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The effects of toxic pollutants on freshwater bacterial communities

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by

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To my Mother and Father

Summary of Thesis submitted for Ph.D. degree

by Carl Russell Milner, B.Sc.,

on

The effects of toxic pollutants on freshwater bacterial communities.

The effects of heavy metals, and phenolic compounds and phenol derivatives, on freshwater bacterial communities was studied. An effluent containing copper, zinc, cadmium and lead was found to have little or no toxic effect on planktonic bacterial communities in the River Aire, except for a localised inhibition of bacterial heterotrophic activity immediately below the discharge. The rapid complexation, adsorption and dilution of metals in the river were thought to account for the absence of bacterial inhibition.

The presence of phenolic compounds and phenol derivatives in a small stream, Sugden Beck, caused major alteration in the planktonic and sediment bacterial communities, although this depended on pH. In comparison with a control stream, Stubs Beck, which did not contain phenolic compounds or phenol derivatives, the bacterial community in Sugden Beck had fewer percentage chromogenic bacteria, reduced species diversity and increased viable counts on Pseudomonas-selective-media (PSM agar). A taxonomic study at these two sites showed that the phenolic compounds and phenol derivatives, in Sugden Beck, caused a shift in species composition away from a diverse bacterial community, containing a wide range of Gram-negative and Gram-positive genera, to one comprised of Gram-negative bacteria belonging to the genus Pseudomonas. Major differences were also found between Sugden Beck and Stubs Beck, in the morphology of planktonic bacteria and the ability to mineralize 2,4-dichlorophenoxyacetic acid (2,4D). Bacteria isolated from Stubs Beck and Sugden Beck were shown to have differing sensitivities to phenolic compounds and phenol derivatives. The toxicity of these compounds to bacteria was found to depend on the compound's structure and also on pH.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1. Role of bacteria in the freshwater environment

Most aquatic bacteria are heterotrophic organisms and therefore, their main function in freshwater ecosystems is the decomposition of organic material, termed mineralization. However, a few aquatic bacteria; the phototrophs and chemoautotrophs, can produce organic compounds, although their overall contribution to primary production is small compared with planktonic algae and macrophytes, as they are normally present only in small numbers.

In unpolluted freshwater systems the release of organic material by primary producers and the leaching of organic material from surrounding soils, provides substrates for heterotrophic bacteria. Bacterial mineralization of these compounds contributes to the cycling of elements in the environment, as the inorganic compounds produced are re-used by primary producers. Aquatic bacteria also produce changes in inorganic compounds e.g. nitrogen fixation, nitrification and denitrification, and sulphur oxidation and reduction (Rheinheimer, 1974) and cause certain specific transformations e.g. methylation of mercury (Grant and Long, 1981).

1.1.i. Self-purification of polluted water.

The release of sewage effluents to receiving waters adds large quantities of organic and inorganic compounds, which provide nutrients for bacterial mineralization. This mineralization of organic material is termed self-purification and results in a reduction in water pollution so that, for example in a river system, the river will normally become clean again within a relatively short distance downstream of a sewage discharge. Bacterial mineralization requires oxygen and during self-purification there is a vigorous oxygen consumption, which may lead to anaerobic

conditions. Organic pollution therefore, affects the aquatic biota by reducing the available oxygen and also by increasing the turbidity of the water and hence reducing photosynthesis. Sewage effluents may also contain human pathogenic bacteria e.g. Salmonella typhi.

As self-purification is essentially a biological process it will be affected by various environmental factors, for example temperature, dissolved oxygen concentration, and pH as well as the composition of the bacterial community. Inhibition or reduction of self-purification may result from the presence of toxic materials in the sewage effluent or receiving water; Rheinheimer (1974) lists heavy metals, cyanides and organic poisons as possible toxic pollutants. In this thesis heavy metals, and phenolic compounds and phenol derivatives are considered as potentially toxic pollutants.

1.2. Pollution by heavy metals

1.2.i. Definition of heavy metals

The heavy metals constitute a diverse group of elements, which characteristically have a density greater than 5g cm^{-3} (Passow et al., 1961) and an atomic number greater than 20, but excluding alkali metals, alkaline earths, lanthamides and actinides (Jones, 1978). The metals most commonly designated as heavy metals are; titanium, vanadium, chromium, manganese, iron, nickel, copper, zinc, arsenic, silver, cadmium, tin, mercury and lead (Murphy and Spiegel, 1983).

The heavy metals studied in this thesis are copper, zinc, cadmium and lead, which are widely used in industry and appear as common pollutants in the environment.

1.2.ii. Heavy metals in the freshwater environment

Heavy metals are ubiquitously distributed in the freshwater environment at low levels. For example, Bowen (1979) gives the median

concentrations of copper, zinc, cadmium and lead in various rivers as $3\mu g l^{-1}$ (range 0.2-30), $15\mu g l^{-1}$ (range 0.2-100), $0.1\mu g l^{-1}$ (range 0.01-3) and $3\mu g l^{-1}$ (range 0.06-120) respectively. The natural background levels of heavy metals in river water, depends on the local geology in the catchment area and on climatic factors, as metals are released by the erosion of soils and minerals. However, the release of domestic and industrial effluents to river water, results in the addition of large quantities of heavy metals.

1.2.iii. Environmental chemistry of the heavy metals

Heavy metals in the freshwater environment are present as a number of different physico-chemical forms or species, which may be classified in the following groups (Hart and Davies, 1978);

a. Free metal ions e.g. $Cu(H_2O)_6^{2+}$

b. Inorganic complexes e.g. $Cu_2(OH)_2^{2+}$, $Pb(CO_3)_2^{2-}$

c. Organic complexes e.g. Zn-fulvic acid

d. Metals associated with colloidal and particulate materials such as clays, hydrous iron and manganese oxides, organic material (e.g. living and dead algae) and precipitates.

The distribution of a heavy metal between these different forms will depend on the chemistry of the metal and on environmental factors.

The speciation of copper, zinc, cadmium and lead in water samples from an Australian river and reservoir, was studied by Florence (1977). Zinc and cadmium had a similar speciation, as these metals existed primarily in the ionic form, with little association with organic chelators or colloids. In contrast both inorganic and organic lead species were present and copper was mainly associated with organic material. Chau and Lum-Shue-Chan (1974) showed that in various Canadian lakes, copper and lead were mostly present as strongly bound forms, whereas zinc was mainly present as labile forms, which corresponded to the ionic form

and simple inorganic and organic complexes. Stiff (1971) showed that in two English rivers, the ionic form of copper comprised only a small percentage of the total soluble copper i.e. copper which passed through a 0.45μm filter and included the free cupric ion and soluble inorganic and organic complexes. In both cases the complexed forms of copper accounted for over 97% of the total soluble copper. In the same study, Stiff (1971) added relatively large amounts of copper (800-900$\mu g l^{-1}$) to water samples from various English rivers. Only a small percentage of the total soluble copper existed as the free ion (0.1-1.4%) with the majority complexed with organic compounds, although inorganic complexes were important (5.4-54.4% of total soluble copper).

Environmental variables are also important in determining the speciation of heavy metals, particularly pH, concentration of the major anions and cations, the type and amount of the organic compounds, and the type and amount of the colloidal and particulate matter present (Hart and Davies, 1978).

1.2.iv. Toxicity of heavy metals

Heavy metals have their main toxic effect through disruption of enzyme structure and function, as they readily form complexes with thiol and other groups on protein molecules, although several heavy metals are regarded as essential elements e.g. copper and zinc.

Sub-lethal concentrations of heavy metals have been shown to retard growth and reduce heterotrophic activity of bacterial cultures. Mitra et al. (1975) showed that cells of Escherichia coli exhibited an abnormally long lag-phase when inoculated into a glucose and salts medium containing either cadmium or zinc. However, the normal pattern of growth was observed after each of the extended lag-phases. Albright et al. (1972) studied the effect of heavy metals on the heterotrophic activity,

as measured by mineralization of ^{14}C glucose, of bacteria in samples from a freshwater environment. The effect of adding, separately, sub-lethal concentrations of copper, zinc, cadmium and lead was to cause a marked reduction in utilization rate and increase in turnover time of the glucose.

Singleton and Guthrie (1977) showed that differences in the sensitivity of bacterial species to heavy metals, may have pronounced effects in the aquatic environment. They compared the effects of adding copper to water samples from two aquatic systems, with untreated control samples. Over a 14 day incubation period the treated samples showed an increase in total colony forming units on agar plates, but a reduction in the number of different colony types. The addition of copper to the aquatic system caused a destabilisation of the natural bacterial community.

Therefore, low levels of heavy metals in the freshwater environment may have only minor effects on viable and total counts of bacteria, but may have major effects on the species composition and activity of the bacterial community.

1.2.v. Speciation and heavy metal toxicity

Heavy metal toxicity in the environment depends on both the intrinsic toxicity of the metal and its availability to aquatic organisms. Heavy metals exist in the freshwater environment as a number of different species, not all of which, however, are toxic. There is considerable evidence to suggest that it is chiefly the ionic form which is toxic, and that removal of the free ion from solution by complexation, adsorption or precipitation will result in reduced toxicity to aquatic organisms.

Brown et al. (1974) showed that the toxicity of copper to fish depended on the concentration of organic material present. The addition of treated sewage effluent and humic substances increased the median survival time of rainbow trout exposed to $2mgl^{-1}$ copper. The toxicity of copper was inversely related to the concentration of treated sewage

effluent and humic substances. This demonstrated that the complexed forms of copper had negligible toxicity to fish and that the reduction in toxicity of copper resulted from the decrease in free-ion concentration.

Andrew et al. (1977) determined the median survival time of Daphnia magna exposed to 5μM copper (317.7μg Cu l^{-1}) with various concentrations of bicarbonate. An increase in bicarbonate caused an increase in the median survival time of Daphnia magna, by increasing the carbonate complexation of ionic copper.

Allen et al. (1980) demonstrated the toxicity of the free ion to algae by measuring the growth of Microcystis aeruginosa in a defined medium containing a toxic concentration of zinc. By adding different concentrations of chelating compounds a significant correlation was shown between the calculated free zinc concentration and the number of cells present after 5 days of growth. Toxicity was dependent on the concentration of the free metal ion rather than the total metal concentration.

The free ionic form has also been shown to be toxic to bacteria, as complexation of the heavy metal by constituents of nutrient media or chelating agents reduces toxicity. Zevenhuizen et al. (1979) showed that copper sensitive bacteria; Klebsiella aerogenes, Escherichia coli and an Alcaligenes sp, could tolerate high copper levels when grown in complex media, when most of the copper was complexed with organic media-constituents. Loveless and Painter (1968) showed that addition of EDTA to a liquid medium, removed the toxic effect of copper on the growth of Nitrosomonas europea. The growth of this organism was inhibited over a range of copper concentrations of 0.05-0.56mgl^{-1}, however, inhibition of growth was removed by the addition of 5mgl^{-1} EDTA.

Clearly, metal complexation with inorganic or organic ligands, absorption onto colloidal and particulate materials and precipitation of insoluble compounds will reduce the toxicity of metals to aquatic bacteria. Environmental factors such as pH and the concentration of chelating agents

will affect the potential toxicity of the heavy metal through their efforts on complexation, adsorption and precipitation.

1.3. Pollution by phenolic compounds and phenol derivatives

1.3.i. Definition of phenolic compounds and phenol derivatives

Phenolic compounds are defined by Buikema et al. (1979) as any aromatic nucleus bearing a hydroxyl group; which includes di- and trihydric phenols, hydroxybenzoic acids, nitro-, chloro-, amino-, methoxy-, phenoxy- and alkyl phenols. The phenolic compounds studied in this thesis are chlorophenols and nitrophenols (See Appendix 1 for structural examples).

The phenol derivatives studied in this thesis are phenoxyalkanoic acids (see Appendix 1 for structural examples). These compounds have an hormone-like ability to regulate plant growth and are widely used as selective herbicides to control a range of broad-leaved weeds in cereals and grassland. Two of the nitrophenols studied; 2-methyl-4,6-dinitrophenol (DNOC) and 2-sec-butyl-4,6-dinitrophenol (DNBP) are also used as contact herbicides.

1.3.ii. Sources and fate of phenolic compounds and phenol derivatives in the freshwater environment

Many phenolic compounds occur naturally in the freshwater environment, where they are derived from the decomposition of leaf litter, lignin and humic acids. Larson (1978) identified several phenolic compounds in stream water, including ferulic acid, gallic acid, salicylic acid, sinapic acid and syringic acid, by gas chromatography. Over a period of 8 months the concentration of phenolic compounds, in stream water, varied from 0.40-4.21mgl^{-1}.

The presence of phenoxyalkanoic acids in the freshwater environment may result directly from their use to control aquatic macrophytes. For example, Hellawell and Bryan (1982) suggest that the

amine formulation of 2,4-dichlorophenoxyacetic acid may be used for aquatic weed control at a concentration of $5mgl^{-1}$ active ingredient.

The source of both phenolic compounds and phenol derivatives studied in this thesis, were from the direct introduction of industrial waste products into a freshwater environment.

Unlike the heavy metals, the phenolic compounds and phenol derivatives are biodegradable, and once these compounds enter the freshwater environment, they will provide a source of organic nutrient for bacterial mineralization. Degradation will result in a reduction of the concentration in water of phenolic compounds and phenol derivatives, and hence toxicity, although other factors such as dilution and adsorption may be important.

Aly and Faust (1964) determined the adsorption of the sodium salt of 2,4-dichlorophenoxyacetic acid (2,4D), various esters of 2,4D and 2,4-dichlorophenol on three clay minerals, in aqueous solution. The sorbed amounts of the 2,4D compounds and 2,4-dichlorophenol, at applied concentrations of $5mgl^{-1}$, ranged between 0.02-0.14mg g^{-1} of clay. These workers concluded that suspended clays in surface waters would not remove significant amounts of either 2,4D or 2,4-dichlorophenol.

1.3.iii. Environmental degradation of phenolic compounds and phenol derivatives

The degradation of phenolic compounds and phenol derivatives in the freshwater environment may occur by biological or non-biological reactions.

a. Non-biological

As well as providing a suitable medium for many chemical reactions e.g. oxidation, reduction and hydrolysis, water is itself chemically reactive. When supplied with energy, usually by absorption of ultraviolet radiation (UV radiation), the phenolic compounds and phenol derivatives are known to undergo photochemical reactions.

Omura and Matsuura (1971) showed that UV irradiation (wavelength 253.7nm) of 4-chlorophenol in aqueous alkaline solution gave rise to various products, following homolytic cleavage of the carbon-halogen bond. These products included; phenol, hydroquinone, 2,4-dihydroxydiphenyl and 2,4-dihydroxy-4-chlorodiphenyl. Irradiation of 3-chlorophenol and 2-chlorophenol, under the same conditions, gave as products of photolysis resorcinol and a resinous mixture, respectively. Aly and Faust (1964) studied the photochemical degradation of the sodium salt of 2,4D, various esters of 2,4D and 2,4-dichlorophenol, in distilled water. Irradiation of these compounds, using UV radiation with a peak wavelength of 253.7nm, caused a rapid photodecomposition, especially under alkaline pH conditions. Degradation of the 2,4D compounds proceeded via 2,4-dichlorophenol.

Clearly, the absorption of UV radiation by phenolic compounds and phenol derivatives leads to the induction of various chemical reactions. However, there is little available evidence for these reactions taking place in the natural environment. Solar UV radiation has wavelengths in the range 290-400nm; the levels of UV radiation below 290nm are limited due to atmospheric absorbance by ozone. Many of the phenolic compounds and all of the phenol derivatives have their maximum UV absorption below 290nm and therefore will only absorb UV radiation weakly at the wavelengths found in the environment. Ultraviolet radiation with wavelengths in the range 290-400nm will also be less energetic than the UV radiation used in the above experiments (253.7nm) and may not induce these reactions in the natural environment. In addition UV radiation will be absorbed by suspended matter and organic material present in the freshwater environment. Aly and Faust (1964) concluded that UV radiation from sunlight was an insignificant factor in the decomposition of 2,4D in surface waters.

b. Biological

Aquatic bacteria can degrade a wide range of natural substrates, ranging from relatively simple compounds such as amino acids and proteins

to more complex plant residues like cellulose and lignin. Degradation of phenolic compounds and phenol derivatives to their constituent inorganic compounds will remove their toxic effect from the aquatic environment, although intermediates may be toxic.

Many workers have demonstrated the biodegradation of phenolic compounds and phenol derivatives. Tabak et al. (1964) isolated bacteria from various aquatic environments which were capable of growing in a simple mineral salts medium, containing various phenolic compounds as sole sources of carbon and energy. Under the experimental conditions employed, bacterial cultures isolated from river mud degraded 95% of $250mgl^{-1}$ 2,4,6-trinitrophenol (picric acid) in 3-6 days and $300mgl^{-1}$ 2,4-dinitrophenol in 7-10 days. Bacterial cultures isolated from the sediment of a petroleum refinery waste lagoon, degraded 95% of $150mgl^{-1}$ 3-chlorophenol and $300mgl^{-1}$ 4-chlorophenol in 3-6 days and $200mgl^{-1}$ 2,4-dichlorophenol in 7-10 days. Aly and Faust (1964) showed that 2,4-dichlorophenol, at an initial concentration of $100\mu gl^{-1}$, in various lake waters was degraded completely within 9 days at $25^{o}C$. Higher initial concentrations of 2,4-dichlorophenol, 500 and $1000\mu gl^{-1}$, were 97.5% degraded within 30 days. These workers also showed that samples of lake mud, which were suspended in distilled water containing $20mgl^{-1}$ 2,4D, were capable of completely degrading the 2,4D within 35-65 days. However, in lake mud samples treated with sodium azide, which inhibited bacteria, there was no loss of 2,4D. Nesbitt and Watson (1980a) studied the biodegradation of 2,4D in an Australian river, Avon River, by measuring the decrease of ultraviolet absorbance. The half-life of $50mgl^{-1}$ 2,4D, added to river water samples, varied from 10 to >50 days. Spain and Van Veld (1983) showed that there was a rapid degradation of 2,4D in ecocores, which were test systems filled with river sediment and water samples. Degradation was determined by measuring the concentration of 2,4D in the water, after 100 hours incubation

there was approximately 10% degradation of 238.8μgl^{-1} 2,4D added to the ecocores.

As degradation of phenolic compounds and phenol derivatives in the aquatic environment occurs mainly by biological processes, degradation will be dependent on environmental factors. Baker et al. (1980) showed that the biodegradation of various chlorophenols in sediment samples, from a small Canadian stream, was dependent on temperature. The degradation of 100mgl^{-1} 3-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol added to suspended samples, in distilled water, was more rapid at 20^{o}C compared with 0^{o}C. At 20^{o}C, 100% of 4-chlorophenol and 56% of 3-chlorophenol were degraded within 30 days, compared with 60% and 10% degradation, respectively at 0^{o}C. With 2,4-dichlorophenol, 73% was degraded at 20^{o}C within 15-30 days compared with 45% at 0^{o}C. Nesbitt and Watson (1980a) determined the effect of environmental factors on the degradation of 2,4D (50mgl^{-1}), in water samples from an Australian river. Significant negative correlations ($p < 0.05$) were found between the half life of 2,4D and both the dissolved organic matter content and the suspended sediment load of the water samples. A further study on the same river (Nesbitt and Watson, 1980b) showed that temperature was also an important factor in the degradation of 2,4D. Water samples were incubated with 50mgl^{-1} 2,4D at various temperatures, after 30 days incubation at 21^{o}C and 25^{o}C approximately 50% of the 2,4D was degraded, compared with 10% at 15^{o}C and 0% at 6^{o}C.

1.3.iv. Toxicity of phenolic compounds and phenol derivatives

The toxic nature of phenolic compounds and phenol derivatives results from their ability to act as non-specific metabolic inhibitors, although few studies have investigated the effects on aquatic microbial populations (Buikema et al., 1979). However, toxicity is dependent upon the compound's structure and on environmental factors such as pH.

Liu et al. (1981) studied the toxicity of various halogenated phenols to bacteria, by measuring the concentration of compound causing a 50% inhibition of bacterial dehydrogenase activity. The toxicity of chlorophenols to bacteria (Bacillus sp. isolated from domestic activated sludge) increased with the degree of chlorination; 2,4-dichlorophenol was approximately 6 times more toxic compared to 3-chlorophenol or 4-chlorophenol. Differences in toxicity were also found between isomers of chlorophenols, as 3,5-dichlorophenol was approximately 22 times more toxic to bacteria compared with 2,6-dichlorophenol.

Zetterberg et al. (1977) showed that the survival of cells of Salmonella typhimurium, exposed to buffered solutions of 2,4D (500mg 2,4D l^{-1}) for 3 hours, was dependent on pH. At pH 6.8 there was no effect of the 2,4D on survival of Salmonella typhimurium, after plating onto nutrient agar, however the toxic effect of 2,4D increased with decreasing pH.

1.4. Outline of thesis

This thesis investigates the effects of potentially toxic pollutants; heavy metals and phenolic compounds and phenol derivatives, on freshwater bacterial communities at two sites suggested by the Yorkshire Water Authority. The work presented in the initial part of this thesis reports on the methods involved in; and results obtained from; routine sampling programmes at the two sites. This is followed by a study on the toxicity of phenolic compounds and phenol derivatives towards bacterial communities and their response to the presence of these compounds.

CHAPTER 2

MATERIALS AND METHODS USED IN ROUTINE SAMPLING OF WATERS POLLUTED BY HEAVY METALS, AND PHENOLIC COMPOUNDS AND PHENOL DERIVATIVES

The following bacteriological and environmental variables were determined.

2.1. Bacteriological variables

2.1.i. Bacterial activity

Bacterial heterotrophic activity was determined by measuring the uptake of ^{14}C glucose added to water samples. However, the addition of substrate interferes with the uptake of natural glucose and hence, natural activity. In this study a single high concentration of glucose ($>100\mu gl^{-1}$) was used, therefore, the potential ability of bacteria to metabolise glucose, rather than the natural activity, was determined (Wright and Burnison, 1979).

Bacterial mineralization i.e. the conversion of ^{14}C glucose to $^{14}CO_2$, was used as a measure of glucose uptake, although some ^{14}C glucose would be retained in the bacterial cell (assimilation). To determine activity 20ml of water sample was added to each of five 125ml serum bottles, followed by 0.1ml of $1.0\mu Ciml^{-1}$ D-[U-^{14}C] glucose, and 0.1ml of 0.22μm membrane filtered $20mgl^{-1}$ glucose. This gave a final combined glucose concentration of $c.100\mu gl^{-1}$. A control was prepared by adding 2ml of 2.5M H_2SO_4 to one of the serum bottles. The bottles were then sealed with a rubber serum-cap to which was attached a glass rod and cup, containing a folded square of Whatman No.1 chromatography paper (see Plate 2.1.) and incubated for 1 hour, at $10^{o}C$, in a rotary incubator (100 r.p.m.). The incubation was terminated by injecting 2ml of 2.5M H_2SO_4 through the serum cap, which also released dissolved $^{14}CO_2$. The $^{14}CO_2$ was absorbed overnight

Plate 2.1. Serum bottle apparatus used for the determination of bacterial activity, by ^{14}C glucose mineralization. Also shown are glass rod and cup, rubber serum cap and folded Whatman No.1. chromotography paper square.

by injecting 0.25ml of 2-phenylethylamine onto the filter paper, which was then transferred to a disposable scintillation vial containing 10ml scintillation fluid (Appendix 2). Radioactivity, as counts per minute (c.p.m.), was assayed by liquid scintillation counting. The number of c.p.m. added initially to each serum bottle was measured by adding 0.1ml of the ^{14}C glucose solution to five scintillation vials and determining their radioactivity by liquid scintillation counting. The counting efficiency was determined by spiking a representative number of vials with an internal standard of ^{14}C-hexadecane; disintegrations per minute (d.p.m.) were then calculated. The fraction of available substrate mineralized (f) represented the potential ability of natural bacteria to metabolize the added glucose and was used as an indication of bacterial activity. The value of f is given by the following equation;

$$f = \frac{\text{mean d.p.m. sample - blank d.p.m.}}{\text{mean d.p.m. isotope added to each bottle}}$$

As only a single glucose concentration was used this method allowed the processing of a large number of samples compared with kinetic methods (Wright and Hobbie, 1966), which required a range of added substrate concentrations.

