0.027	0	0.6	8.84	2420.0	101	0	0	0
C28	0.030	0	0.7	8.71	2628.8	37	0	0	0
C29	0.030	0	0.6	8.79	2778.6	54	0	0	0
C30	0.033	0	1.0	8.78	2301.2	28	17	0	0

3. SITE C – MANGROVE MUD FLATS AREA

(contd.)

Site	Total Hg µg/g	Methyl Hg µg/g	% Organic Content	pH	Sulphate mg/L	Faecal Coliforms /g DW	Esch. coli /g DW	*Cl. perfringens* /g DW	*D. desulfuricans* /g DW
C31	0.030	0	0.8	8.89	2917.5	23	14	0	0
C32	0.032	0	0.7	8.94	3379.7	96	0	0	0
C33	0.033	0	0.8	8.82	3029.4	15	8	0	0
C34	0.039	0	0.9	8.90	3139.4	88	0	0	0
C35	0.033	0	0.9	9.06	3066.9	22	4	4	0
C36	0.033	0	1.0	8.62	3245.0	29	12	0	0
C37	0.030	0	1.0	7.84	3041.6	7	4	0	0
C38	0.033	0	1.0	8.94	3168.5	23	0	0	0
C39	0.036	0	1.0	9.02	3168.5	19	0	0	0
C40	0.024	0	1.0	7.95	3193.3	4	4	0	0
C41	0.032	0	0.9	9.25	3148.4	8	0	55	0
C42	0.035	0	1.0	9.10	2206.3	176	105	55	0
C43	0.054	0	1.0	9.12	2498.9	224	179	58	0
C44	0.050	0	1.0	9.12	2090.7	206	206	86	0
C45	0.037	0	2.0	9.07	2162.9	101	20	27	0
C46	0.040	0	1.0	9.23	2396.0	307	61	59	0
C47	0.034	0	1.0	9.06	2162.9	71	29	63	0
C48	0.041	0	1.0	9.20	2308.2	59	35	59	0
C49	0.027	0	1.0	9.06	2249.9	117	23	49	0
C50	0.014	0	1.0	9.26	1804.8	262	105	52	0
C51	0.013	0	1.0	9.03	2025.3	59	12	133	0
C52	0.034	0	1.0	9.06	1978.2	195	0	40	0
C53	0.019	0	2.0	9.09	2013.6	126	25	43	0
C54	0.034	0	2.0	8.82	2025.3	24	9	28	0
C55	0.033	0	0.9	9.13	1978.2	81	16	33	0
C56	0.073	0	1.0	9.05	1859.4	28	0	52	0
C57	0.041	0	2.0	9.89	2246.4	32	19	97	0
C58	0.038	0	2.0	9.07	2095.7	109	22	69	0
C59	0.041	0	2.0	9.09	2177.1	32	0	200	0
C60	0.042	0	1.0	9.09	2200.2	24	15	57	0