2.1.ii. Enumeration of the total numbers of bacteria

The total number of bacteria present in a water sample was determined by epifluorescence microscopy, using an acridine-orange direct-count (AODC) technique. This technique involved the staining of bacteria with a fluorochrome dye, acridine orange, which binds to nucleic acid. Subsequent illumination with blue light caused stained bacteria to fluoresce, green or orange. The AODC technique does not differentiate between viable and inactive bacterial cells.

Water samples collected for total-count determinations were

preserved by the addition of 0.22μm membrane-filtered formaldehyde, to a final concentration of 2% v/v. Daley and Hobbie (1975) showed that preservation with formaldehyde permitted the storage of water samples for 1-2 weeks without significant changes in bacterial numbers occurring. In this study counts were usually carried out within 3 days of sampling. Bacteria in the preserved samples were then stained with $10mgl^{-1}$ acridine orange for 10 minutes. A known volume (0.1 - 1.0ml) was added to a filtering funnel, which contained sufficient 0.22μm membrane-filtered distilled-water to give a final volume of 6ml to ensure an even distribution of bacteria on the filter. Filtration, using gentle suction, was through a black (dyed with Dylon ebony black, see Jones and Simon, 1975 for method) 25mm diameter, 0.22μm pore-size Millipore cellulose acetate/cellulose nitrate membrane filter. This filter was then transferred to a microscope slide and covered with a drop of low-fluorescent immersion oil. The microscope used was a Zeiss Photomicroscope fitted with the 3RS epi-condenser, with a 200W mercury super-pressure lamp as a light source. Excitation was through a 3mm BG18 red-suppression filter and a 3mm BG12 (transmission peak 405nm) exciter filter. A chromatic beam splitter was used, and the image was viewed through a 2mm barrier filter 50 (500nm cut off). A 100x oil-immersion objective was used, and counting was at a total magnification of x1600 (field area = $0.0064mm^2$). Any green or orange fluorescing bacterial-shaped objects were counted in each of 30 fields of view. A blank filtration of dilution water was also viewed, to check that any bacteria introduced through contamination were not significant.

The concentration of total bacteria, in the original sample, was calculated from the total number of bacteria in the filtered sample (arithmetic mean x filtering area/field area) after allowing for volume filtered and any dilution used.

2.1.iii. Enumeration of viable bacteria

A surface spread plate method, on casein-peptone-starch agar (see Appendix 2), was used to estimate the number of viable bacteria in water samples. Casein-peptone-starch (CPS) agar was recommended for counting heterotrophic bacteria in freshwater by Jones (1970), and Staples and Fry (1973).

Water samples were diluted using sterile 1/4 strength Ringer solution and a small volume (0.1 - 0.25ml) was spread over the surface of each of ten agar plates, which were incubated for 7 days at 20°C. The numbers of bacterial colonies were then counted, and the concentration of colony-forming units (CFU) in the initial water sample calculated. The number of chromogenic colonies and the number of morphologically different colony types were also recorded from the CPS plates. These values were used to calculate the concentration of chromogenic bacteria and diversity of the bacterial community, in the water samples. Bacterial diversity was calculated by $1000 \times S/\sqrt{N}$, where S equalled the number of different colony types and N equalled the concentration of viable bacteria (Griffiths et al., (1975).

The number of colony forming units was also determined on Pseudomonas-selective-media (see Appendix 2), by the spread plate technique (see above). Pseudomonas-selective-media (PSM) is a selective medium used for the isolation of Pseudomonas species.

The number of chromogenic bacteria, bacterial diversity and the viable count on PSM agar, were used to monitor changes in the composition of freshwater heterotrophic bacterial communities, caused by either heavy metals or phenolic compounds and phenol derivatives.

2.2. Environmental variables

2.2.i. Biochemical oxygen demand

Bacterial mineralization of organic material in water samples consumes oxygen; the biochemical oxygen demand (BOD) is the amount of oxygen consumed during a 5-day incubation period, and is an approximate measure of the amount of degradable organic matter present. Polluted waters have a high BOD, although a low BOD in such an environment may result from bacterial inhibition by toxic compounds.

To determine BOD, a water sample (pH adjusted to 6.5 - 8.5, if required) or a sample diluted (5 - 100 times) with sterile distilled water was aerated overnight and its oxygen concentration measured, using a Kent EIL dissolved-oxygen meter. The sample was then placed in two 250ml, tightly stoppered, reagent bottles and incubated in the dark for 5 days, at 20^{o}C. The oxygen concentration was then remeasured and the difference between the two readings, after taking into account any dilution, was the BOD (mg oxygen l^{-1}). Dilution of the water sample was necessary to maintain excess dissolved oxygen over the incubation period, and was chosen so that over 30% of the saturation oxygen concentration remained after 5 days (Department of the Environment, 1972).

2.2.ii. Turbidity

The relative turbidity of water samples was measured using a Corning-EEL nephelometer. The nephelometer was calibrated, with a blank filter, against distilled water for zero and the manufacturer's arbitrary, ground-glass, standard for full-scale deflection.

On site determinations were made of the following variables;

2.2.iii. pH

pH measurements were made using an E.I.L. portable pH meter, model 30C, which was calibrated in the field using pH 4.0 and 9.0 buffers.

2.2.iv. Temperature

Temperature was measured using a 0 - 50°C mercury-in-glass thermometer, calibrated at 0.5°C intervals.

2.2.v. Dissolved-oxygen concentration

Dissolved-oxygen concentration was measured using a Kent EIL portable, digital, oxygen-meter. This instrument was calibrated prior to field excursions using a 5% sodium sulphite solution for zero oxygen concentration and aerated deionised-water for a fully saturated reading. On site, the oxygen probe was swirled gently in the water until a steady reading was obtained.

2.2.vi. Conductivity

Conductivity was measured using a portable Duran Conductivity Bridge.

2.3. Use of an artificial substratum

The effects of potentially toxic pollutants on sediment bacterial communities were also investigated. As sediment provides a stationary environment for bacteria, these organisms will have a longer contact time with toxic pollutants compared with planktonic bacteria, which are continuously displaced downstream away from the source of pollution. Under such circumstances sediment bacterial communities may provide a more easily detected response to toxic pollutants.

To ensure that sediment taken from all sites was consistent, and for ease of collection and subsequent manipulation, an artificial substratum of aquarium gravel retained in polyester-mesh bags was used. The gravel, which was crushed, calcined (heated) flint (> 99% silica), was prepared for use by washing in 10% HCl followed by frequent washes in distilled water and then dry sterilized at 160°C for 3 hours. Fifty-five

grams of treated gravel were then added to a polyester-mesh bag (10 x 15cm), prepared from polyester-mesh (Henry Simon Ltd., Stockport, aperture 1.8mm, 4.4 meshes cm^{-1}, open area 61%), which were stitched on a domestic sewing machine with polyester cotton. The bags were then transported to site and secured, so as to rest submerged on the stream bed, with nylon fishing line (0.55mm diameter) for a period of 1 month. The bags were then returned to the laboratory in sterile plastic-bags, and assessed for bacteriological variables.

To measure bacterial activity 10g of gravel was placed into each serum bottle followed by 20ml of 1/4 strength Ringer solution. A cylindrical aluminium scoop (internal diameter and height 22mm) was used to measure 10g of gravel, when filled level with the top of the scoop, it contained 10.0g ($\pm$ 0.117g). Non-radioactive glucose and ^{14}C glucose were then added as described above, and the procedure followed as outlined in 2.1.i. To provide samples for total and viable counts, 1g of gravel was added to a sterile glass vial followed by 1g of sterile sand and 10ml 1/4 strength Ringer solution. The bacteria were then removed by shaking the vial on a Mickle shaker (Mickle Laboratory Engineering Co., Gomshall, Surrey) for 5 minutes. Total and viable bacterial counts were determined as outlined in 2.1.ii. and 2.1.iii., except that viable counts on PSM agar were not determined. Shaking gravel in this way only released a proportion of the bacteria associated with the gravel; the results were, however, used for comparisons between sites.

2.4. Heavy-metal analysis

The concentration of copper, zinc, cadmium and lead in water samples was determined using differential pulsed anode stripping voltammetry (DPASV). An advantage of this DPASV technique was that it allowed the direct determination of heavy metals at the low levels commonly found in the freshwater environment (see Introduction 1.2.ii.).

2.4.i. Principles of DPASV

DPASV is an electrochemical technique in which the current at a hanging mercury drop electrode is measured as a function of an applied potential. Characteristically DPASV is a two stage process; the first stage involves the electrodeposition of metals from solution onto the mercury electrode by reduction, followed by a second stage where the metals are re-oxidised and stripped from the electrode back into solution.

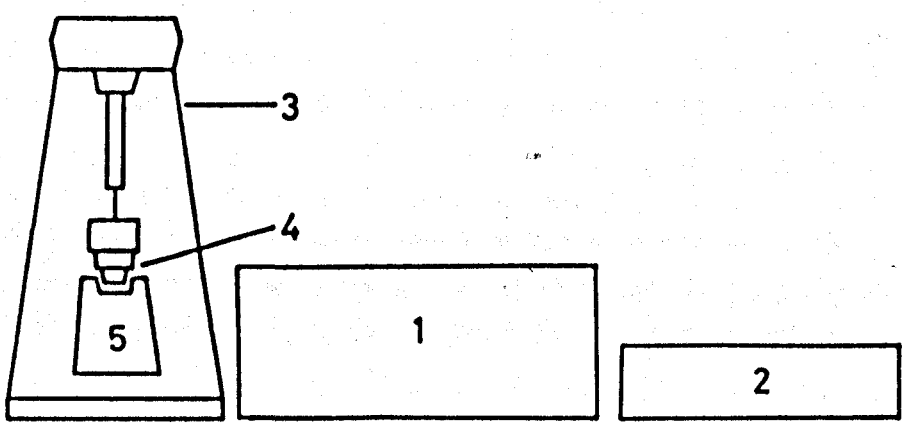
Electrodeposition of heavy metals occurs when a potential, which is more negative than the reduction potentials of the metals is applied to the mercury electrode. Under such conditions the mercury acts as a cathode and metals are deposited onto its surface by reduction. By applying an increasing positive potential the mercury electrode acts as an anode, and metals are sequentially oxidised when their individual redox potential is reached. Oxidation causes a current to flow; the oxidation peak current (Faradaic current) is directly proportional to the metal concentration in solution.

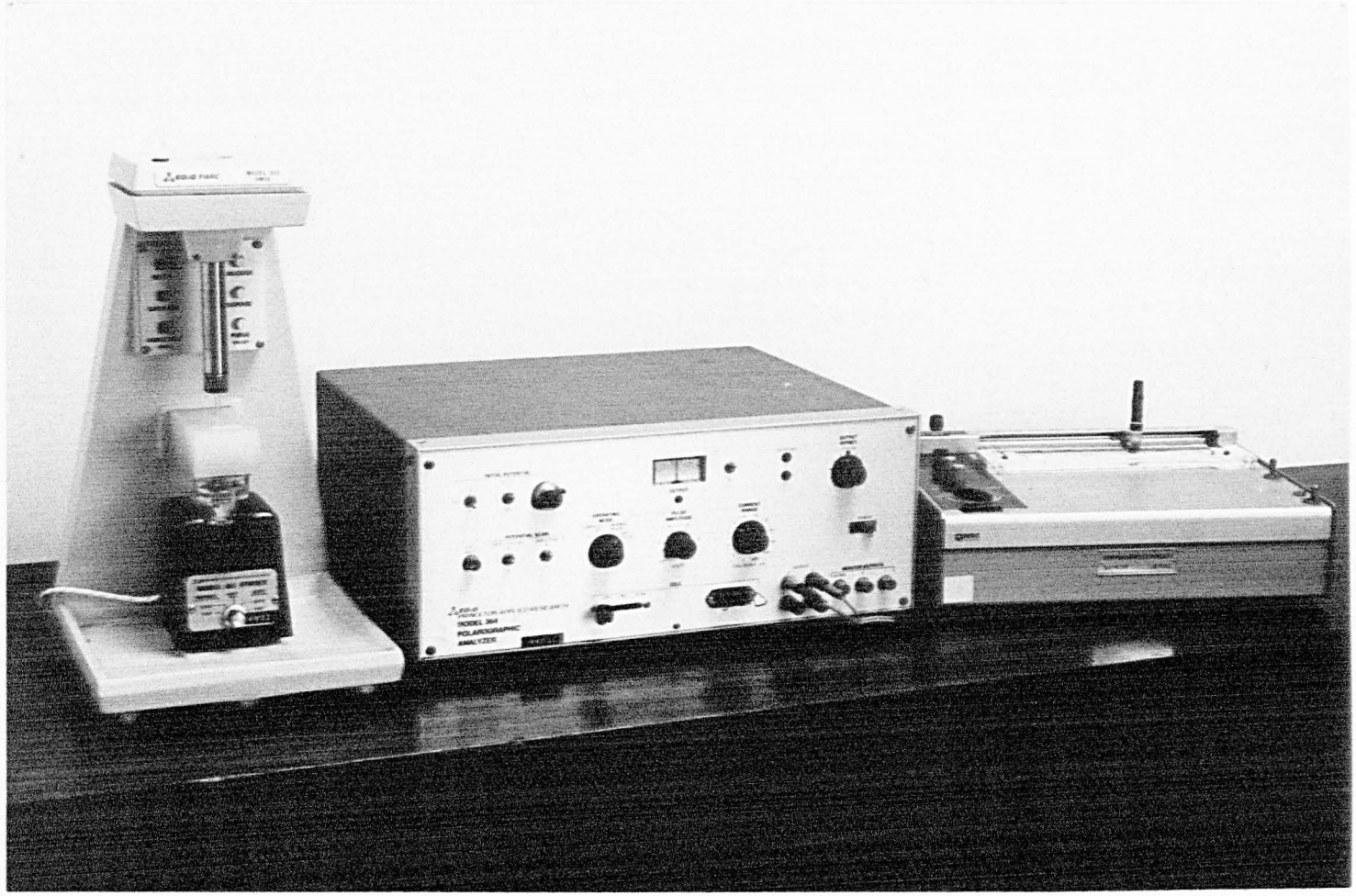
2.4.ii. Equipment

The apparatus used comprised a model 303 static mercury drop electrode, model 305 stirrer and model 364 polarographic analyser (EG and G, Princeton Applied Research) together with a servoscribe chart recorder (model 1s) to record output (see Plate 2.2.). Analysis of the heavy metals was carried out in a borosilicate glass cell, which contained three types of electrode; the working electrode (hanging mercury drop), a platinum counter electrode and a reference electrode. During DPASV current passes between the counter electrode and the working electrode. The cell also contained a polythene tube, which carried oxygen-free nitrogen for sample deoxygenation.

Plate 2.2. Apparatus used for the determination of heavy metals in water samples by differential pulsed anode stripping voltammetry;

1. Polarographic analyser
2. Chart recorder
3. Static mercury drop electrode
4. Borosilicate glass cell
5. Stirrer




MODEL 364
POLAROGRAPHIC
ANALYZER

2.4.iii. Collection and treatment of water samples

Water samples were collected in 2 litre polythene containers, which were rinsed with river water before filling. Polythene containers were found by Batley and Gardner (1977), and Laxen and Harrison (1981) to reduce the problems of leaching of metals from container walls and the reverse process of adsorption from the sample. Before collection of water samples the containers were acid-washed (10% HNO_3) followed by repeated rinsing with double-distilled water. Water samples were stored at $4^{o}C$ prior to analysis.

As DPASV only measured metals that were in solution, sample digestion was required to release complexed or adsorbed metals. Sample digestion was carried out by gently heating (c.$50^{o}C$) a known volume (1 - 10ml) of water sample with 2ml of concentrated HNO_3 (BDH, Aristar grade) in a borosilicate-glass cell, to dryness (Anon., 1976). After allowing the cell to cool 2.5ml, pH 4.8, 2M acetate buffer (BDH, Aristar grade sodium acetate and acetic acid) and 7.5ml of triple-distilled water were added to the digested sample.

The acetate buffer was used as a supporting electrolyte, as DPASV requires a conductive medium (minimum 10^{-3}M, Anon., 1980), in addition the buffer maintained pH during analysis. Hart and Davies (1978) recommended a pH greater than 3.0 for the simultaneous determination of copper, zinc, cadmium and lead, as zinc was not easily determined at low pH due to the proximity of the hydrogen reduction wave. Buffers affect the redox potential of the metals, Chau and Lum-Shue-Chan (1974) reported the following peak potentials of the four metals (vs. Ag/AgCl, sat. KCl), using a similar acetate buffer; Zn - 0.965V, Cd - 0.540V, Pb - 0.362V and Cu + 0.046V.

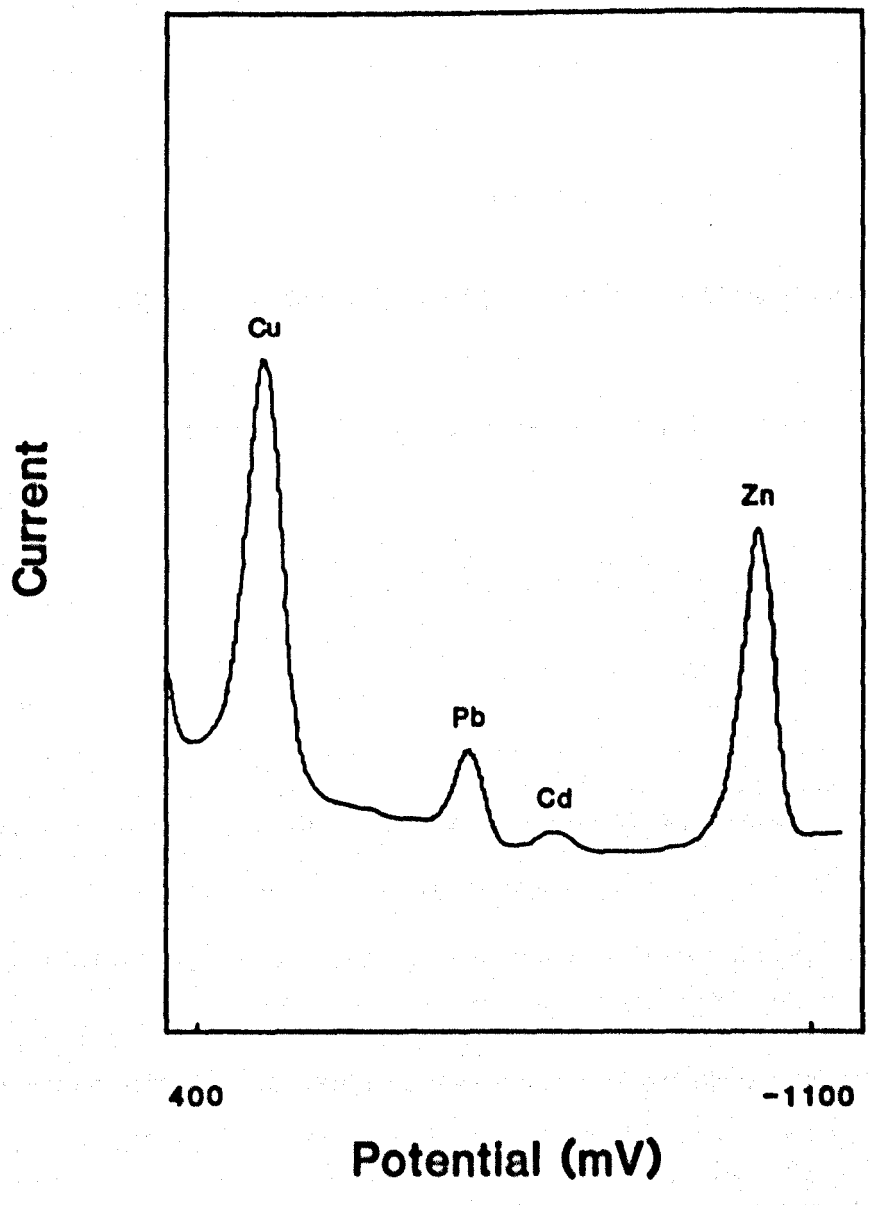
2.4.iv. Simultaneous determination of copper, zinc, cadmium and lead

The borosilicate-glass cell, containing a magnetic stirrer bar, was placed in contact with the electrodes and the sample bubbled with oxygen-free nitrogen for 12 minutes. This removes oxygen from the sample which would otherwise interfere with DPASV of the metals, as oxygen is a reducible species. To prevent re-entry of oxygen during analysis, nitrogen was diverted over the surface of the sample.

For the simultaneous determination of copper, zinc, cadmium and lead deposition was carried out an an initial potential of -1100mV. The metals were usually deposited for 3 minutes, without stirring for the last 30s of the deposition, using a 'small' mercury drop (area $0.0096cm^2$). The metals were then stripped-off the mercury drop by applying an increasing positive potential at a scan rate of $5mVs^{-1}$ over a range of 1500mV i.e. scan ended at +400mV. The most sensitive range of the polarographic analyser was 0.5 - 2μA, therefore, if the sample contained a high concentration of metals a deposition time of 30s without stirring was used (Anon., 1974). Fig. 2.1. shows a typical trace obtained from DPASV of a water sample containing copper, zinc, cadmium and lead.

The peaks were quantified using a method of standard addition. Stock concentrations of $2mgl^{-1}$ Cu, $5mgl^{-1}$ Zn, $0.5mgl^{-1}$ Cd and $2mgl^{-1}$ Pb were prepared from copper sulphate (BDH, Analar grade), zinc sulphate (Fisons, Analytical reagent), cadmium sulphate (Hopkins and Williams, Analar grade) and lead nitrate (Hopkins and Williams, Analar grade), dissolved in 0.08M HNO_3 (BDH, Aristar grade). These stock solutions were stored in acid washed polythene bottles. The cell was spiked with 50μl or 100μl of these solutions to give between 50 - 500% increase in peak height (Anon., 1976). After addition of the spike the sample had nitrogen passed through it, which also aided mixing. A fresh mercury drop was then dispensed and the initial plating procedure repeated. The amount of each metal ion was calculated from the following equation (Anon, 1976);

Fig. 2.1. Typical trace obtained from differential pulsed anode stripping voltammetry of a water sample containing copper, zinc, cadmium and lead.


Current
Cu
Pb
Cd
Zn
400
-1100
Potential (mV)

$$Cu = \frac{i_1 \; v \; Cs}{i_2 v + (i_2 - i_1) V}$$

where Cu = unknown concentration ($\mu g l^{-1}$)

Cs = concentration of standard ($\mu g l^{-1}$)

i_1 = sample peak height (mm)

i_2 = sample plus spike peak height (mm)

v = volume of standard added (ml)

V = volume of sample before digestion (ml)

A blank of triple-distilled water was taken through the entire digestion and metal determination procedure, and the subsequent blank values for the metals subtracted from the experimental values. Sources of contamination in the blank included metals added as a result of digestion with HNO_3 and metals present in the buffer.

As DPASV is a very sensitive technique the following precautions were taken to avoid contamination;

a. Triple-distilled water was used throughout.

b. The purest possible grades of chemicals were used i.e. Aristar grade, BDH or if not available Analar grade.

c. Triple-distilled mercury was used, which was further cleaned by passing oxygen through the mercury whilst covered with, first 10% NaOH and then 10% HNO_3 for 24 hours, followed by numerous washes with triple-distilled water.

d. Borosilicate-glass cells and magnetic stirrers were cleaned prior to use by overnight soaking in 6M HNO_3, followed by frequent washes in triple-distilled water. All glass pipettes were soaked in 10% HNO_3 for 48 hours and then washed with triple-distilled water. Both treatments removed metals adsorbed onto surfaces.

e. The reference electrode and counter electrode were soaked overnight in dilute HNO_3 followed by a thorough washing with triple-

distilled water prior to use and between each determination.

f. At the end of the working day the working electrode was removed and cleaned by aspirating concentrated HCl through it, followed by triple-distilled water. The electrode was then left to dry overnight at 50°C.

2.5. Analysis of phenolic compounds and phenol derivatives

The concentration of identified phenolic compounds and phenol derivatives was determined using high-performance liquid-chromatography (HPLC).

2.5.i. Principles of HPLC

The partition of solute molecules between a mobile-phase and a stationary-phase, during high-performance liquid-chromatography, allows the separation of phenolic compounds and phenol derivatives.

HPLC was preferred to gas-chromatography as it allowed the direct determination of phenolic compounds and phenol derivatives, after concentration, without solvent extraction or derivatization (chemical modification to enhance detection).

2.5.ii. Equipment

The apparatus used comprised two high-pressure pumps (model 750/03) run by a deci-linear gradient programmer (model 750/36, all supplied by Applied Chromatography Systems) connected to a 10cm Hypersil ODS5 column (Shandon), which had octadecyl (C_{18}) groups attached to 5μm diameter spherical silica. Detection of separated phenolic compounds and phenol derivatives was by UV absorbance at 280nm, measured using a LC-UV detector (Pye Unicam). Absorbance sensitivity ranged from 0.04 - 0.16 AUFS (absorbance units for full-scale deflection) depending on the sample. Output was recorded on a Linseis recorder (model LS4), with a chart

speed of 200mm hr^{-1} (see Plate 2.3).

2.5.iii. Collection and treatment of water samples

Water samples were collected in, acetone washed, 300ml glass bottles, which were rinsed with stream water before filling. The samples were then preserved by acidification (1ml conc. HCl/300ml sample) and stored at 4°C prior to analysis. Storage in this way was found not to affect significantly the quantity of phenolic compounds and phenol derivatives present.

Water samples were prepared for analysis by filtration through a 0.22µm pore size, 47mm diameter, cellulose acetate membrane filter, to remove suspended particulates which may block the column. A volume of 250ml of the filtrate was then concentrated to 2 - 3ml using a rotary evaporator, with a water bath at 75°C. The concentrated sample was added to a glass vial followed by, 2.5ml of a saponification mixture, containing an internal standard. The saponification mixture was 0.2M KOH, prepared from equal volumes of isopropanol and double-distilled water, and contained 500mgl^{-1} 4-bromophenol as an internal standard.

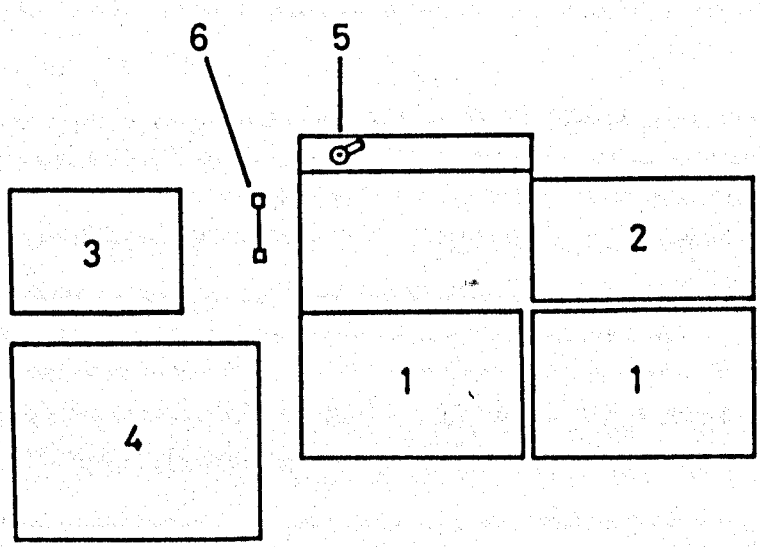
Saponification was required to convert ester or amine forms of the phenoxyalkanoic acids present in the water sample, to the acid form, so that only a single peak was obtained on subsequent analysis. Skelly et al. (1977) used a similar saponification mixture to convert ester and amine forms of 2,4D to the acid form, saponification was complete after 95s at 25°C.

2.5.iv. Analysis of phenolic compounds and phenol derivatives

The mobile-phase used for the separation of phenolic compounds and phenol derivatives was composed of two solvents; 0.1M pH 3.0 citrate buffer (prepared using double-distilled water) and acetonitrile (HPLC grade). The citrate buffer was used to maintain pH in the mobile-phase,

Plate 2.3. Apparatus used for the determination of phenolic compounds and phenol derivatives in water samples by high-performance liquid-chromatography;

1. High-pressure pump
2. Deci-linear gradient programmer
3. LC-UV detector
4. Chart recorder
5. Injection port
6. Hypersil ODS5 column (10cm)



although the mixing of an aqueous buffer with acetonitrile will alter the initial pH. As the phenolic compounds and phenol derivatives were weak acids, the ionisation of these compounds, and hence retention times, was dependent upon pH; a pH 3.0 citrate buffer was found to give the best separation of these compounds.

Both the pH 3.0 citrate buffer and acetonitrile were degassed, using helium, and then connected to the high-pressure pumps. The proportion of citrate buffer to acetonitrile, in the mobile-phase, was determined by the deci-linear gradient programmer. The following programme, with a flow rate of 3ml $minute^{-1}$, was found to give the best separation of phenolic compounds and phenol derivatives;

10% acetonitrile for 10 minutes
Increase acetonitrile at 1% $minute^{-1}$, for 10 minutes
Hold acetonitrile concentration constant for 10 minutes
Increase acetonitrile at 1% $minute^{-1}$, for 20 minutes
Hold acetonitrile concentration until run completed.

(final acetonitrile concentration 40%, run time 1 hour)

This programme was activated on injecting 20µl of the saponified sample onto the column. During the initial part of the programme the mobile-phase had a mainly polar character, and therefore, the first compounds eluted were the more water soluble compounds i.e. nitrophenols, which tended to be more water soluble compared with chlorophenols and phenoxyalkanoic acids. With increasing acetonitrile concentration the non-polar character of the mobile-phase increased causing the more hydrophobic chlorophenols and phenoxyalkanoic acids to elute. Fig. 2.2. shows a typical trace obtained from HPLC analysis of a water sample containing phenolic compounds and phenol derivatives.

A blank of double-distilled water was carried through the

the concentration and saponification stage and then injected onto the column.

2.5.v. Peak identification and quantification

Peaks were tentatively identified by comparing the retention time of compounds, in the samples, with known standards, under the same experimental conditions. In this way it was possible to identify fourteen compounds present in the water samples (see Appendix 1) and as can be seen from the trace (see Fig. 2.2.) several unidentified compounds were also present.

The concentration of the identified compound in the original water sample was calculated from the peak height, concentration of the water sample and the instrument's response factor to the different compounds, compared with 4-bromophenol. The response factor was measured by injecting 20µl of $100mgl^{-1}$ of test compound and 4-bromophenol, in the saponification mixture, onto the column using the programme shown in 2.5.iv.

This method had the following detection limits; $20\mu gl^{-1}$ nitrophenol, $100\mu gl^{-1}$ chlorophenol and $300\mu gl^{-1}$ phenoxyalkanoic acids.

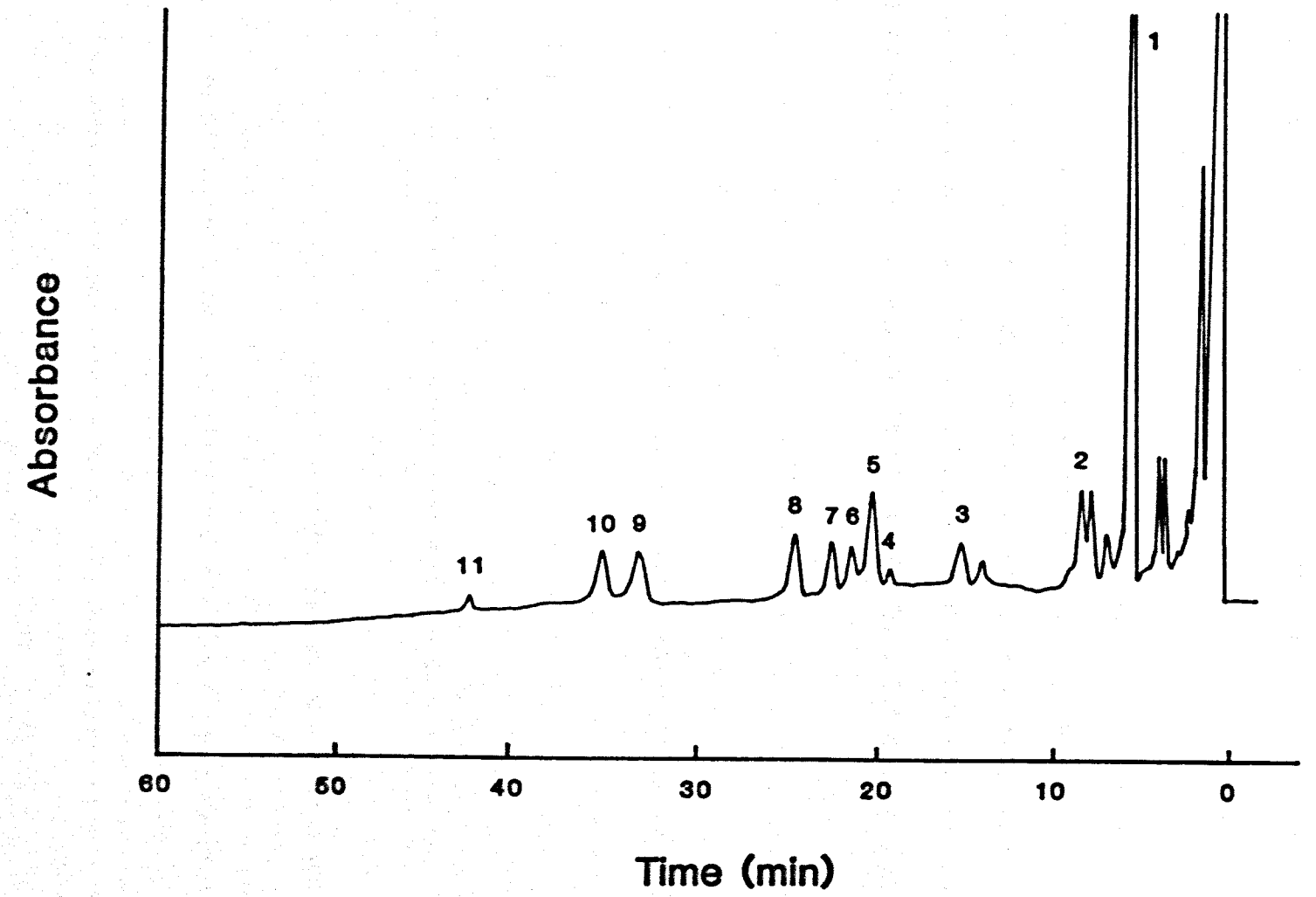
2.5.vi. Column performance

Between each sample injection, the column was cleaned by passing acetonitrile, followed by citrate buffer and finally 10% acetonitrile through the column for 5 - 10 minutes. However, with continued use the column performance decreased, which led to alterations in retention times of the compounds. Repacking of the top of the column was found to return normal performance, but for prolonged use a guard column is suggested.

Fig. 2.2. Typical trace obtained from analysis of a water sample containing phenolic compounds and phenol derivatives by high-performance liquid-chromatography (HPLC). The identified compounds present are (see Appendix 1 for chemical structures);

1. 2,4,6-trinitrophenol
2. 2,4-dinitrophenol
3. 4-bromophenol (internal standard)
4. 2-methyl-4,6-dinitrophenol
5. 2,4-dichlorophenoxyacetic acid
6. 4-chloro-2-methylphenol
7. 2-methyl-4-chlorophenoxyacetic acid
8. 2,4-dichlorophenol
9. 2-(2,4-dichlorophenoxy)-proprionic acid
10. 2-(2-methyl-4-chlorophenoxy)-proprionic acid
11. 4-(2-methyl-4-chlorophenoxy)-butyric acid

unlabelled peaks are unidentified compounds


Absorbance
1
2
3
4
5
6
7
8
9
10
11
60
50
40
30
20
10
0
Time (min)

2.6. Analysis of data

Statistical analysis of field data was carried out using SPSS; statistical package for the social sciences (Nie et al., 1975 and SPSS update, Hull and Nie, 1979) on computing facilities provided by the University of Hull computer centre.

Significant differences in bacteriological variables between sites and streams, were determined using the following parametric tests; t-test and oneway analysis of variance. Equality of variance was determined using an F-test (Sokal and Rohlf, 1969 p.186) for the t-test and by Bartlett's test (Sokal and Rohlf, 1969 p.370) for oneway analysis of variance. A $\log_{10}(x)$ or $\sqrt{x}$ (or $\log_{10}$ x+1, $\sqrt{x+0.5}$ if the data contained zero values) transformation was carried out on data with heteroscedastic variances, if this failed to give homogenous variances the following non-parametric tests were applied; Mann-Whitney U-test (Sokal and Rohlf, 1969 p.392) and the Kruskal-Wallis test (Sokal and Rohlf, 1969 p.388) in place of the t-test and oneway analysis of variance, respectively. When oneway analysis of variance showed significant differences between means, the Student-Newman-Keuls test (Sokal and Rholf, 1969 p.329) was used to compare bacteriological-variable means. Unfortunately no similar procedure was available to compare means which were significantly different by the Kruskal-Wallis test.

Relationships between the bacteriological and environmental variables were examined by calculating Pearson's product-moment correlation coefficients (r, using a two-tailed test of significance). The correlation coefficients indicated the degree to which variation in bacteriological variables was related to variation in environmental variables, although they did not necessarily demonstrate causal relationships. In order to calculate correlation coefficients, values of both variables must follow a normal distribution. The Kolmogorov-

Smirnov goodness of fit test (Sokal and Rohlf, 1969 p.571) was used to determine whether or not the values followed a normal distribution. Variables which were significantly different from a normal distribution ($p<0.05$) were transformed, usually by a $\log_{10}(x)$ transformation or a $\log_{10}(x+1)$ if the data contained zero values or occasionally a $\sqrt{x}$ transformation.

CHAPTER 3

THE EFFECT OF HEAVY METALS ON FRESHWATER BACTERIA

The effect of heavy metals on freshwater bacterial communities was studied in the River Aire, near Leeds, West Yorkshire.

3.1. Site description

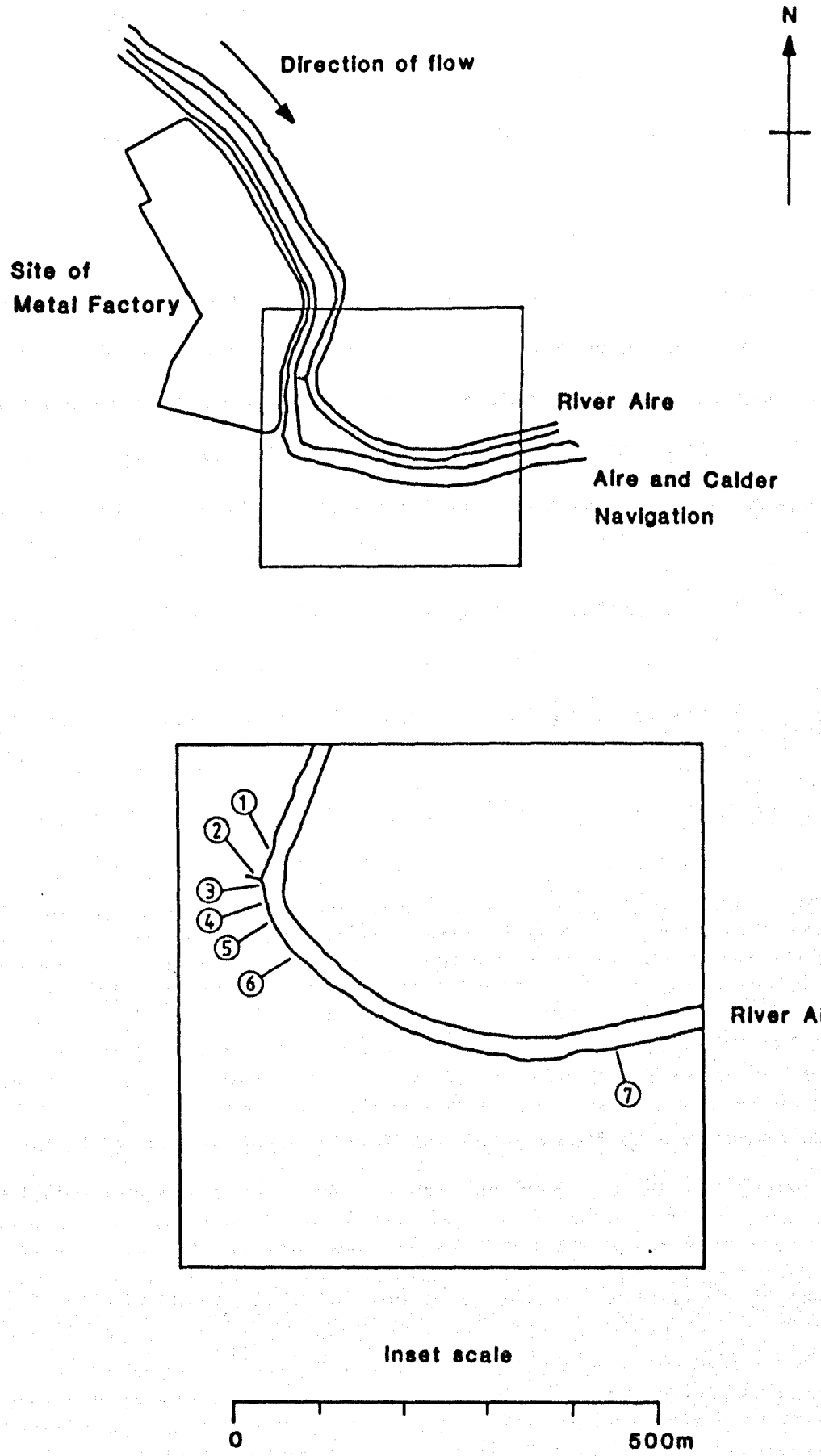
The River Aire rises in the Pennines near Malham, North Yorkshire and flows in a roughly South East direction for approximately 90Km, before joining the River Calder near Castleford, West Yorkshire. The geology of the catchment area (105,000 hectares) varies from Carboniferous limestone near the origin, through millstone grit to productive coal measures in the Leeds area. The lower part of the River Aire flows through industrial West Yorkshire and receives substantial quantities of treated sewage effluent, which originate from the processing of domestic and industrial wastes.

At Leeds, the River Aire had a mean daily flow rate of $20.3 m^3 s^{-1}$ (range $5.3 - 97.3 m^3 s^{-1}$) on the twelve 1981 sampling dates, and over the period 1981-1982 a mean bicarbonate alkalinity of 2.3 m equivalents l^{-1} ($n = 46$, range 1.1 - 3.0 m equivalents l^{-1}) - data supplied by the Yorkshire Water Authority.

The effects of heavy metals on freshwater bacteria were studied at Stourton in S.E. Leeds (SE 335 303), where the River Aire receives a metal-containing effluent, which apparently originates from the site of a non-ferrous metal factory. The effluent is carried by culvert under the nearby Aire and Calder navigation, into the River Aire. The main river was sampled at six points; one 30m upstream of the discharge; and then at various distances downstream: 5m, 25m, 50m, 100m and 500m (see Fig.3.1.). These sites represented partial (5m) to complete (500m) mixing of the effluent with river water.

Fig. 3.1. Position of sampling sites on the River Aire;

1. 30m upstream
2. Culvert
3. 5m downstream
4. 25m downstream
5. 50m downstream
6. 100m downstream
7. 500m downstream


N
Direction of flow
Site of
Metal Factory
River Aire
Aire and Calder
Navigation
1
2
3
4
5
6
7
River Aire
Inset scale
0
500m

3.2. Sampling programme

The River Aire was visited monthly for a period of one year (24 January to 17 December 1981). Surface water samples were collected from each site, 1m from the S.W. bank, and used for determination of the bacteriological and environmental variables described in 2.1., 2.2. and 2.4. Water for bacteriological analysis and determination of BOD was collected in sterile 300ml glass bottles; water for heavy metal analysis and determination of turbidity was collected in acid-washed 2 litre polythene containers. Both were rinsed with river water prior to filling. In addition a water sample was collected from the culvert to determine environmental variables in the effluent.

The artificial, acid-washed gravel-substratum was not successfully used in the River Aire, as many of the net bags were washed away during periods of high flow rates. In addition bacterial diversity, on CPS agar, was not determined.

3.3. Results and discussion

A summary of the results obtained from the River Aire sampling is shown in Tables 3.1.a and 3.1.b., the raw data are shown in Appendix 3. All of the bacteriological variables, at each of the sampling sites, had equal variances.

Analysis of the effluent and river water by DPASV (see 2.4) showed that copper, zinc, cadmium and lead were present in varying quantities (see Tables 3.1.b and 3.2.). Copper was the most abundant of the four metals in the effluent and at 5m and 25m downstream of the discharge, whereas zinc was the most abundant elsewhere. Metals were present in relatively high concentrations in the effluent from the culvert, with maximum values recorded of; Cu 3240μgl^{-1} (5.1×10^{-5}M), Zn 595μgl^{-1} (9.1×10^{-6}M), Cd 20μgl^{-1} (1.8×10^{-7}M) and Pb 281μgl^{-1} (1.4×10^{-6}M),

in addition other metals may be present. The metal concentrations immediately below the discharge were much higher than the concentrations upstream, however these high metal concentrations decreased rapidly with increasing distance downstream, presumably by dilution.

Bowen (1979) lists the following median concentrations of the four metals from various rivers as; copper $3\mu gl^{-1}$ (range 0.2 - 30), zinc $15\mu gl^{-1}$ (range 0.2 - 100), cadmium $0.1\mu gl^{-1}$ (range 0.01 - 3) and lead $3\mu gl^{-1}$ (range 0.06 - 120). The metal concentrations in the River Aire, even upstream of the discharge, were higher than these median values, which probably reflected the discharge of sewage effluents and other industrial wastes to the river.

Initially the effect of the metal-containing effluent on the bacterial community was examined by comparing bacteriological variables from the 5m downstream site with corresponding variables from the 30m upstream site. A t-test showed that there were no significant differences (at $p<0.05$) for any of the bacteriological variables (see Table 3.3.), although activity was significantly different at $p = 0.11$. The non-parametric Mann-Whitney U-test showed that there were no significant differences (at $p<0.05$) for any of the bacteriological variables, except that bacterial activity was significantly greater at the 30m upstream site compared with the 5m downstream site (Umin = 30.0 n_1, n_2 = 12,12 $p = 0.015$).

One way analysis of variance showed no significant differences ($p>0.05$) between the sampling sites on the River Aire, for any of the bacteriological variables (see Table 3.3.).

Clearly, there were few significant effects of metals on bacteriological variables in the River Aire, although bacterial activity was apparently the most sensitive bacteriological variable. However, the effect of the effluent on bacterial activity was restricted to the site immediately below the discharge. The lowest value for activity was

recorded at the 5m downstream site on nine out of the twelve sampling occasions (see raw data, Appendix 3). A plot (Fig. 3.2.) of the means of bacterial activity and total metal concentrations illustrates clearly the localised inhibition of activity at the 5m downstream site.

Heavy metals have been reported by other workers to inhibit bacterial heterotrophic activity and reduce viable counts. Albright et al. (1972) studied the effect of adding copper, zinc, cadmium and lead, separately, to bacteria in water samples from a freshwater environment. They found that exposure, prior to plating on nutrient agar, to concentrations of Cu^{2+} $10\mu gl^{-1}$, Zn^{2+} $100\mu gl^{-1}$, Cd^{2+} $100\mu gl^{-1}$, and Pb^{2+} $100\mu gl^{-1}$ had no effect on viable numbers of bacteria. However, there was a reduction in bacterial heterotrophic activity, as V_{max} was reduced and there was an increased turnover time of a ^{14}C glucose substrate. Mills and Colwell (1977) showed that the addition of cadmium or lead, at concentrations of $10mgl^{-1}$ and $100mgl^{-1}$ caused an inhibition of bacterial ^{14}C glucose oxidation in estuarine water and sediment samples. The presence of high levels of copper, zinc, cadmium and lead, in water samples from the Humber Estuary, were found by Goulder et al (1979) to reduce bacterial activity (f value determined, see 2.1.i.) and viable counts on agar plates. The maximum levels of the metals recorded were; Cu $63.5mgl^{-1}$, Zn $49.5mgl^{-1}$, Cd $7.5mgl^{-1}$ and Pb $2.2mgl^{-1}$.

In general, the levels of metals in the above studies were very much greater than the metal levels recorded in the River Aire, although toxic effects of heavy metals have been recorded at lower metal concentrations. Goulder et al. (1980) showed that the addition of $64\mu gl^{-1}$ copper to water samples, from the Humber Estuary, inhibited bacterial heterotrophic activity. The added copper caused a reduction in V_{max} values, as determined by mineralization of ^{14}C glucose, of between 35 - 41%, compared with control samples without added copper.

Zevenhuizen et al. (1979) showed that concentrations of copper, in the range 10^{-8} - 10^{-6} M Cu^{2+} ($\sim$ 0.6 - 60μgl^{-1}), inhibited the growth of Klebsiella aerogenes, Escherichia coli and an Alcaligenes sp. in liquid medium.

Other workers have shown that the presence of heavy metals in the aquatic environment may cause a destabilisation of the bacterial community. Singleton and Guthrie (1977) compared the effects of adding copper ($2mgl^{-1}$) to water samples from two aquatic systems, with untreated control samples. Over a 14 day incubation period, the treated samples had higher viable counts on agar plates, but a reduced number of different colony types. These differences in bacterial communities were explained by differences in bacterial-species sensitivities to heavy metals. Several workers have found that the sensitivity of bacteria to heavy metals varies. Zevenhuizen et al. (1979) showed that the growth of copper sensitive bacteria, such as Klebsiella aerogenes, Escherichia coli and an Alcaligenes sp. was inhibited at 10^{-8} - 10^{-6} M Cu^{2+} ($\sim$ 0.6 - 60$\mu gCu^{2+}l^{-1}$), whereas more resistant bacteria tolerated up to 10^{-3} M Cu^{2+} ($\sim$ 60$mgCu^{2+}l^{-1}$). Doyle et al. (1975) found that the growth of bacteria, in complex liquid-medium, was inhibited by the presence of cadmium. The growth of Lactobacillus acidophilus, Staphylococcus aureus and Streptococcus faecalis was inhibited at 40 and $80mgl^{-1}$ Cd, whereas Escherichia coli and Bacillus cereus grew well at these concentrations.

However, there was no evidence for the destabilisation of the bacterial community in the River Aire, by heavy metals in the effluent. There were no significant differences ($p > 0.05$) between sites for viable counts on CPS agar or PSM agar, or the number of chromogenic bacteria. These were variables which would be expected to change significantly if the heavy metals had caused a bacterial-species selection.

The toxicity of heavy metals to aquatic biota, including bacteria, is related to the concentration of the free ion (see Introduction, 1.2.iv).

Complexation of heavy metals with organic or inorganic ligands, and adsorption onto particulates, decreases the toxicity of the metal by reducing the free ion concentration. The River Aire results suggest that metals were present at the site immediately below the discharge in sufficient concentration, and with appropriate speciation to reduce bacterial activity, but not to affect other bacteriological variables.

The River Aire had a relatively high bicarbonate alkalinity and contained large amounts of sewage-derived organic material (note BODs, Table 3.1.b.), which would encourage the complexation and adsorption of copper, zinc, cadmium and lead (see Introduction, 1.2.iii.). Therefore, the overall absence of bacterial inhibition, in the River Aire, may be explained by metal complexation and adsorption, and hence a reduction in toxicity, together with a rapid decrease in metal concentration through dilution below the discharge.

Pearson's product-moment correlation coefficients were calculated, using combined data from all sites, to examine any relationships between bacteriological and environmental variables in the River Aire (see Table 3.4.). The following variables, from the combined River Aire sites, were found to follow a distribution which was significantly different ($p < 0.05$) from a normal distribution; bacterial activity, number of chromogenic bacteria, viable count on PSM agar, turbidity, flow rate and all the metal variables. A $\log_{10}(x)$ transformation of the values for these variables, prior to calculating correlation coefficients, normalised the data, except for bacterial activity which required a $\sqrt{x}$ transformation.

There was only one significant negative correlation between the bacteriological variables and metal variables. Bacterial activity was significantly negatively correlated with total copper concentration; $r = -0.31$, $n = 72$, $p = 0.008$. This suggests that copper may be the most toxic of the metals present in the effluent towards bacteria. When the

correlation coefficient was recalculated, omitting the 5m downstream data, there was no significant relationship ($r = 0.0029$, $n = 60$, $p = 0.983$); this emphasizes the local nature of any inhibition brought about by copper. Clearly, the general absence of significant negative correlations suggests that the heavy metals had little or no toxic effect on bacteria in the River Aire.

There were significant positive correlations between viable counts on CPS agar and the number of chromogenic bacteria with total cadmium and lead levels. These correlations were thought to reflect relationships in common with other environmental variables, rather than a stimulatory effect by these metals.

All of the bacteriological variables in the River Aire were significantly positively correlated with BOD. As BOD was a measure of the quantity of organic material in the river, a positive relationship with bacteriological variables was expected. Similar correlations between bacteriological variables and indicators of organic content have been shown by other workers. Geesey and Costerton (1979) found that the total number of bacteria, as measured by AODC (see 2.1.ii.), was positively correlated ($r = 0.86$, $p < 0.05$) with total organic carbon concentrations in a Canadian river. Nuttall (1982a) showed that both heterotrophic activity (V_{max} determined by ^{14}C acetic acid uptake) and the viable bacterial count on CPS agar, were higher at a polluted site on the River Dee, compared with a relatively unpolluted site on the same river. Albright and Wentworth (1973) found a positive correlation between heterotrophic activity (V_{max} determined by uptake and mineralization of ^{14}C glucose) with soluble carbon ($r = 0.90$), particulate carbon ($r = 0.78$) and BOD ($r = 0.51$) in five Canadian rivers.

Table 3.5 compares the viable and total bacterial counts in the River Aire with those of other U.K. rivers. This table shows that viable and total bacterial counts in the River Aire were similar to counts

obtained by Nuttall (1982a) from a polluted site on the Welsh River Dee. Viable bacterial counts in the River Aire were much higher than in cleaner rivers e.g. River Hull (Goulder, 1980), River Frome and Tadnoll Brook (Baker and Farr, 1977). There was, however, less variation in total bacterial counts between polluted and unpolluted waters.

All of the bacteriological variables in the River Aire, with the exception of total bacterial count, were significantly positively correlated with turbidity. As aquatic bacteria can utilise suspended organic material as a source of nutrients a positive relationship with turbidity was not surprising. Baker and Farr (1977) found that viable counts, on CPS agar, from the River Frome and Tadnoll Brook were very highly correlated with suspended solids ($p < 0.001$) over a two year sampling period. Nuttall (1982b) showed that bacterial heterotrophic activity, as determined by uptake of ^{14}C acetic acid, was significantly positively correlated with turbidity at a relatively unpolluted site on the Welsh River Dee. However, there was no significant correlation with turbidity at two polluted sites on the same river.

There was no consistent effect of flow rate on bacteriological variables in the River Aire; there were significant positive correlations with bacterial activity and viable count on PSM agar and a significant negative correlation with bacterial total count. Nuttall (1982b) showed a positive relationship between flow rate and environmental variables representing particulate and organic matter in the River Dee. Therefore, a positive relationship between flow rate and bacteriological variables would be expected. Baker and Farr (1977) found a positive correlation between discharge and the numbers of viable bacterial counts on CPS agar, in the River Frome and Tadnoll Brook. However, other workers have shown negative relationships between bacteriological variables and flow rate. Daubner (1969) reported a negative relationship between flow rate, in the polluted River Danube, and viable counts on agar plates. Goulder (1980)

showed a negative correlation between total bacterial counts, as determined by AODC (see 2.1.ii.), and discharge at a site on the River Hull. Both workers explained these negative relationships as a dilution effect.

Significant correlations were found between total bacterial counts with pH and dissolved oxygen concentration; bacterial total counts were positively correlated with pH and negatively correlated with dissolved oxygen. As it is probable that neither pH nor dissolved oxygen concentrations were limiting in the River Aire, the ecological significance of these relationships was unclear. Similarly, the cause of the correlations of conductivity with bacterial activity and total bacterial count was unknown. Temperature did not appear to affect any of the bacteriological variables in the River Aire.

In all, the results suggest that BOD, turbidity and to a lesser extent flow rate were amongst the most important environmental variables affecting bacteria in the River Aire, and that the metal containing effluent caused little or no inhibition of self-purification.

Table 3.1.a. River Aire, January to December 1981; summary of bacteriological variables.

Site: 30m upstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	29.0	10.2-88.8	12	84.0
Total count x $10^{-6}ml^{-1}$	5.9	2.0-14.7	12	56.7
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	5.7	0.7-12.1	11	64.3
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	13.3	0.6-34.9	11	88.2
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	5.2	1.1-15.6	12	82.2

Site: 5m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	14.3	0.5-63.0	12	134.3
Total count x $10^{-6}ml^{-1}$	5.8	2.1-13.4	12	56.1
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	6.1	0.3-13.2	11	73.9
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	13.2	0.6-29.3	11	75.5
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	3.2	0.6-10.1	12	82.5

Site: 25m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	28.5	1.0-80.6	12	81.3
Total count x $10^{-6}ml^{-1}$	5.7	2.0-12.1	12	51.4
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	5.6	0.7-13.0	11	80.2
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	13.3	0.6-45.9	11	106.8
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	4.2	1.2-12.6	12	84.5

Table 3.1.a. (continued)

Site: 50m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	29.7	0.9-87.0	12	90.2
Total count x $10^{-6}ml^{-1}$	5.6	2.0-11.2	12	49.8
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	6.3	0.9-16.9	11	91.8
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	18.2	1.4-64.2	11	115.5
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	4.6	0.5-15.9	12	102.5

Site: 100m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	27.2	0.3-70.1	12	75.2
Total count x $10^{-6}ml^{-1}$	5.6	2.1-12.6	12	52.8
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	6.7	0.4-17.7	11	77.3
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	18.6	0.4-59.3	11	99.1
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	4.5	0.2-19.6	12	119.3

Site: 500m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}h^{-1}$	30.9	1.1-77.6	12	84.8
Total count x $10^{-6}ml^{-1}$	5.9	1.9-12.3	12	51.5
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	6.2	1.0-21.2	11	95.4
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	15.0	0.8-61.7	11	119.6
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	4.7	0.3-19.2	11	121.4

Table 3.1.b. River Aire, January to December 1981; summary of environmental variables and metal concentrations.

Site: 30m upstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.6	7.1-8.1	12	4.1
Temperature (^{o}C)	15.1	8.0-25.0	12	42.2
Dissolved oxygen (mgl^{-1})	9.9	7.8-12.4	12	14.4
BOD (mg oxygen l^{-1})	14.9	4.3-28.5	8	57.1
Conductivity (μScm^{-1})	526	272-715	11	28.8
Turbidity (Arbitrary units)	10	4-35	12	83.8
Flow rate (m^3s^{-1})*	20.3	5.3-97.3	12	142.5
Total copper (μgl^{-1})	23.9	8.3-63.9	12	64.2
Total zinc (μgl^{-1})	48.7	15.5-119	12	66.5
Total cadmium (μgl^{-1})	1.2	0.3-4.0	12	80.1
Total lead (μgl^{-1})	15.2	4.8-32.1	12	53.8
Total metal concentration (μgl^{-1})	90.0	43.2-183	12	52.8

Site: Water sample taken from culvert

Variable	Mean	Range	n	c.v.(%)
pH	7.2	6.0-7.8	12	6.7
Temperature (^{o}C)	15.0	6.5-21.0	12	35.9
Dissolved oxygen (mgl^{-1})	7.8	5.2-9.7	12	20.0
BOD (mg oxygen l^{-1})	16.4	9.5-28.2	6	46.7
Conductivity (μScm^{-1})	996	686-1430	11	20.4
Turbidity (Arbitrary units)	35	9-85	11	57.6
Total copper (μgl^{-1})	988	169-3236	12	92.3
Total zinc (μgl^{-1})	278	98.1-595	12	48.8
Total cadmium (μgl^{-1})	10.8	3.3-20.0	12	48.3
Total lead (μgl^{-1})	111	54.7-281	12	61.6
Total metal concentration (μgl^{-1})	1390	338-3740	12	139.1

*Flow rate data for the River Aire was provided by the Yorkshire Water Authority, gauged at Armley, Leeds.

Table 3.1.b. (continued)

Site: 5m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.3	6.4-7.8	12	5.7
Temperature (^{o}C)	14.8	8.0-22.0	12	41.0
Dissolved oxygen (mgl^{-1})	9.0	6.3-11.5	12	19.6
BOD (mg oxygen l^{-1})	15.9	5.9-26.5	9	40.5
Conductivity (μScm^{-1})	719	358-1144	11	29.5
Turbidity (Arbitrary units)	20	6-38	12	45.9
Total copper (μgl^{-1})	324	36.2-921	12	87.8
Total zinc (μgl^{-1})	129	46.7-219	12	36.1
Total cadmium (μgl^{-1})	6.4	1.8-15.9	12	57.2
Total lead (μgl^{-1})	66.8	19.9-159	12	61.2
Total metal concentration (μgl^{-1})	527	148-1260	12	60.8

Site: 25m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.6	7.2-7.9	12	3.1
Temperature (^{o}C)	15.2	8.0-23.0	12	42.2
Dissolved oxygen (mgl^{-1})	9.8	8.0-12.2	12	13.4
BOD (mg oxygen l^{-1})	12.6	6.5-25.8	8	50.8
Conductivity (μScm^{-1})	543	257-701	11	28.0
Turbidity (Arbitrary units)	12	4-36	12	69.4
Total copper (μgl^{-1})	55.3	13.4-175	12	89.9
Total zinc (μgl^{-1})	50.4	30.0-93.9	12	41.3
Total cadmium (μgl^{-1})	2.3	0.2-5.3	12	75.2
Total lead (μgl^{-1})	27.3	8.1-73.1	12	84.5
Total metal concentration (μgl^{-1})	135	60.1-272	12	56.9

Table 3.1.b. (continued)

Site: 50m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.6	7.2-8.0	12	3.6
Temperature (^{o}C)	15.2	8.0-23.0	12	41.9
Dissolved oxygen (mgl^{-1})	9.8	7.8-12.6	12	15.3
BOD (mg oxygen l^{-1})	13.3	5.5.-22.5	9	44.0
Conductivity (μScm^{-1})	527	243-657	11	27.7
Turbidity (Arbitrary units)	12	5-35	12	72.7
Total copper (μgl^{-1})	41.7	13.6-118	12	67.9
Total zinc (μgl^{-1})	47.5	14.9-85.5	12	49.4
Total cadmium (μgl^{-1})	1.0	0.3-3.2	12	91.4
Total lead (μgl^{-1})	19.3	5.3-70.3	12	89.9
Total metal concentration (μgl^{-1})	109	34.3-208	12	50.2

Site: 100m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.6	7.2-8.0	12	3.8
Temperature (^{o}C)	15.3	8.0-23.0	12	42.0
Dissolved oxygen (mgl^{-1})	9.9	8.0-12.2	12	14.0
BOD (mg oxygen l^{-1})	12.7	6.0-23.1	8	57.3
Conductivity (μScm^{-1})	517	243-645	11	27.0
Turbidity (Arbitrary units)	11	5-34	12	70.7
Total copper (μgl^{-1})	34.9	12.5-78.5	12	64.3
Total zinc (μgl^{-1})	41.9	21.5-92.3	12	53.2
Total cadmium (μgl^{-1})	0.8	0.2-1.6	12	53.5
Total lead (μgl^{-1})	15.8	6.5-38.7	12	54.9
Total metal concentration (μgl^{-1})	93.4	46.6-150	12	41.2

Table 3.1.b. (continued)

Site: 500m downstream of discharge

Variable	Mean	Range	n	c.v.(%)
pH	7.6	7.2-8.0	12	3.8
Temperature (^{o}C)	15.2	8.0-23.0	12	41.7
Dissolved oxygen (mgl^{-1})	9.9	8.0-12.2	12	13.9
BOD (mg oxygen l^{-1})	14.3	5.4-25.5	9	53.0
Conductivity (μScm^{-1})	514	243-644	11	27.1
Turbidity (Arbitrary units)	11	3-40	12	90.6
Total copper (μgl^{-1})	23.9	8.5-73.2	12	76.8
Total zinc (μgl^{-1})	44.5	10.4-84.7	12	51.5
Total cadmium (μgl^{-1})	0.6	0.1-1.1	12	62.2
Total lead (μgl^{-1})	13.5	5.3-25.9	12	46.1
Total metal concentration (μgl^{-1})	82.5	34.2-127	12	39.4

Table 3.2. Summary of mean metal molarities at the River Aire sampling sites, January to December 1981, all values are 10^{-8}M.

Distance from discharge	Copper	Zinc	Cadmium	Lead
30m upstream	38	75	1	7
Culvert	1553	426	10	54
5m downstream	510	198	6	32
25m downstream	87	77	2	13
50m downstream	66	73	1	9
100m downstream	55	64	1	8
500m downstream	38	68	1	7

Table 3.3. Summary of t-values from t-test of bacteriological variables between 30m upstream site and 5m downstream site, and summary of variance ratios from oneway analysis of variance (NS=non-significant)

t-values

Variable	t-value	n	probability	
Activity	1.65	22	0.11	NS
Total Count	0.12	22	0.90	NS
Viable count on CPS agar	0.21	20	0.84	NS
Number of chromogenic bacteria	0.02	20	0.99	NS
Viable count on PSM agar	1.38	22	0.18	NS

Variance ratios

Variable	F-value	V_1, V_2	probability	
Activity	0.824	5,66	0.54	NS
Total count	0.026	5,66	0.99	NS
Viable count on CPS agar	0.074	5,60	0.99	NS
Number of chromogenic bacteria	0.271	5,60	0.93	NS
Viable count on PSM agar	0.270	5,65	0.93	NS

Table 3.4. Relationships between bacteriological and environmental variables in the River Aire (24 January-17 December 1981). Values are Pearson's product-moment correlation coefficients (r), a minus sign indicates a negative correlation, NS = non-significant ($p>0.05$), figures in brackets refer to significance level (1) = $p<0.05$, (2) = $p<0.01$. (n ranged from 60-72, except for BOD's where n ranged from 45-51).

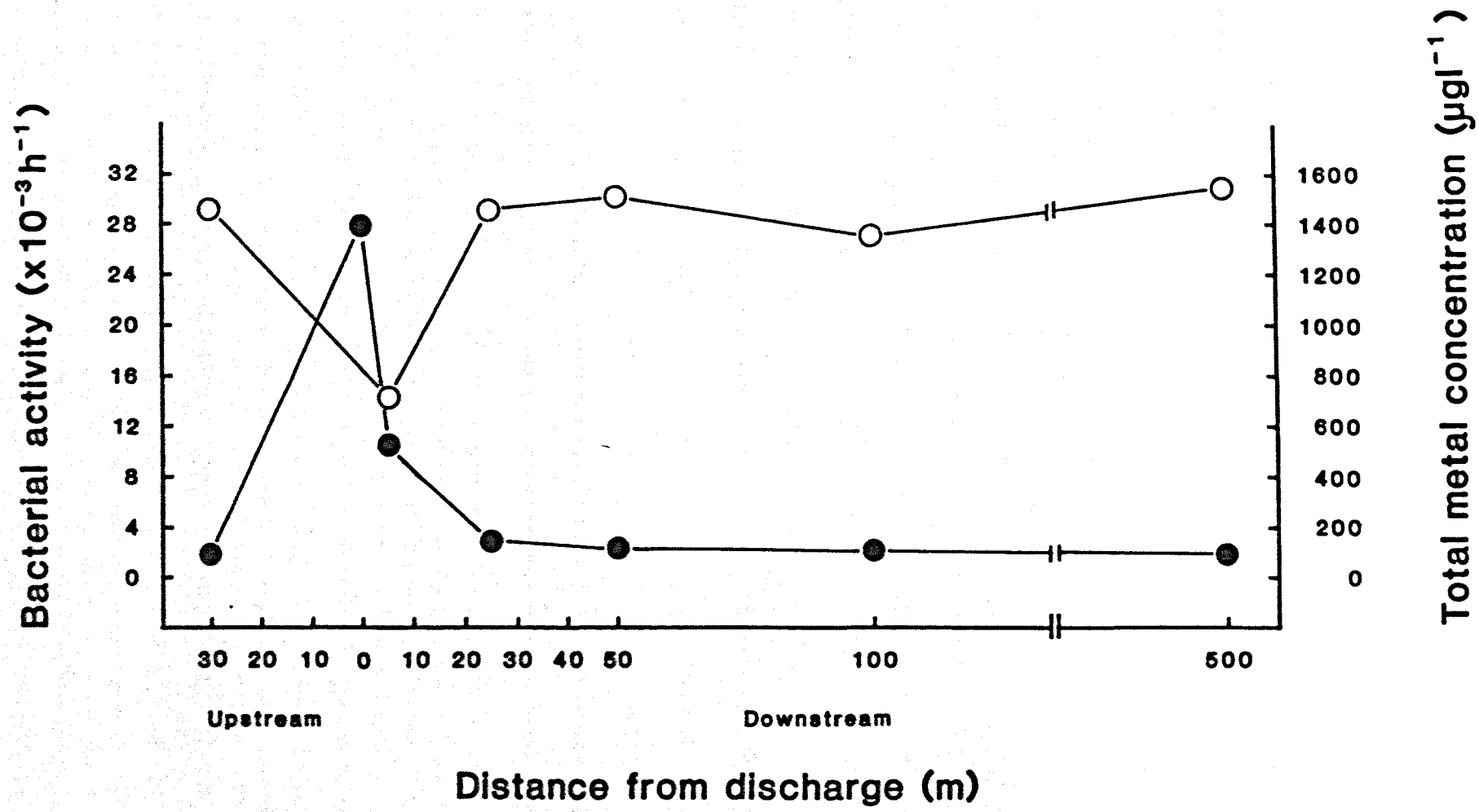
Environmental variables and metal variables	Activity	Total count	Viable count on CPS agar	Number of chromogenic bacteria	Viable count on PSM agar
pH	NS	0.31(2)	NS	NS	NS
Temperature	NS	NS	NS	NS	NS
Dissolved oxygen	NS	-0.41(3)	NS	NS	NS
BOD	0.39(2)	0.38(2)	0.34(1)	0.31(1)	0.45(2)
Conductivity	-0.39(2)	0.29(1)	NS	NS	NS
Turbidity	0.44(3)	NS	0.29(1)	0.31(1)	0.46(3)
Flow rate	0.54(3)	-0.33(2)	NS	NS	0.33(2)
Total copper	-0.31(2)	NS	NS	NS	NS
Total zinc	NS	NS	NS	NS	NS
Total cadmium	NS	NS	0.25(1)	0.36(2)	NS
Total lead	NS	NS	0.25(1)	0.32(2)	NS
Total metal concentration	NS	NS	NS	NS	NS

Table 3.5. Viable and total counts of bacteria from various U.K. rivers

River	Viable count ($x10^{-4}$ CFU ml^{-1})		Total count ($x10^{6}$ ml^{-1})	
	Mean	Range	Mean	Range
Aire	61.0	2.9-212.0	5.8	1.9-14.7
Dee[1], polluted site	20.0	1-100	-	1.5-5.0
unpolluted site	1.2	0.1-10	-	0.5-1.5
Hull[2], Wansford	-	0.4-12.8	-	0.3-2.9
Bethells Bridge	-	1.2-24.2	-	0.3-4.4
Frome[3]	5.2	1-40	-	-
Tadnoll Brook[3]	2.9	0.5-30	-	-

1. Nuttall, 1982 a.
2. Goulder, 1980.
3. Baker and Farr, 1977.

Fig. 3.2. River Aire, January to December 1981; variation of mean bacterial activity (O) and the mean total metal concentration (●).


Bacterial activity (x10⁻³h⁻¹)
32
28
24
20
16
12
8
4
0
1600
1400
1200
1000
800
600
400
200
0
Total metal concentration (µgl⁻¹)
30 20 10 0 10 20 30 40 50 100 500
Upstream
Downstream
Distance from discharge (m)

CHAPTER 4

THE EFFECTS OF PHENOLIC COMPOUNDS AND PHENOL DERIVATIVES ON FRESHWATER BACTERIA

The effects of phenolic compounds and phenol derivatives on freshwater bacterial communities were studied in Sugden Beck, near Cleckheaton, West Yorkshire.

4.1. Site description (see Fig.4.1.)

Sugden Beck is a small stream rising from coal measures near Wyke (SE 162 273). This beck flows in an easterly direction, partly in culvert, for approximately 2.5Km, where it then joins the larger Hunsworth Beck. Near the origin, Sugden Beck receives contaminated groundwater originating from a manufacturer of agricultural herbicides and other phenolic compounds. These phenolic compounds and phenol derivatives are leached from old mine workings beneath the site of the present factory, and give Sugden Beck water a distinct yellow appearance and phenolic odour.

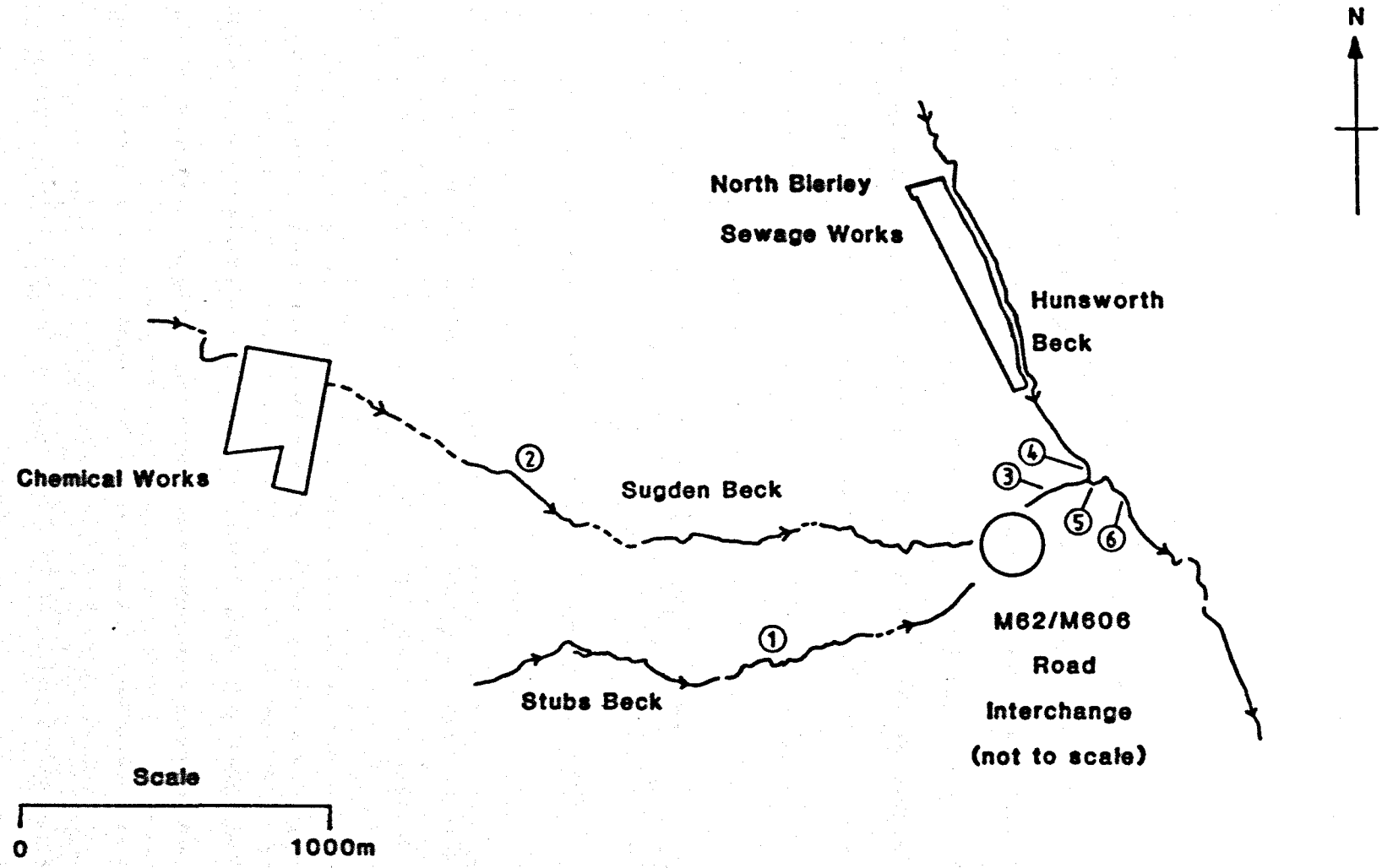
Stubs Beck is located about 500m to the South of Sugden Beck. Stubs Beck is a small, clean stream similar in many ways to Sugden Beck, except that it does not receive any man-made phenolic compounds or phenol derivatives. Throughout the sampling programme and subsequent experiments, Stubs Beck was used as a control stream. Stubs Beck joins Sugden Beck underneath the nearby M62/M606 road interchange.

Hunsworth Beck is a much larger stream, which flows in a south-easterly direction, eventually forming Spen River, which joins the River Calder near Ravensthorpe (SE 231 204). Approximately 500m upstream of the confluence with Sugden Beck, Hunsworth Beck receives sewage effluent from North Bierley sewage works.

There were two sampling stations on Sugden Beck, one about 500m downstream of the chemical works (Sugden Beck Upper) and one 40m before

Fig. 4.1. Position of Sugden Beck/Hunsworth Beck sampling sites. Broken lines represent underground culvert.

1. Stubs Beck sampling site
2. Sugden Beck Upper
3. Sugden Beck Lower
4. Hunsworth Beck 17m upstream of Sugden Beck confluence
5. Hunsworth Beck 25m downstream of Sugden Beck confluence
6. Hunsworth Beck 110m downstream of Sugden Beck confluence.


N
North Bierley
Sewage Works
Hunsworth
Beck
Chemical Works
②
Sugden Beck
④
③
⑤
⑥
①
Stubs Beck
M62/M606
Road
Interchange
(not to scale)
Scale
0
1000m

the stream joins Hunsworth Beck (Sugden Beck Lower). Stubs Beck was sampled about 500m before it joined Sugden Beck. Hunsworth Beck was sampled 17m upstream of the confluence of Sugden Beck and then 25m and 110m downstream.

4.2. Sampling programme

The Sugden Beck site was visited monthly for a period of thirteen months (6 April 1981 to 6 April 1982). Surface water samples were collected from mid-stream in Sugden Beck and Stubs Beck and at a distance of 1m from the bank in Hunsworth Beck. Water for bacteriological analysis and analysis of phenolic compounds and phenol derivatives was collected in separate, sterile 300ml glass bottles. Both were rinsed with stream-water prior to filling. Polyester-mesh bags containing gravel, which had been on the stream bed for one month, were removed from each site and returned to the laboratory in sterile polythene bags.

The water and gravel samples were then used to determine the bacteriological and environmental variables outlined in 2.1., 2.2., 2.3. and 2.5. An estimate of the flow-rate was made 60m upstream of the Sugden Beck Lower sampling site, as here the stream passed through an open culvert, which had a uniform width. By measuring the length of time taken for a body of water to travel 5m and the average depth of water, it was possible to estimate flow-rate in m^3s^{-1}. In addition the level of Hunsworth Beck was measured at a convenient gauging-point between the 25m and 110m downstream sites. Stubs Beck was found to be dry on two occasions (9 July 1981 and 9 September 1981) during the sampling programme.

4.3. Results and discussion

4.3.i. Effect of phenolic compounds in Sugden Beck and comparison with Stubs Beck

A summary of the results obtained from the Sugden Beck sites and

Stubs Beck site are shown in Table 4.1.a. and 4.1.b., the raw data are shown in Appendix 4.

Analysis of Sugden Beck water by HPLC (see 2.5.) showed that a total of fourteen, identifiable, phenolic compounds and phenol derivatives were present, in addition several unidentified compounds were present. The fourteen identified compounds may be classified in the following groups (see Appendix 1 for chemical structures);

Chlorophenols (abbreviations used in brackets)

3-chlorophenol (3CHLR)

4-chlorophenol (4CHLR)

2,4-dichlorophenol (2,4-DCP)

4-chloro-2-methyl-phenol (PCOC)

Nitrophenols

2,4-dinitrophenol (2,4-DNP)

2,4,6-trinitrophenol (picric acid)

2-methyl-4,6-dinitrophenol (DNOC)

2-sec-butyl-4,6-dinitrophenol (DNBP)

Phenoxyalkanoic acids

2,4-dichlorophenoxyacetic acid (2,4D)

2-methyl-4-chlorophenoxyacetic acid (MCPA)

2-(2,4-dichlorophenoxy)-proprionic acid (2,4DP)

2-(2-methyl-4-chlorophenoxy)-proprionic acid (CMPP)

4-(2,4-dichlorophenoxy)-butyric acid (2,4DB)

4-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB)

The levels of the above compounds were usually less at the Sugden Beck Lower site compared with the Sugden Beck Upper sites, which reflected dilution from the input of Stubs Beck between the Sugden Beck sites. No phenolic compounds or phenol derivatives were detected in Stubs Beck.

The means of all the bacteriological variables and the sum of all the identified phenolic compounds and phenol derivatives are plotted as histograms in Fig. 4.2., to illustrate the differences between sites.

The following untransformed bacteriological variables had equal variances; water total counts, gravel activity and gravel bacterial diversity. The following transformed variables had equal variances; $\log_{10}$ (x) of water bacterial activity and the square root of the gravel viable counts. The remaining water and gravel bacteriological variables were heteroscedastic, even after transformation. Table 4.2. shows a summary of the variance ratios, from oneway analysis of variance, together with corrected H values from the Kruskal-Wallis test.

There were similar significant differences between sites (determined using the Student-Newman-Keuls test, see 2.6.), for bacteriological variables, in both water and gravel samples. Bacterial activity was significantly greater at the Stubs Beck and Sugden Beck Lower sites than at Sugden Beck Upper, although there was no significant difference between the Stubs Beck and Sugden Beck Lower sites.

There were no significant differences between sites for bacterial total and viable counts, except that the viable count in Sugden Beck Lower gravel samples was significantly greater than the viable count at Sugden Beck Upper. The highest bacterial total and viable counts were found at the Sugden Beck Lower site.

In common with the bacterial total and viable counts the highest number of chromogenic bacteria were found at the Sugden Beck Lower site. There were significant differences between the sites for the number of chromogenic bacteria using the Kruskal-Wallis test. The use of the Student-Newman-Keuls test (see 2.6.) on the heteroscedastic data, although not strictly correct, suggested that the number of chromogenic bacteria in gravel samples was significantly greater at Sugden Beck Lower compared to Stubs Beck and Sugden Beck Upper. There were no suggested differences between the sites for the number of chromogenic bacteria in water samples. Table 4.3. illustrates the differences between

sites, when the number of chromogenic bacteria were expressed as a percentage of the total viable count on CPS agar. This table showed that Stubs Beck had the highest percentage of chromogenic bacteria, in both water and gravel samples, compared to the Sugden Beck sites.

There were significant differences between sites, for bacterial diversity, which was greatest at the Stubs Beck site. For gravel samples, bacterial diversity was significantly greater at Stubs Beck, compared with either Sugden Beck Upper or Sugden Beck Lower, although there was no significant difference between the Sugden Beck sites. Similar differences in bacterial diversity were suggested, when the Student-Newman-Keuls test was applied to the heteroscedastic, water sample data.

Finally, there were no significant differences between the sites, for viable counts on PSM agar, although very much increased counts were obtained at both the Sugden Beck sites compared with the Stubs Beck site. The absence of significant differences may have resulted from the ranking procedure used in the Kruskal-Wallis test, as several zero counts were recorded at the Sugden Beck sites.

Comparison of the values of the bacteriological variables at Stubs Beck, with corresponding values at the Sugden Beck sites, showed major differences in water and gravel bacterial communities. Compared with Stubs Beck, the Sugden Beck sites had reduced bacterial diversity, a lower percentage of chromogenic bacteria and very much increased counts on PSM agar. The decrease in percentage chromogenic bacteria, together with an increase in the viable counts on PSM agar, represent changes in composition of the bacterial communities. Clearly, environmental factors in Sugden Beck caused a selection of bacterial species resulting in an overall reduction in bacterial species diversity.

The percentage of chromogenic bacteria has been used, by other workers, to study changes in aquatic bacterial communities. Guthrie et al.

(1974) showed, in two American lakes, that the percentage of chromogenic bacteria was reduced in water samples from a lake receiving municipal and industrial waste, compared with an unpolluted lake. In the polluted lake, the percentage of chromogenic bacteria was 2 - 12% (mean 7%) of the total viable count, on standard plate count agar, whereas in the unpolluted lake the percentage of chromogenic bacteria varied from 15 - 71% (mean 44%) of the total viable count. Cherry et al. (1977) studied the recovery of a bacterial community, in a stream, following chemical pollution with terchlorethylene, trichlorethylene and methanol. They showed that, although the number of colony forming units and colony diversity, in water samples, were similar to a nearby control stream, the percentage of chromogenic bacteria was much lower. In the polluted stream the chromogenic bacteria comprised 15% of the total viable count compared to 28% in the control stream, which did not receive any chemical pollution. As recovery of the bacterial community progressed, the percentage of chromogenic bacteria was found to increase.

Pearson's product-moment correlation coefficients were calculated to examine any relationships between bacteriological and environmental variables (see Table 4.4.a. and 4.4.b.). In the case of Sugden Beck, data from both sites were combined. The following bacteriological variables, from the combined Sugden Beck sites, were significantly different from a normal distribution ($p<0.05$); water activity, number of chromogenic bacteria in water and gravel samples and the viable count on PSM agar. A $\log_{10}(x)$ transformation of the values for these variables, except for the viable counts on PSM agar, normalised the data, prior to calculating correlation coefficients. The viable count on PSM agar required a $\sqrt{x + 0.5}$ transformation, as the values of this variable included a number of zero counts. All of the other bacteriological and environmental variables were not significantly different from a normal

distribution ($p > 0.05$), and therefore, did not require transformation.

Possibly the single, most important, environmental factor controlling bacteriological variables in Sugden Beck was pH (see Table 4.4.a.). All of the bacteriological variables, with the exception of bacterial diversity in both water and gravel samples, were significantly positively correlated with pH. Bacterial diversity, in Sugden Beck, was not significantly correlated with pH, and tended to remain low under all pH conditions.

Table 4.1.b. showed that the pH, at the Sugden Beck sites, ranged from 2.7 - 7.6, which was much wider than the optimum pH range for aquatic bacteria, given by Rheinheimer (1974) as 6.5 - 8.5. Clearly, over such a wide range of environmental pH values, pH would be expected to have a marked positive effect on bacteriological variables.

The positive effect of pH, in Sugden Beck, was readily illustrated by comparing the means of bacteriological variables under neutral and acidic pH conditions. Table 4.5. shows the mean values of bacterial activity and viable counts, at Sugden Beck Upper, under conditions of neutral and acidic pH. This table showed that under neutral pH conditions, a larger and more active bacterial community was present in both water and gravel samples. Fig. 4.3. shows the dependence of these bacteriological variables on pH, at the Sugden Beck Upper site, during the sampling programme.

There were significant correlations between bacteriological variables and phenolic compounds and phenol derivatives in Sugden Beck. All of the bacteriological variables, except for total counts and bacterial diversity in water and gravel samples, were significantly negatively correlated with the total amount of phenolic compounds and phenol derivatives. These negative correlations suggested a toxic effect of the phenolic compounds and phenol derivatives on bacteria, which was dependent upon concentration. The total counts of bacteria in Sugden Beck were not

significantly correlated with the total amount of phenolic compounds and phenol derivatives. This was not surprising, as the acridine orange direct count method (AODC, see 2.1.ii.) did not differentiate between living and dead bacteria.

There were no significant correlations between bacterial diversity, in water and gravel samples, with the total amount of phenolic compounds and phenol derivatives present in Sugden Beck. The bacterial diversity, however, was significantly lower at the Sugden Beck sites compared to the Stubs Beck site, which contained no phenolic compounds or phenol derivatives. Clearly, the presence of these compounds, in Sugden Beck, caused a selection of a few resistant bacterial species, which were not affected by the concentration of phenolic compounds.

Fewer negative correlations were found between bacteriological variables and the total quantity of phenoxyalkanoic acids, than either chlorophenols or nitrophenols. Phenoxyalkanoic acid compounds were, however, present in higher concentrations than either chlorophenols or nitrophenols, suggesting that chlorophenols and nitrophenols were relatively more toxic to bacteria than were phenoxyalkanoic acids.

There were significant negative correlations between many of the bacteriological variables at the Sugden Beck sites with conductivity and BOD. These negative relationships involved the interaction of several environmental factors. Water which had a high conductivity and BOD usually contained high levels of phenolic compounds and phenol derivatives, which in turn, coincided with conditions of low pH and hence, reduced levels of bacteriological variables. Significant negative correlations were also found between temperature and the number of chromogenic bacteria, at the Sugden Beck sites, although the ecological significance of this relationship was unclear.

There were significant positive correlations, in Sugden Beck,

between certain bacteriological variables and the estimated flow rate. The effect of flow rate was probably due to its interrelationship with pH, as pH was more likely to be neutral when the flow rate was high. The calculated correlation coefficient between flow rate and pH was positive, although not significant ($r = 0.51$, $n = 14$, $p = 0.065$).

There were few significant correlations between bacteriological and environmental variables in Stubs Beck (see Table 4.4.b.). The number of chromogenic bacteria in water and the total count of bacteria from gravel samples were positively correlated with BOD. In water samples bacterial activity was positively correlated with turbidity and the viable count on PSM agar was positively correlated with the estimated flow rate in Sugden Beck. This environmental variable was used as a measure of the flow rate in Stubs Beck, as both streams arose in the same catchment area.

Both negative and positive correlations were found with pH in gravel samples from Stubs Beck; there was a positive correlation with bacterial activity and a negative correlation with total count. The ecological significance of these relationships was unclear, since the pH values were never extreme.

Table 4.1.a. Summary of bacteriological variables from Stubs Beck and Sugden Beck sampling sites, April 1981 to April 1982.

Site: Stubs Beck

Variable	Mean	Range	n	c.v. (%)
Water				
Activity x $10^{4}h^{-1}$	22.6	4.2-74.2	11	97.8
Total count x $10^{-5}ml^{-1}$	17.3	5.4-48.1	11	83.9
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	6.0	1.2-14.8	11	66.4
Number of chromogenic bacteria x $10^{-3}CFUml^{-1}$	15.6	2.5-29.7	11	60.1
Bacterial diversity	32.9	17.1-48.9	8	35.8
Viable count on PSM agar $CFUml^{-1}$	97.2	0-417	11	119.1
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	14.9	9.8-21.7	6	27.2
Total count x $10^{-7}g^{-1}$	2.5	1.6-2.9	6	20.2
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	9.0	1.3-17.7	6	62.6
Number of chromogenic bacteria x $10^{-5}CFUg^{-1}$	29.4	5.6-77.1	6	84.1
Bacterial diversity	10.4	7.4-15.8	5	32.3

Table 4.1.a. (continued)

Site: Sugden Beck Upper

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{4}h^{-1}$	2.7	0.3-18.5	13	183.8
Total count x $10^{-5}ml^{-1}$	16.5	2.4-49.2	13	84.8
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	7.7	0.002-46.7	13	175.7
Number of chromogenic bacteria x $10^{-3}CFUml^{-1}$	12.1	0.001-109	13	247.7
Bacterial diversity	12.5	2.4-28.6	9	64.5
Viable count on PSM agar $CFUml^{-1}$	2900	0-18000	13	176.3
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	2.8	0.006-8.2	11	122.9
Total count x $10^{-7}g^{-1}$	3.0	0.2-11.0	11	113.1
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	9.7	0.0003-54.2	11	165.5
Number of chromogenic bacteria x $10^{-5}CFUg^{-1}$	2.5	0.0005-7.1	11	114.6
Bacterial diversity	3.2	0.1-9.0	9	86.4

Table 4.1.a. (continued)

Site: Sugden Beck Lower

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{4}h^{-1}$	16.8	0.2-74.8	12	129.4
Total count x $10^{-5}ml^{-1}$	25.8	3.1-73.7	13	76.8
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	25.0	0.001-79.4	12	110.0
Number of chromogenic bacteria x $10^{-3}CFUml^{-1}$	34.4	0.002-118	11	122.4
Bacterial diversity	11.1	7.4-18.3	8	32.8
Viable count on PSM agar $CFUml^{-1}$	16600	0-84300	12	149.8
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	15.2	0.0004-25.1	10	58.6
Total count x $10^{-7}g^{-1}$	4.5	1.0-13.2	10	80.0
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	33.3	0.004-90.9	9	88.0
Number of chromogenic bacteria x $10^{-5}CFUg^{-1}$	87.3	0.02-182	7	90.4
Bacterial diversity	4.5	2.3-9.5	6	60.4

Table 4.1.b. Summary of environmental variables and concentration of phenolic compounds and phenol derivatives from Stubs Beck and Sugden Beck sampling sites, April 1981 to April 1982.

Site: Stubs Beck

Variable	Mean	Range	n	c.v.(%)
pH	7.5	7.0-8.0	11	4.6
Temperature (^{o}C)	7.6	1.0-17.0	11	60.0
Dissolved oxygen (mgl^{-1})	10.0	7.7-12.0	11	13.7
BOD (mg oxygen l^{-1})	6.7	1.7-10.8	11	47.0
Conductivity (μScm^{-1})	512	329-1001	11	39.0
Turbidity (Arbitrary units)	6	2-18	11	88.2

No phenolic compounds or phenol derivatives were detected in Stubs Beck.

Table 4.1.b. (continued)

Site: Sugden Beck Upper

Variable	Mean	Range	n	c.v.(%)
pH	4.9	2.7-7.4	13	37.5
Temperature (^{o}C)	9.9	1.0-19.0	13	49.2
Dissolved oxygen (mgl^{-1})	10.3	8.2-12.1	13	11.8
BOD (mg oxygen l^{-1})	255	70.0-520	9	51.6
Conductivity (μScm^{-1})	237	529-6292	13	77.0
Turbidity (Arbitrary units)	16	3-36	13	51.0
Phenolic compounds and phenol derivatives (μgl^{-1})				
Chlorophenols				
3CHLR	667	0-2900	13	132.6
4CHLR	569	0-1900	13	97.2
2,4DCP	3459	0-19000	13	145.9
PCOC	2908	0-14800	13	153.4
Total chlorophenols	7602	0-34600	13	123.2
Nitrophenols				
Picric acid	1961	360-4400	13	60.5
2,4DNP	72	0-240	13	109.3
DNOC	32	0-90	13	101.2
DNBP	8	0-50	13	206.6
Total nitrophenols	2072	390-4690	13	60.9
Phenoxyalkanoic acids				
2,4D	2069	0-6400	13	91.3
MCPA	2079	0-9000	13	135.4
2,4DP	7419	650-17300	13	76.5
CMPP	8131	0-21600	13	66.3
2,4DB	472	0-1300	13	108.3
MCPB	155	0-1200	13	226.5
Total phenoxyalkanoic acids	20324	4400-53420	13	68.5
Total of phenolic compounds and phenol derivatives	30000	4790-69690	13	60.3

Table 4.1.b. (continued)

Site: Sugden Beck Lower

Variable	Mean	Range	n	c.v.(%)
pH	6.4	3.3-7.6	13	25.6
Temperature (^{o}C)	9.2	2.0-19.0	13	50.1
Dissolved oxygen (mgl^{-1})	10.4	8.5-12.6	13	12.1
BOD (mg oxygen l^{-1})	198	60.0-510	8	96.1
Conductivity (μScm^{-1})	1506	429-4576	13	88.0
Turbidity (Arbitrary units)	16	2-41	13	79.4
Estimated flow rate at Sugden Beck Lower (x $10^{3}m^{3}s^{-1}$)	48	6-135	7	96.0
Phenolic compounds and phenol derivatives (μgl^{-1})				
Chlorophenols				
3CHLR	412	0-2200	13	179.3
4CHLR	302	0-2000	13	182.1
2,4DCP	1639	0-8600	13	142.2
PCOC	1532	0-10600	13	192.8
Total chlorophenols	3885	0-21490	13	149.9
Nitrophenols				
Picric acid	827	160-1900	13	72.0
2,4DNP	50	0-290	13	158.5
DNOC	19	0-90	13	148.1
DNBP	3	0-20	13	244.1
Total nitrophenols	899	160-2240	13	75.3
Phenoxyalkanoic acids				
2,4D	936	0-3700	13	120.9
MCPA	906	0-3700	13	131.9
2,4DP	3584	0-7600	13	65.4
CMPP	4636	0-17000	13	89.9
2,4DB	175	0-1100	13	206.8
MCPB	108	0-600	13	198.7
Total phenoxyalkanoic acids	10345	770-25700	13	64.8
Total of phenolic compounds and phenol derivatives.	15130	940-48300	13	78.0

Table 4.2. Summary of variance ratios from oneway analysis of variance and corrected H values from Kruskal-Wallis oneway analysis of variances, for significant differences in bacteriological variables between Stubs Beck and Sugden Beck sampling sites (NS = non-significant).

Variable	variance ratio (F)	v_1, v_2	p
Water			
Activity	11.680	2,33	<0.001
Total count	1.162	2,34	0.325 NS
Gravel			
Activity	12.932	2,24	<0.001
Viable count on CPS agar	3.813	2,23	0.037
Bacterial diversity	10.412	2,17	0.001

Variable	corrected H value	cases	p
Water			
Viable count on CPS agar	3.717	36	0.156 NS
Number of chromogenic bacteria	6.324	35	0.042
Bacterial diversity	12.750	25	0.002
Viable count on PSM agar	5.207	36	0.074 NS
Gravel			
Total count	3.787	27	0.151 NS
Number of chromogenic bacteria	13.168	24	0.001

Table 4.3. Chromogenic bacteria expressed as a percentage of the viable count on CPS agar, from Stubs Beck and Sugden Beck sampling sites.

Site	Water	Gravel
Stubs Beck	26.0	32.7
Sugden Beck Upper	15.7	2.6
Sugden Beck Lower	13.8	26.2

Table 4.4.a. *Relationships between bacteriological and environmental variables in Sugden Beck (6 April 1981 - 6 April 1982).* Values are Pearson's product-moment correlation coefficients (r), a minus sign indicates a negative correlation, NS = non-significant correlation ($p > 0.05$), figures in brackets refer to significance level (1) = $p < 0.05$ (2) = $p < 0.01$ (3) = $p < 0.001$. (n varied from 11-26).

i. Water data

Variable	Activity	Total count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity	Viable count on PSM agar
pH	0.86(3)	0.59(2)	0.65(3)	0.96(3)	NS	0.74(3)
Temperature	NS	NS	NS	-0.63(2)	NS	-0.46(1)
Dissolved oxygen	NS	NS	NS	NS	NS	NS
BOD	NS	NS	NS	-0.80(3)	NS	-0.58(1)
Conductivity	-0.65(3)	-0.54(2)	-0.51(2)	-0.87(3)	-0.49(1)	-0.60(2)
Turbidity	NS	NS	NS	NS	NS	NS
Estimated flow rate in Sugden Beck	0.62(1)	NS	0.68 (1)	0.63(1)	NS	NS
Total amount of phenoxyalkanoic acids	-0.42(1)	NS	-0.42(1)	NS	NS	NS
Total amount of chlorophenols	-0.51(2)	NS	-0.44(1)	-0.51(1)	NS	-0.48(1)
Total amount of nitrophenols	-0.67(3)	NS	-0.54(2)	-0.69(3)	NS	-0.59(2)
Total amount of phenolic compounds and phenol derivatives	-0.59(2)	NS	-0.54(2)	-0.47(1)	NS	-0.48(1)

ii. Gravel data

Variable	Activity	Total Count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity
pH	0.72(3)	0.46(1)	0.66(2)	0.92(3)	NS
Temperature	NS	NS	NS	-0.49(1)	NS
Dissolved oxygen	NS	NS	NS	NS	NS
BOD	NS	NS	-0.56(1)	-0.62(1)	NS
Conductivity	-0.57(2)	-0.45(1)	-0.51(1)	-0.75(3)	NS
Turbidity	NS	NS	NS	NS	NS
Estimated flow rate in Sugden Beck	NS	NS	NS	NS	NS
Total amount of phenoxyalkanoic acids	NS	NS	NS	NS	NS
Total amount of chlorophenols	-0.50(1)	NS	NS	-0.53(1)	NS
Total amount of nitrophenols	-0.62(2)	NS	-0.60(2)	-0.68(2)	NS
Total amount of phenolic compounds and phenol derivatives	-0.56(2)	NS	-0.52(1)	-0.56(1)	NS

Table 4.4.b. *Relationships between bacteriological and environmental variables in Stubs Beck (6 April 1981 - 6 April 1982). Values are Pearson's product-moment correlation coefficients (r), a minus sign indicates a negative correlation, NS = non-significant correlation (p>0.05), figures in brackets refer to significance level (1) = p<0.05, (2) = p<0.01. (n varied from 5-11).*

i. Water data

Variable	Activity	Total Count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity	Viable count on PSM agar
pH	NS	NS	NS	NS	NS	NS
Temperature	NS	NS	NS	NS	NS	NS
Dissolved oxygen	NS	NS	NS	NS	NS	NS
BOD	NS	NS	NS	0.71(1)	NS	NS
Conductivity	NS	NS	NS	NS	NS	NS
Turbidity	0.69(1)	NS	NS	NS	NS	NS
Estimated flow rate in Sugden Beck	NS	NS	NS	NS	NS	0.83(1)

ii Gravel data

Variable	Activity	Total Count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity
pH	0.85(1)	-0.92(1)	NS	NS	NS
Temperature	NS	NS	NS	NS	NS
Dissolved oxygen	NS	NS	NS	NS	NS
BOD	NS	0.88(1)	NS	NS	NS
Conductivity	NS	NS	NS	NS	NS
Turbidity	NS	NS	NS	NS	NS
Estimated flow rate in Sugden Beck	NS	NS	NS	NS	NS

Table 4.5. Means of selected bacteriological variables from Sugden Beck Upper under neutral and acid pH conditions, April 1981 to April 1982.

Variable	Mean	Range	n	c.v.(%)
Neutral pH				
pH	6.8	6.3-7.4	6	6.3
Water				
Activity x $10^{4}h^{-1}$	5.4	1.2-18.5		124.2
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	16.6	2.3-46.7	6	96.8
Viable count on PSM agar $CFUml^{-1}$	6280	600-18000	6	97.3
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	5.1	0.3-8.2	6	60.2
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	17.8	3.3-54.2	6	104.4
Acid pH				
pH	3.4	2.7-4.8	7	22.4
Water				
Activity x $10^{4}h^{-1}$	0.5	0.3-1.0	7	58.3
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	0.02	0.004-0.05	7	114.2
Viable count on PSM agar $CFUml^{-1}$	0	0	6	0
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	0.02	0.006-0.07	5	148.2
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	0.03	0.0003-0.07	5	136.0

Fig. 4.2. Histograms showing means of bacteriological variables and total levels of phenolic compounds and phenol derivatives from the Stubs Beck and Sugden Beck sampling sites.

Key for histograms;

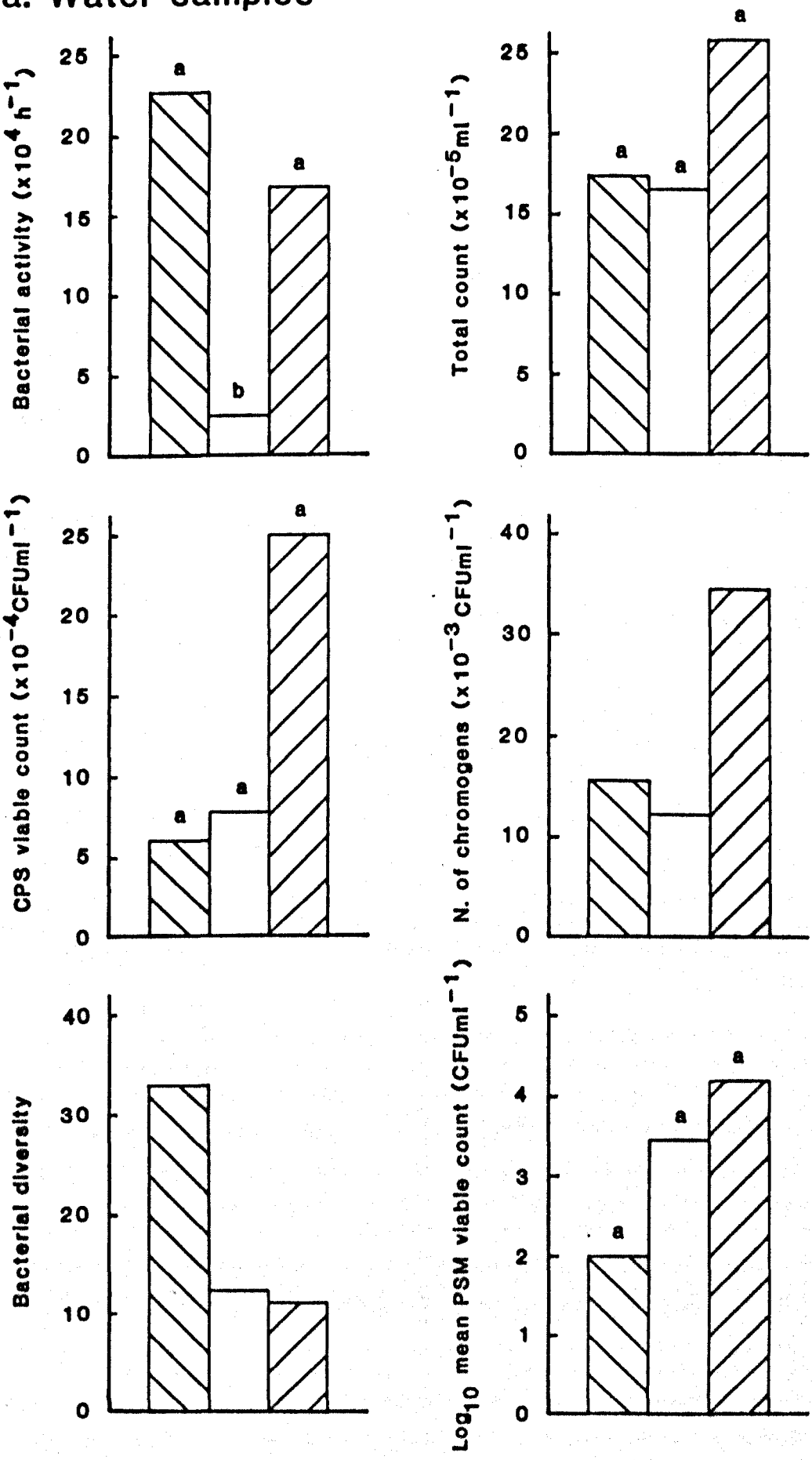
 = Stubs Beck

 = Sugden Beck Upper

 = Sugden Beck Lower

There is no significant difference ($p > 0.05$) between columns with the same superscript (anova or Kruskal-Wallis test), columns with different superscripts are significantly different ($p < 0.05$, SNK test). Where no superscripts are shown there are significant differences between columns ($p < 0.05$, Kruskal-Wallis test) but significant differences between particular individual columns are not identified because of the non-availability of a non-parametric equivalent of the SNK test. In the case of concentration of phenolic compounds and phenol derivatives no statistical analysis was required because no phenolic compounds or phenol derivatives were detected in Stubs Beck.

a. Water samples



b. Gravel samples

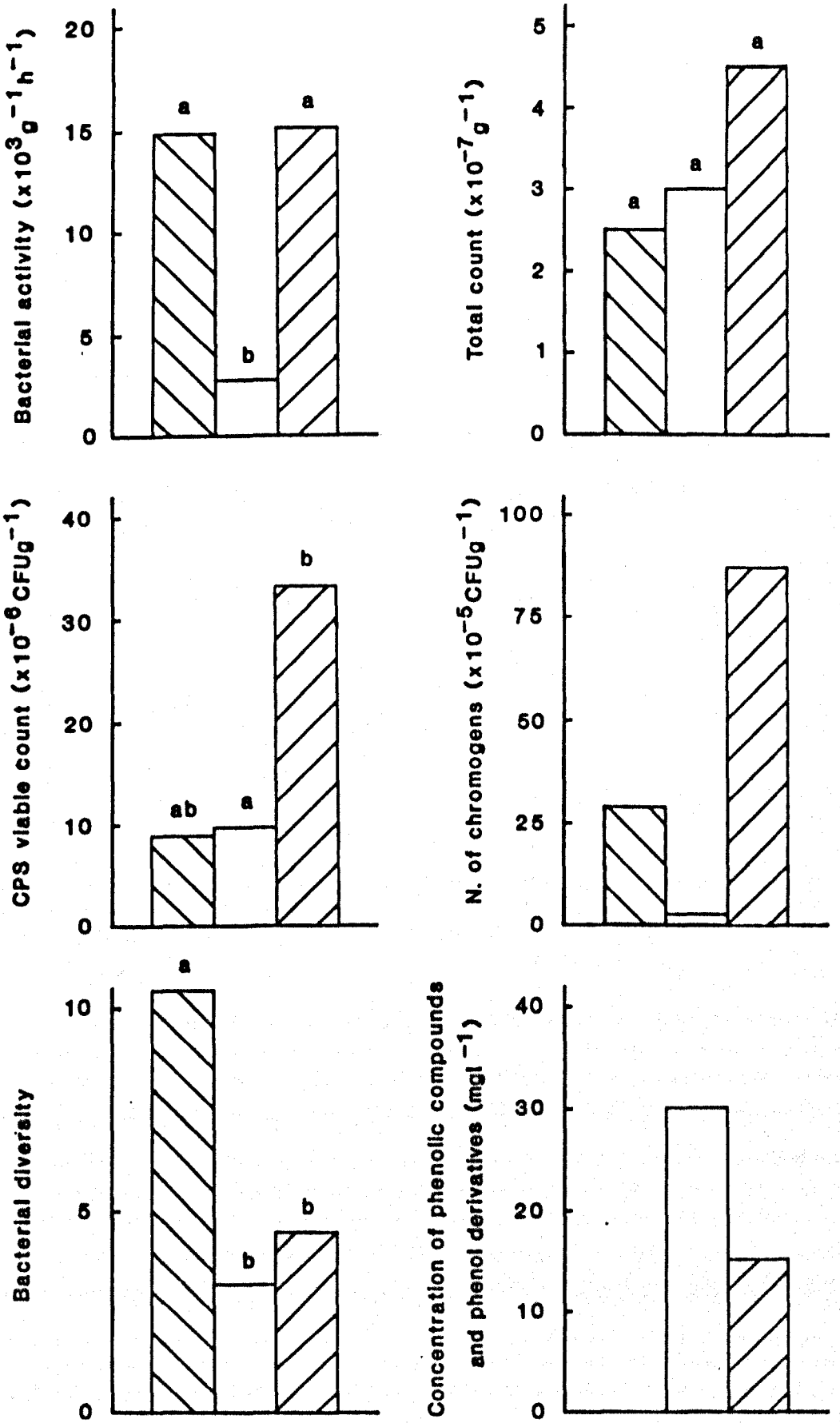
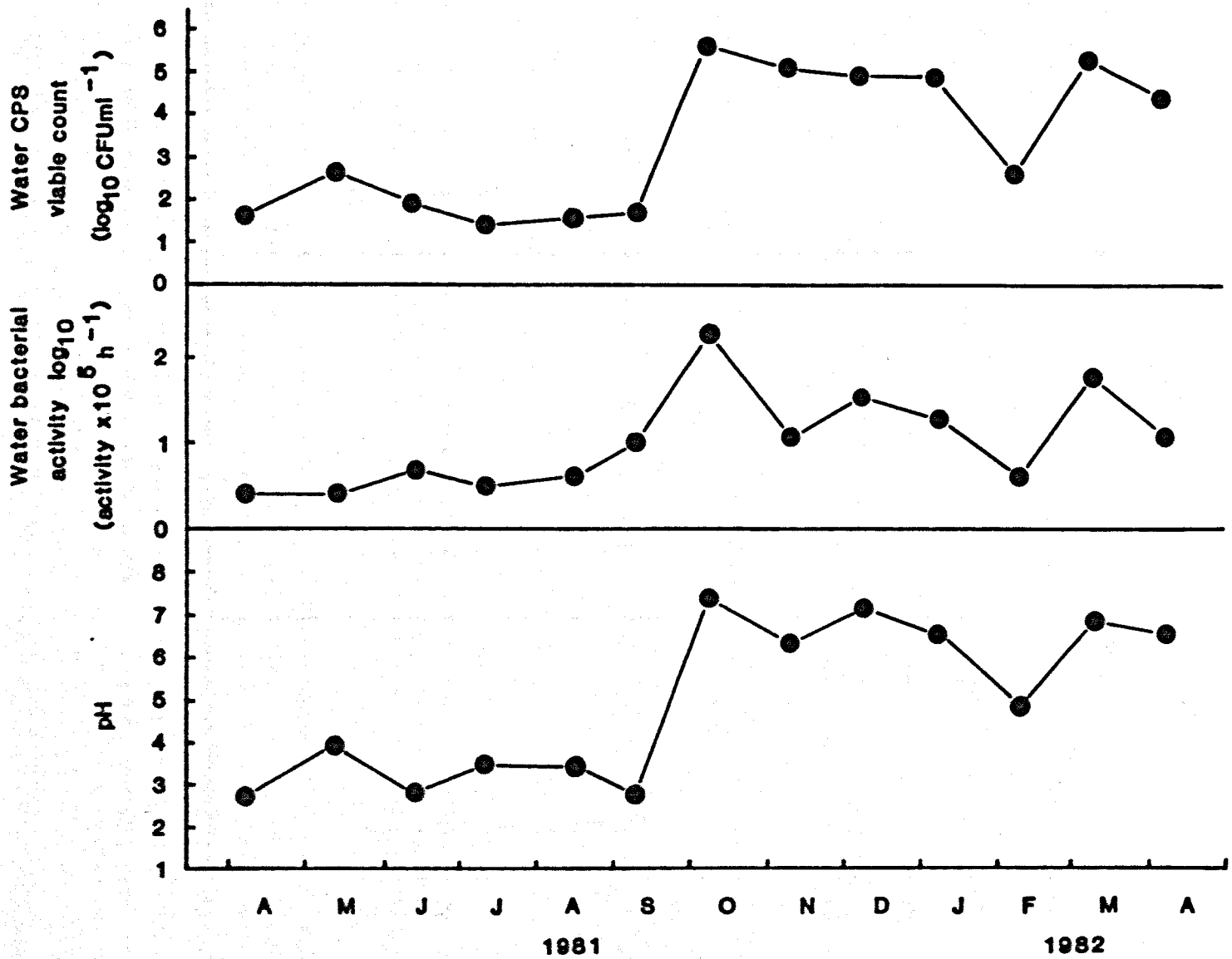
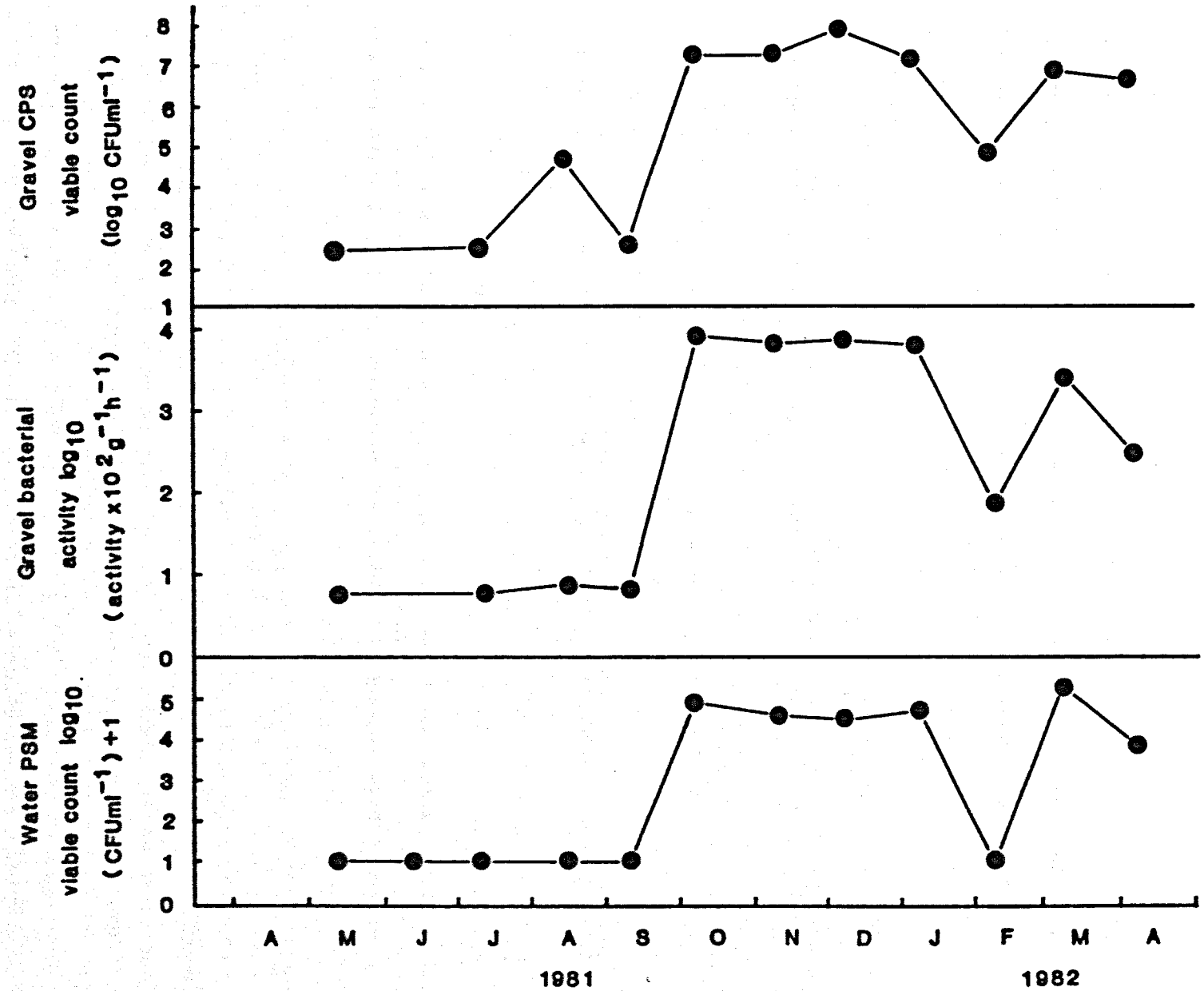


Fig. 4.3. Relationship between selected bacteriological variables and water pH, at the Sugden Beck Upper sampling site, during the routine sampling programme (6 April 1981 to 6 April 1982).


Water CPS
viable count
(log_{10} CFUml^{-1})
6
5
4
3
2
1
0
Water bacterial
activity log_{10}
(activity x10^{5} h^{-1})
2
1
0
pH
8
7
6
5
4
3
2
1
A
M
J
J
A
S
O
N
D
J
F
M
A
1981
1982


Gravel CPS
viable count
(log_{10} CFUml^{-1})
8
7
6
5
4
3
2
1
Gravel bacterial
activity log_{10}
(activity x10^2g^{-1}h^{-1})
4
3
2
1
0
Water PSM
viable count log_{10}
(CFUml^{-1}) +1
6
5
4
3
2
1
0
A
M
J
J
A
S
O
N
D
J
F
M
A
1981
1982

4.3.ii. Effect of phenolic compounds in Sugden Beck under neutral pH conditions and comparison with Stubs Beck.

The correlation coefficients shown in Table 4.4.a. suggested that bacteria in Sugden Beck were influenced by both pH and the concentration of phenolic compounds and phenol derivatives present. In order to consider the effects of phenolic compounds and phenol derivatives independent of the influence of pH, data from the Sugden Beck sites, when $pH \geq 6.0$, were considered separately. This removed the effect of extreme pH values.

The values of bacteriological and environmental variables from the Sugden Beck sites, when $pH \geq 6.0$, are shown in Table 4.6.a. and 4.6.b. This table showed that under neutral pH conditions, there was an increase in the mean values of all the bacteriological variables in Sugden Beck, except for gravel bacterial diversity at Sugden Beck Lower which remained constant. The mean values of the bacteriological variables, when $pH \geq 6.0$, from each site are plotted as histograms in Fig. 4.4.

The following untransformed bacteriological variables had equal variances; water total counts, gravel activity and bacterial diversity. The following $\log_{10}(x)$ transformed variables had equal variances; water activity, viable count, number of chromogenic bacteria, bacterial diversity and viable counts on PSM agar, together with, gravel viable counts and the number of chromogenic bacteria. The variance of the transformed and untransformed gravel total counts was heteroscedastic. Table 4.7. shows a summary of the variance ratios, from oneway analysis of variance, and the corrected H value from the Kruskal-Wallis test.

There were similar significant differences between sites (determined using the Student-Newman-Keuls test, see 2.6.), for bacteriological variables, in both water and gravel samples. Bacterial activity was

significantly greater at the Stubs Beck and Sugden Beck Lower sites compared to Sugden Beck Upper. There was no significant difference in bacterial activity between the Stubs Beck and Sugden Beck Lower sites in water samples, although in gravel samples bacterial activity was significantly greater at Sugden Beck Lower.

There were no significant differences between the sites for both total and viable bacterial counts, except the viable counts at Sugden Beck Lower were significantly greater than the counts at Stubs Beck. The highest total and viable bacterial counts were at Sugden Beck Lower, followed by Sugden Beck Upper and finally, Stubs Beck.

There were no significant differences between the sites for the number of chromogenic bacteria, except that the number of chromogenic bacteria, in gravel samples, at Stubs Beck and Sugden Beck Lower were significantly greater than at Sugden Beck Upper. In common with total and viable bacterial counts, the highest number of chromogenic bacteria were at Sugden Beck Lower. Table 4.8.illustrates the differences between sites, when the number of chromogenic bacteria were expressed as a percentage of the total viable count. This table showed that, even under neutral pH conditions, Stubs Beck had the highest percentage of chromogenic bacteria, in both water and gravel samples, compared to the Sugden Beck sites.

Bacterial diversity was significantly greater at the Stubs Beck site compared to the Sugden Beck sites, although there was no significant differencein bacterial diversity between Sugden Beck Upper and Sugden Beck Lower.

There were significant differences between the sites for the viable count on PSM agar. Both the Sugden Beck sites had significantly greater counts on PSM agar compared with Stubs Beck, although there was no significant difference between the Sugden Beck sites.

Selection of data from the Sugden Beck sites, to exclude the

effect of extreme pH, allowed a more direct comparison to be made between sites. Compared with the Stubs Beck site, both Sugden Beck sites had a reduced percentage of chromogenic bacteria, lower bacterial diversity and very much increased viable counts on PSM agar. The highest values of all the bacteriological variables including the Stubs Beck site, except for bacterial diversity, were found at the Sugden Beck Lower site. These results suggest that providing the pH remained neutral, a relatively large and active bacterial community could develop in Sugden Beck. However, the presence of the phenolic compounds and phenol derivatives caused a change in composition of the bacterial community by selection of a few resistant bacterial species.

Compared to Sugden Beck Upper, the Sugden Beck Lower site had higher values for all of the bacteriological variables, except for gravel bacterial diversity. These differences in the Sugden Beck sites, under neutral pH conditions, were probably explained by the input of Stubs Beck water, which diluted phenolic compounds and phenol derivatives and added viable bacteria to the system. Under all pH conditions the input of Stubs Beck water, buffered the extreme pH values in Sugden Beck. For example low pH values ($pH < 6.0$) were recorded on seven out of the thirteen sampling occasions at Sugden Beck Upper, compared with only three occasions at Sugden Beck Lower, on two of which Stubs Beck was dry. The higher values for bacteriological variables at Sugden Beck Lower, compared to Sugden Beck Upper, readily illustrated this effect (see Table 4.1.a.).

To examine the relationships between bacteriological variables and phenolic compounds and phenol derivatives in Sugden Beck, when $pH \geq 6.0$, Pearson's product-moment correlation coefficients were calculated using combined data from the Sugden Beck Upper and Sugden Beck Lower sites (see Table 4.9.). All of the bacteriological and environmental variables were not significantly different from a normal distribution ($p > 0.05$) and

therefore, did not require transformation.

Under neutral pH conditions there were fewer significant negative correlations between bacteriological variables and phenolic compounds and phenol derivatives, compared to all pH conditions (see Table 4.4.a.). Significant negative relationships were found between the total amount of phenolic compounds and phenol derivatives and the number of chromogenic bacteria in water samples and between the total amount of nitrophenols and bacterial activity, viable counts and the number of chromogenic bacteria in gravel samples. Significant positive relationships were found between bacterial diversity in water samples with the total amount of phenoxyalkanoic acids, nitrophenols and phenolic compounds and phenol derivatives. Clearly, water pH was an important factor determining the toxicity of these compounds to bacterial communities in Sugden Beck.

Table 4.6.a. Summary of bacteriological variables from Sugden Beck sampling sites, April 1981 to April 1982, when pH was greater than or equal to 6.0.

Site: Sugden Beck Upper

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{4}h^{-1}$	5.4	1.2-18.5	6	124.2
Total count x $10^{-5}ml^{-1}$	25.6	12.6-49.2	6	56.6
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	16.6	2.3-46.7	6	96.8
Number of chromogenic bacteria x $10^{-3}CFUml^{-1}$	26.2	2.5-109	6	158.0
Bacterial diversity	16.8	12.3-28.6	6	38.1
Viable count on PSM agar $CFUml^{-1}$	6280	600-18000	6	97.3
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	5.1	0.3-8.2	6	60.2
Total count x $10^{-7}g^{-1}$	4.6	1.4-11.0	6	85.3
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	17.8	3.3-54.2	6	104.4
Number of chromogenic bacteria x $10^{-5}CFUg^{-1}$	4.5	0.4-7.1	6	50.2
Bacterial diversity	4.5	2.1-9.0	6	54.0

Table 4.6.a. (continued)

Site: Sugden Beck Lower

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{4}h^{-1}$	24.7	5.2-74.8	8	92.5
Total count x $10^{-5}ml^{-1}$	31.0	13.9-73.7	9	63.5
Viable count on CPS agar x $10^{-4}CFUml^{-1}$	37.3	5.8-79.4	8	69.9
Number of chromogenic bacteria x $10^{-3}CFUml^{-1}$	47.3	1.4-118	8	90.6
Bacterial diversity	11.6	7.4-18.3	7	31.6
Viable count on PSM agar $CFUml^{-1}$	24700	1556-84300	8	109.8
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	19.6	12.7-25.1	7	22.4
Total count x $10^{-7}g^{-1}$	5.1	2.4-13.2	7	72.4
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	41.9	7.8-90.9	7	65.2
Number of chromogenic bacteria x $10^{-5}CFUg^{-1}$	102	13.9-182	6	74.1
Bacterial diversity	4.5	2.3-9.5	6	60.4

Table 4.6.b. Summary of environmental variables and concentration of phenolic compounds and phenol derivatives from Sugden Beck sampling sites, April 1981 to April 1982, when pH was greater than or equal to 6.0.

Site: Sugden Beck Upper

Variable	Mean	Range	n	c.v.(%)
pH	6.8	6.3-7.4	6	6.4
Temperature (^{o}C)	6.7	1.0-9.0	6	47.4
Dissolved oxygen (mgl^{-1})	10.2	9.3-11.4	6	7.8
BOD (mg oxygen l^{-1})	185	70.0-230	6	32.3
Conductivity (μScm^{-1})	825	529-1230	6	34.1
Turbidity (Arbitrary units)	14	3-19	6	40.3
Phenolic compounds and phenol derivatives (μgl^{-1})				
Chlorophenols				
3CHLR	125	0-750	6	245.0
4CHLR	405	0-1030	6	101.8
2,4DCP	1878	0-4400	6	106.6
PCOC	383	0-2300	6	245.0
Total chlorophenols	2792	0-6100	6	95.0
Nitrophenols				
2,4DNP	15	0-50	6	156.4
Picric acid	1115	360-1700	6	43.7
DNOC	20	0-70	6	137.8
DNBP	5	0-30	6	245.0
Total nitrophenols	1155	390-1780	6	42.3
Phenoxyalkanoic acids				
2,4D	2267	0-6400	6	102.4
MCPA	2130	0-6400	6	106.5
2,4DP	6758	650-17300	6	100.4
CMPP	8167	0-21600	6	92.8
2,4DB	420	0-1200	6	126.4
MCPB	287	0-1200	6	172.1
Total phenoxyalkanoic acids	20028	4400-53420	6	91.9
Total of phenolic compounds and phenol derivatives	23975	4790-56310	6	78.8

Table 4.6.b. (continued)

Site: Sugden Beck Lower

Variable	Mean	Range	n	c.v.(%)
pH	7.4	7.1-7.6	9	2.5
Temperature (^{o}C)	8.1	2.0-19.0	9	60.3
Dissolved oxygen (mgl^{-1})	10.2	8.5-11.7	9	11.8
BOD (mg oxygen l^{-1})	156	60.0-510	7	103.0
Conductivity (μScm^{-1})	896	429-1859	9	52.2
Turbidity (Arbitrary units)	16	2-41	9	89.3
Flow rate at Sugden Beck Lower ($x\ 10^{3}m^{3}s^{-1}$)	55	18-135	6	84.0
Phenolic compounds and phenol derivatives (μgl^{-1})				
Chlorophenols				
3CHLR	42	0-380	9	300.0
4CHLR	283	0-2000	9	231.1
2,4DCP	679	0-2300	9	132.7
PCOC	212	0-1500	9	236.4
Total chlorophenols	1217	0-3800	9	110.5
Nitrophenols				
2,4DNP	19	0-80	9	146.4
Picric acid	610	160-1400	9	68.8
DNOC	4	0-20	9	198.5
DNBP	4	0-20	9	198.5
Total nitrophenols	638	160-1480	9	70.3
Phenoxyalkanoic acids				
2,4D	1250	0-3700	9	99.7
MCPA	1276	0-3700	9	100.3
2,4DP	2899	0-6600	9	80.3
CMPP	3341	0-6800	9	65.9
2,4DB	44	0-400	9	300.0
MCPB	67	0-600	9	300.0
Total phenoxyalkanoic acids	8877	770-16900	9	65.9
Total of phenolic compounds and phenol derivatives	10731	940-20080	9	64.1

Table 4.7. Summary of variance ratios from oneway analysis of variance and corrected H value from Kruskal-Wallis oneway analysis of variance, for significant differences in bacteriological variables between Stubs Beck and Sugden Beck sampling sites when pH $\geq$ 6.0 (NS = non-significant).

Variable	variance ratio (F)	v_1, v_2	p
Water			
Activity	6.912	2,22	0.005
Total count	1.752	2,23	0.196 NS
Viable count on CPS agar	10.022	2,22	0.001
Number of chromogenic bacteria	0.869	2,22	0.433 NS
Bacterial diversity	16.292	2,18	$<$ 0.001
Viable count on PSM agar	54.719	2,21	$<$ 0.001
Gravel			
Activity	22.797	2,16	$<$ 0.001
Viable count on CPS agar	4.996	2,16	0.021
Number of chromogenic bacteria	13.036	2,15	0.001
Bacterial diversity	7.826	2,14	0.005

Variable	corrected H value	cases	p
Gravel			
Total count	4.967	19	0.083 NS

Table 4.8. Chromogenic bacteria expressed as a percentage of the viable count on CPS agar, from Stubs Beck and Sugden Beck sampling sites when pH 6.0.

Site	% Chromogenic bacteria	
	Water	Gravel
Stubs Beck	25.9	32.7
Sugden Beck Upper	15.8	2.5
Sugden Beck Lower	12.7	24.3

Table 4.9. Relationships between bacteriological variables and phenolic compounds and phenol derivatives in Sugden Beck (6 April 1981-6 April 1982), when pH≥6.0. Values are Pearson's product-moment correlation coefficients (r), a minus sign indicates a negative correlation, NS = non-significant correlation ($p>0.05$), figures in brackets refer to significance level (1) = $p<0.05$ (2) = $p<0.01$, (3)= $p<0.001$. (n varied from 12-15).

i. Water data

Variable	Activity	Total count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity	Viable count on PSM agar
Total amount of phenoxyalkanoic acids	NS	NS	NS	NS	0.71(2)	NS
Total amount of chlorophenols	NS	NS	NS	NS	NS	NS
Total amount of nitrophenols	NS	NS	NS	NS	0.66(1)	NS
Total amount of phenolic compounds and phenol derivatives	NS	NS	NS	-0.54(1)	0.72(2)	NS

ii. Gravel data.

Variable	Activity	Total count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity
Total amount of phenoxyalkanoic acids	NS	NS	NS	NS	NS
Total amount of chlorophenols	NS	NS	NS	NS	NS
Total amount of nitrophenols	-0.57(1)	NS	-0.57(1)	-0.62(1)	NS
Total amount of phenolic compounds and phenol derivatives	NS	NS	NS	NS	NS

Fig. 4.4. Histograms showing means of bacteriological variables and total level of phenolic compounds and phenol derivatives from the Stubs Beck and Sugden Beck sampling sites when pH $\geq$ 6.0.

Key for histograms;

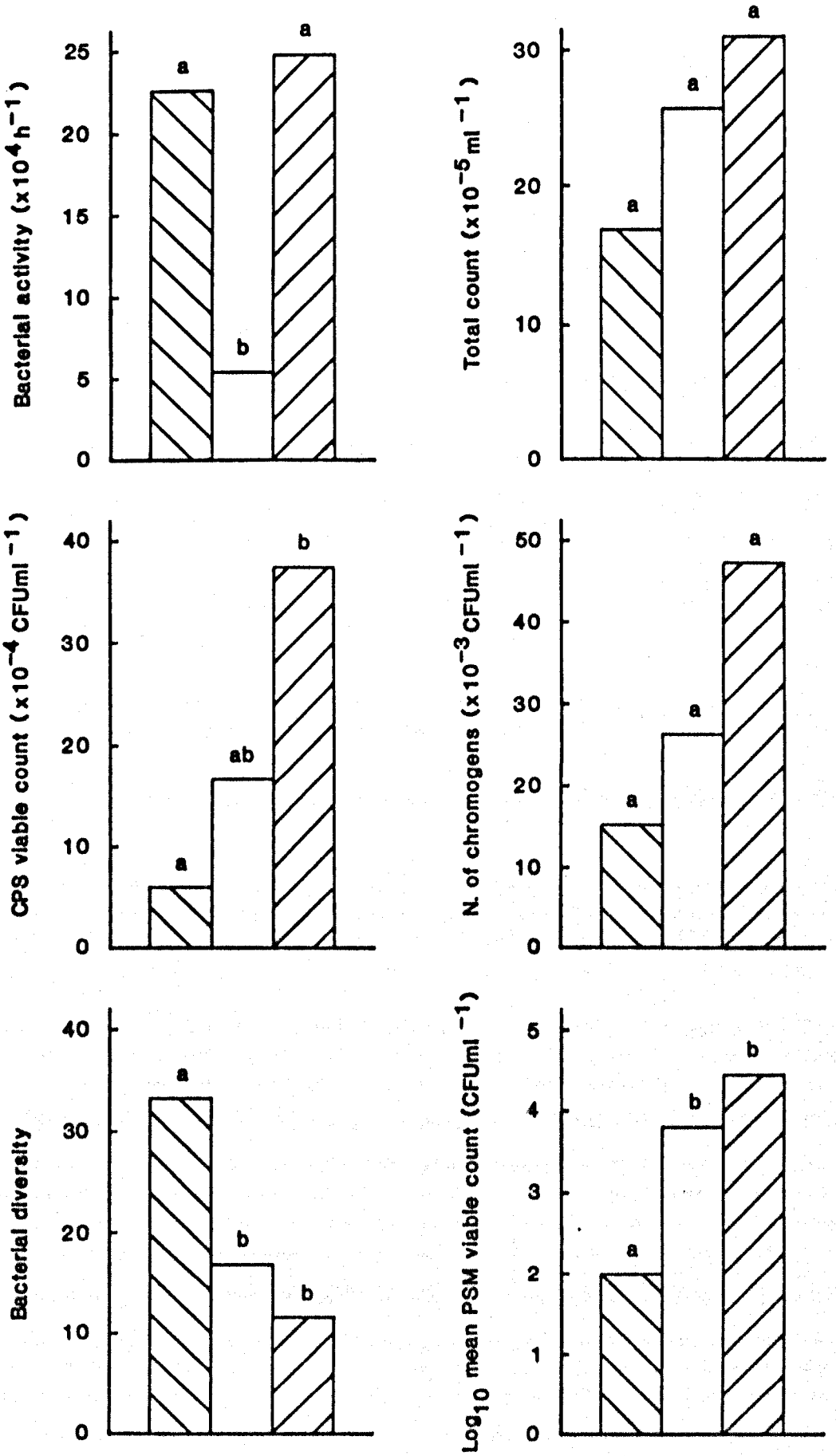
= Stubs Beck

= Sugden Beck Upper

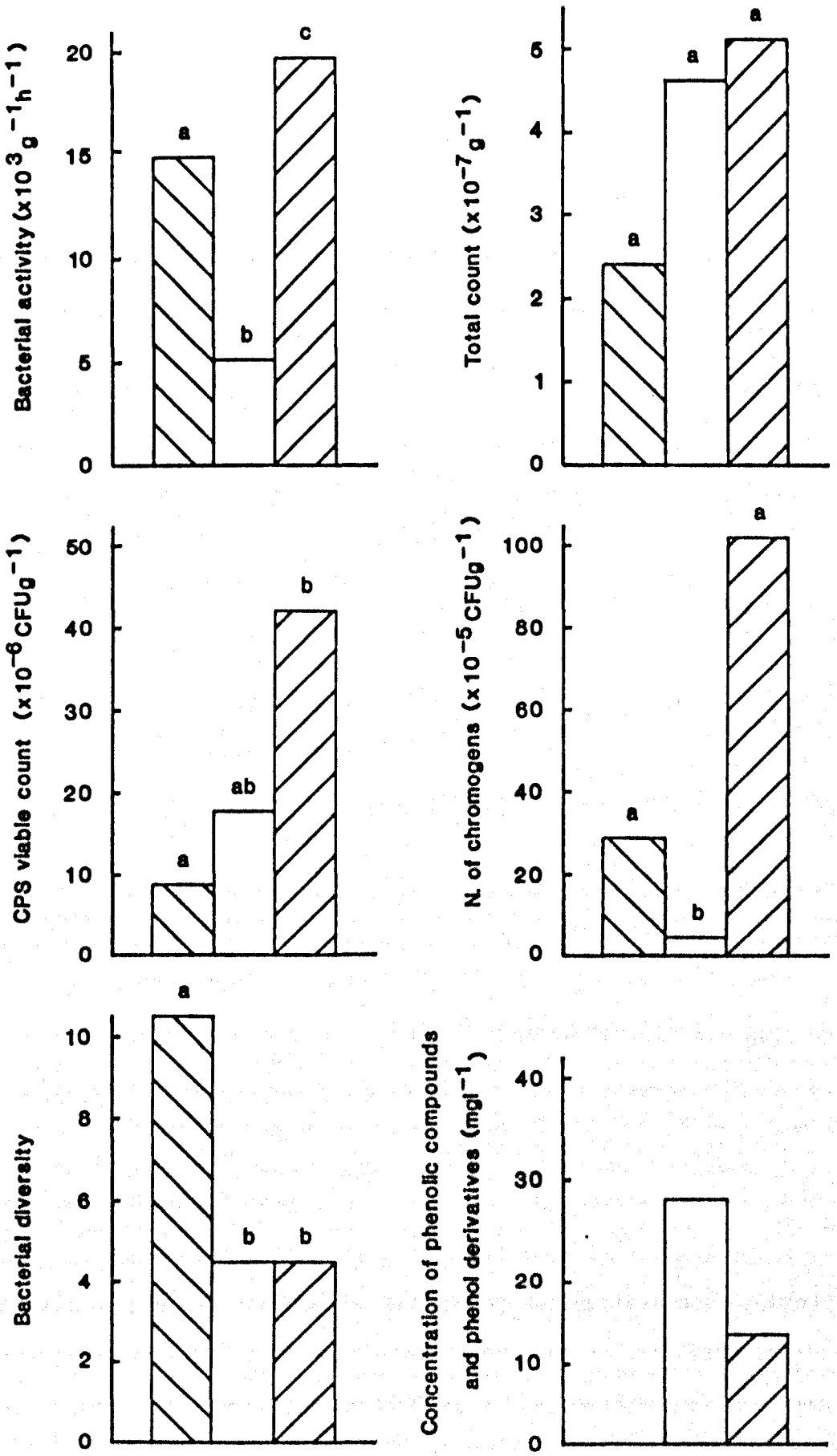
= Sugden Beck Lower

There is no significant difference ($p > 0.05$) between columns with the same superscript (anova or Kruskal-Wallis test), columns with different superscripts are significantly different ($p < 0.05$, SNK test). In the case of concentration of phenolic compounds and phenol derivatives no statistical analysis was required because no phenolic compounds or phenol derivatives were detected in Stubs Beck.

a. Water samples



b. Gravel samples



4.3.iii. Effect of Sugden Beck on Hunsworth Beck.

A summary of the results obtained from the Hunsworth Beck sampling sites is shown in Table 4.10.a. and 4.10.b., the raw data are shown in Appendix 5.

Analysis of Hunsworth Beck water by HPLC (see 2.5.) showed that there were fewer phenolic compounds and phenol derivatives present compared with the Sugden Beck sites and that they were also present in much smaller quantities (see Table 4.1.b.). The following phenolic compounds and phenol derivatives were detected in Hunsworth Beck;

Chlorophenols (abbreviations used in brackets)

2,4-dichlorophenol (2,4DCP)

4-chloro-2-methyl-phenol (PCOC)

Nitrophenols

2,4,6-trinitrophenol (picric acid)

2-sec-butyl-4,6-dinitrophenol (DNBP)

Phenoxyalkanoic acids

2-(2,4-dichlorophenoxy)-proprionic acid (2,4DP)

2-(2-methyl-4-chlorophenoxy)-proprionic acid (CMPP)

4-(2,4-dichlorophenoxy)-butyric acid (2,4DB)

Some phenolic compounds and phenol derivatives were detected in small quantities in Hunsworth Beck upstream of the confluence with Sugden Beck, these compounds were present in the sewage effluent from North Bierley Sewage works.

The means of all the bacteriological variables and the sum of the individual phenolic compounds and phenol derivatives are plotted as histograms in Fig.4.5., to illustrate differences between sites. The following untransformed variables had equal variances; water bacterial activity, total and viable counts, water bacterial diversity and gravel

bacterial diversity. After a $\log_{10}(x)$ transformation the following bacteriological variables had equal variances; number of chromogenic bacteria in water and gravel samples and the gravel total and viable counts. The variance of gravel bacterial activity, both before and after transformation, was heteroscedastic. A summary of the variance ratios, from oneway analysis of variance and the corrected H value from the Kruskal-Wallis test are shown in Table 4.11.

There were no significant differences between the Hunsworth Beck sites for any of the bacteriological variables, except for gravel total counts. The total number of bacteria was significantly greater at the 110m downstream site compared with the 17m upstream site.

The means of the water bacteriological variables were very similar at all sites, however, gravel samples showed higher total counts, viable counts, number of chromogenic bacteria and bacterial activity downstream of the confluence with Sugden Beck. The greatest number of chromogenic bacteria, total and viable bacterial counts occurred at the 110m downstream site. The highest gravel bacterial activity was detected at the 25m downstream site. Only in the case of gravel bacterial diversity was there a reduction downstream of the confluence with Sugden Beck.

Table 4.12. shows the difference between the Hunsworth Beck sites, when the number of chromogenic bacteria were expressed as a percentage of the total viable count. This table showed that there was little difference in percentage chromogenic bacteria between the sites in water samples. In the gravel samples the percentage of chromogenic bacteria was highest at the 110m downstream site and lowest at the 17m upstream site.

Many of the bacteriological variables, from gravel samples in Hunsworth Beck, were higher downstream of the confluence with Sugden Beck, which suggested that the low levels of phenolic compounds and phenol

derivatives present may have had a stimulatory effect on gravel bacterial communities. This effect was not seen with bacteriological variables from water samples, presumably due to the short contact time between the planktonic bacteria and phenolic compounds and phenol derivatives.

To examine the relationship between bacteriological and environmental variables in Hunsworth Beck, Pearson's product-moment correlation coefficients were calculated, using combined data from all sites (see Table 4.13.). All of the bacteriological and environmental variables, except the viable count and number of chromogenic bacteria in water samples, were not significantly different from a normal distribution ($p > 0.05$) and therefore, did not require transformation. A $\log_{10}(x)$ transformation of water viable counts and numbers of chromogenic bacteria normalised the distribution prior to calculating correlation coefficients.

In both, Hunsworth Beck, water and gravel samples there were no significant negative correlations between bacteriological variables and any of the phenolic compounds and phenol derivatives, indeed, there were many positive correlations between these variables. There were significant positive correlations between all of the phenolic compounds and phenol derivatives with the viable count on CPS agar and the number of chromogenic bacteria, in both water and gravel samples. Bacterial activity was significantly positively correlated with the total amount of nitrophenols in water samples, as was the viable count on PSM agar. The bacterial total count in water samples was significantly positively correlated with all the phenolic compounds and phenol derivatives, except the total amount of chlorophenols. In gravel samples the bacterial total count was positively correlated with only the total amount of phenoxyalkanoic acids. There were no significant correlations between any of the phenolic compounds and phenol derivatives with bacterial diversity, in either water or gravel samples.

Clearly, the absence of significant negative correlations, together with the many positive correlations found, suggest that the phenolic compounds and phenol derivatives present had little or no toxic effect on water or gravel bacterial communities in Hunsworth Beck. The toxicity of phenolic compounds and phenol derivatives in Sugden Beck was related to water pH; under neutral pH conditions these compounds were relatively non-toxic. As the pH in Hunsworth Beck was consistently neutral (mean pH 7.1, range 6.6-7.6) and the phenolic compounds and phenol derivatives were present at much lower concentrations, it was not surprising that these compounds had no toxic effect. The positive correlations between bacteriological variables and phenolic compounds and phenol derivatives were rather unexpected and may be due to both bacteriological variables and phenolic compounds and phenol derivatives responding to common independent variables (e.g. physico-chemical factors). Alternatively phenolic compounds and phenol derivatives at the low concentrations found in Hunsworth Beck may have a stimulatory effect on the bacteria.

All of the water bacteriological variables in Hunsworth Beck, except for viable count on PSM agar, were significantly correlated with temperature and dissolved oxygen concentration. There were significant positive correlations of temperature with bacterial activity, total and viable count and the number of chromogenic bacteria. There was a negative correlation between bacterial diversity, in water samples, and temperature. Over the temperature range in Hunsworth Beck (4-19^{o}C), temperature would be expected to have a positive effect on bacteriological variables.

Positive correlations of bacteriological variables with temperature, have also been shown by other workers. Goulder (1980) studied the seasonal variations in heterotrophic activity, viable and total counts, at two sites on the River Hull. The heterotrophic activity of bacteria, as determined by mineralization of a ^{14}C glucose substrate, was significantly

correlated with temperature, as was the total counts of bacteria determined by AODC (see 2.1.i.). However, viable counts of bacteria on CPS agar were not significantly correlated with temperature. Bell et al. (1980) showed that the bacterial heterotrophic activity, as determined by uptake and mineralization of glucose, in two Canadian rivers was positively correlated with temperature. Nuttall (1982a) also found a significant positive correlation of bacterial heterotrophic activity, as determined by uptake of ^{14}C acetic acid, at two polluted sites on the River Dee. Jones (1971) found a positive correlation between temperature and the viable count of bacteria on CPS agar, in water samples from a nutrient-rich English lake, although there was a non-significant relationship between temperature and viable counts in a more nutrient-poor lake. Baker and Farr (1977) also showed that there was no significant relationship between temperature and the viable count on CPS agar, in the River Frome, although the counts were not constant throughout the year. Daubner (1969) showed that in the polluted River Danube, there was an overall positive relationship between temperature and the numbers of heterotrophic bacteria, cultured on agar plates at 20°C and 37°C. However, certain bacterial groups; enterococci and clostridia, which were identified on membrane filters, exhibited a negative relationship with temperature.

In Hunsworth Beck (see Table 4.13) there were significant negative correlations between dissolved oxygen concentration with activity, total and viable counts and the number of chromogenic bacteria. There was a positive correlation between bacterial diversity and oxygen concentration. Clearly, low dissolved oxygen concentrations were correlated with high values for bacteriological variables. As a large and metabolically active bacterial community has a high oxygen requirement, these negative relationships may be explained by the resulting depletion of oxygen. In addition, decreased oxygen solubility in water occurs at high temperatures when the bacterial biomass would be expected to be high.

Nuttall (1982b) found significant negative correlations between heterotrophic activity, as determined by uptake of ^{14}C acetic acid, with dissolved oxygen concentration at two polluted sites on the River Dee. At a third, relatively unpolluted site on the same river, there was no significant correlation with dissolved oxygen concentration.

The ecological significance of the significant correlations of bacterial diversity with temperature and dissolved oxygen concentration, in Hunsworth Beck water samples, was unclear.

Water bacterial activity and the viable count on PSM agar were positively correlated with BOD. As BOD reflected the quantity of organic material present in water samples, a positive relationship with bacteriological variables was expected.

Albright and Wentworth (1973) studied eutrophication in five Canadian rivers by determining the bacterial heterotrophic activity. They showed that heterotrophic activity as measured by uptake and mineralization of ^{14}C glucose, was positively correlated with both soluble and particulate carbon concentration, and BOD. Nuttall (1982a) showed that bacterial heterotrophic activity was higher at two organically polluted sites on the River Dee, compared with a relatively unpolluted site on the same river.

Positive correlations were found between bacterial activity, viable count and the viable count on PSM agar in water samples with turbidity. Baker and Farr (1977) found that, over a two year sampling period, CPS viable counts in the River Frome and Tadnoll Brook were positively correlated with suspended solids ($p < 0.001$).

Positive correlations were found between the viable count on PSM agar with pH and between the total count of bacteria with conductivity, although the ecological significance of these relationships was unclear.

No significant correlations were found between any of the

bacteriological variables from water samples with the level of Hunsworth Beck at the gauging point, which was related to the flow rate in Hunsworth Beck.

These correlations suggested that, planktonic bacteria were more likely to be affected by environmental variables such as temperature, dissolved oxygen concentration and to a lesser extent, turbidity and BOD, rather than the toxicity of phenolic compounds and phenol derivatives.

Few significant correlations were found between any of the gravel bacteriological variables and environmental variables. There were negative correlations between the number of chromogenic bacteria with dissolved oxygen concentration and between bacterial diversity and the level of Hunsworth Beck at the gauging point.

Table 4.10.a. Summary of bacteriological and environmental variables from Hunsworth Beck sampling sites, April 1981 to April 1982.

Site: Hunsworth Beck 17m upstream.

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{2}h^{-1}$	6.4	1.3-15.9	13	72.4
Total count x $10^{-6}ml^{-1}$	13.2	5.4-29.9	13	54.1
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	24.3	5.6-99.8	13	112.4
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	47.4	6.8-293	13	162.2
Bacterial diversity	6.2	2.3-11.4	9	50.3
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	67.6	9.6-222	13	94.0
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	13.9	4.1-20.3	9	36.9
Total count x $10^{-7}g^{-1}$	5.6	3.2-9.0	9	39.5
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	18.8	4.3-41.7	9	64.9
Number of chromogenic bacteria x $10^{-6}CFUg^{-1}$	4.0	1.0-8.0	9	66.3
Bacterial diversity	4.8	3.6-6.2	6	20.8
Environmental variables				
pH	7.1	6.7-7.6	13	3.5
Temperature (^{o}C)	11.6	4.0-19.0	13	41.0
Dissolved oxygen (mgl^{-1})	8.6	6.3-10.8	13	17.5
BOD (mg oxygen l^{-1})	19.0	6.5-41.7	11	50.5
Conductivity (μScm^{-1})	944	400-1287	13	27.4
Turbidity (Arbitrary units)	11	4-33	13	82.3
Level of Hunsworth Beck at gauging point (cm)	44.5	37.0-59.0	8	17.3
Total amount of phenolic compounds and phenol derivatives (μgl^{-1})	194	0-990	12	169.8

Table 4.10.a. (continued)

Site: Hunsworth Beck 25m downstream

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{2}h^{-1}$	5.6	1.4-11.1	13	53.8
Total count x $10^{-6}ml^{-1}$	13.0	4.1-29.5	13	57.6
Viable count on CPS agar x $10^{-5}CFUml^{-1}$	25.6	5.6-134	13	138.0
Number of chromogenic bacteria x $10^{-4}CFUml^{-1}$	59.8	6.5-516	13	230.6
Bacterial diversity	6.1	2.5-11.5	9	50.2
Viable count on PSM agar x $10^{-2}CFUml^{-1}$	79.4	16.3-221	13	69.7
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	17.4	12.8-25.4	11	23.8
Total count x $10^{-7}g^{-1}$	9.0	2.2-19.9	11	67.4
Viable count on CPS agar x $10^{-6}CFUg^{-1}$	36.6	1.4-84.4	11	66.6
Number of chromogenic bacteria x $10^{-6}CFUg^{-1}$	9.3	0.5-22.4	11	81.1
Bacterial diversity	3.9	2.8-5.7	8	24.9
Environmental variables				
pH	7.1	6.7-7.6	13	3.4
Temperature (^{o}C)	11.5	4.0-19.0	13	39.8
Dissolved oxygen (mgl^{-1})	8.5	5.6-11.0	13	17.1
BOD (mg oxygen l^{-1})	21.8	7.8-42.3	9	45.0
Conductivity (μScm^{-1})	989	429-1387	13	28.5
Turbidity (Arbitrary units)	9	4-20	13	50.9
Total amount of phenolic compounds and phenol derivatives (μgl^{-1})	1005	0-6100	13	167.4

Table 4.10.a. (Continued)

Site: Hunsworth Beck 110m downstream

Variable	Mean	Range	n	c.v.(%)
Water				
Activity x $10^{2}h^{-1}$	5.5	1.4-11.9	13	57.1
Total count x $10^{-6}ml^{-1}$	13.0	4.5-31.8	13	60.1
Viable count on CPS agar x10^{-5}CFUml^{-1}	20.9	5.6-73.5	13	99.0
Number of chromogenic bacteria x 10^{-4}CFUml^{-1}	45.9	9.0-225	13	129.3
Bacterial diversity	6.1	2.7-11.6	9	47.8
Viable count on PSM agar x 10^{-2}CFUml^{-1}	70.1	12.6-164	13	66.4
Gravel				
Activity x $10^{3}g^{-1}h^{-1}$	16.6	14.5-20.2	11	11.1
Total count x $10^{-7}g^{-1}$	11.3	5.3-21.7	11	49.6
Viable count on CPS agar x 10^{-6}CFUg^{-1}	58.2	0.8-188	11	84.8
Number of chromogenic bacteria x 10^{-6}CFUg^{-1}	17.5	0.1-72.0	11	112.5
Bacterial diversity	3.8	2.5-4.8	8	21.8
Environmental variables				
pH	7.1	6.6-7.5	13	3.8
Temperature (^{o}C)	11.6	4.0-19.0	13	41.1
Dissolved oxygen (mgl^{-1})	8.5	5.8-10.3	13	18.0
BOD (mg oxygen l^{-1})	23.7	15.0-43.8	11	32.0
Conductivity (μScm^{-1})	977	472-1344	13	25.1
Turbidity (Arbitrary units)	10	5-26	13	63.7
Total amount of phenolic compounds and phenol derivatives (μgl^{-1})	912	0-7720	13	232.6

Table 4.10.b. Summary of individual phenolic compounds and phenol derivatives from Hunsworth Beck, sampling sites, April 1981 to April 1982.

Site: Hunsworth Beck 17m upstream

Variable (μgl^{-1})	Mean	Range	n	c.v.(%)
Chlorophenols				
2,4DCP	0	0	12	0
PCOC	0	0	12	0
Nitrophenols				
Picric acid	30	0-170	12	170.0
DNBP	0	0	12	0
Phenoxyalkanoic acids				
2,4DP	42	0-500	12	364.4
CMPP	64	0-430	12	235.5
2,4DB	58	0-700	12	346.4

Site: Hunsworth Beck 25m downstream

Variable (μgl^{-1})	Mean	Range	n	c.v.(%)
Chlorophenols				
2,4DCP	132	0-1400	13	297.0
PCOC	101	0-1100	13	303.5
Nitrophenols				
Picric acid	93	0-180	13	63.9
DNBP	5	0-30	13	244.1
Phenoxyalkanoic acids				
2,4DP	224	0-1030	13	171.5
CMPP	451	0-2500	13	158.1
2,4DB	0	0	13	0

Table 4.10.b. (continued)

Site: Hunsworth Beck 110m downstream

Variable (μgl^{-1})	Mean	Range	n	c.v.(%)
Chlorophenols				
2,4DCP	154	0-2000	13	361.0
PCOC	132	0-1500	13	314.0
Nitrophenols				
Picric acid	59	0-160	13	99.7
DNBP	0	0	13	0
Phenoxyalkanoic acids				
2,4DP	127	0-960	13	247.9
CMPP	440	0-3100	13	199.5
2,4DB	0	0	13	0

Table 4.11. Summary of variance ratios from oneway analysis of variance and corrected H value from Kruskal-Wallis oneway analysis of variance, for significant differences in bacteriological variables between Hunsworth Beck sampling sites (NS = non-significant).

Variable	variance ratio (F)	v_1, v_2	p
Water			
Activity	0.244	2,36	0.785 NS
Total count	0.001	2,36	0.999 NS
Viable count on CPS agar	0.092	2,36	0.912 NS
Number of chromogenic bacteria	0.105	2,36	0.901 NS
Bacterial diversity	0.000	2,24	0.999 NS
Viable count on PSM agar	0.164	2,36	0.849 NS
Gravel			
Total count	3.744	2,28	0.036
Viable count on CPS agar	1.345	2,28	0.277 NS
Number of chromogenic bacteria	1.445	2,28	0.253 NS
Bacterial diversity	2.304	2,19	0.127 NS

Variable	corrected H value	cases	p
Gravel			
Activity	1.773	31	0.412 NS

Table 4.12. Chromogenic bacteria expressed as a percentage of the viable count on CPS agar, from Hunsworth Beck sampling sites.

	% chromogenic bacteria	
Site	Water	Gravel
Hunsworth 17m Above	19.5	21.3
Hunsworth 25m Below	23.4	25.4
Hunsworth 110m Below	22.0	30.1

Table 4.13. Relationships between bacteriological and environmental variables in Hunsworth Beck (6 April 1981 - 6 April 1982). Values are Pearson's product-moment correlation coefficients (r) calculated using combined data from all Hunsworth Beck sites. A minus sign indicates a negative correlation, NS = non-significant correlation ($p>0.05$), figures in brackets refer to significance level (1) = $p<0.05$, (2) = $p<0.01$, (3) = $p<0.001$. (n varied from 20-39)

i. Water data

	Activity	Total count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity	Viable count on PSM agar
pH	NS	NS	NS	NS	NS	0.41 (1)
Temperature	0.52(2)	0.37(1)	0.72(3)	0.61(3)	-0.83(3)	NS
Dissolved oxygen	-0.38(1)	-0.61(3)	-0.46(2)	-0.40(1)	0.54(2)	NS
BOD	0.44(1)	NS	NS	NS	NS	0.56(2)
Conductivity	NS	0.44(2)	NS	NS	NS	NS
Turbidity	0.55(3)	NS	0.37(1)	NS	NS	0.74(3)
Levels of Hunsworth Beck at gauging point	NS	NS	NS	NS	NS	NS
Total amount of phenoxyalkanoic acids	NS	0.46(2)	0.50(2)	0.55(3)	NS	NS
Total amount of chlorophenols	NS	NS	0.50(2)	0.61(3)	NS	NS
Total amount of nitrophenols	0.34(1)	0.41(1)	0.49(2)	0.49(2)	NS	0.49 (2)
Total amount of phenolic compounds and phenol derivatives	NS	0.40(1)	0.53(2)	0.60(3)	NS	NS

ii. Gravel data

Variable	Activity	Total count	Viable count on CPS agar	Nos. of chromogenic bacteria	Bacterial diversity
pH	NS	NS	NS	NS	NS
Temperature	NS	NS	NS	NS	NS
Dissolved oxygen	NS	NS	NS	-0.41(1)	NS
BOD	NS	NS	NS	NS	NS
Conductivity	NS	NS	NS	NS	NS
Turbidity	NS	NS	NS	NS	NS
Level of Hunsworth Beck at gauging point	NS	NS	NS	NS	-0.51(1)
Total amount of phenoxyalkanoic acids	NS	0.38(1)	0.65(3)	0.73(3)	NS
Total amount of chlorophenols	NS	NS	0.69(3)	0.78(3)	NS
Total amount of nitrophenols	NS	NS	0.40(1)	0.42(1)	NS
Total amount of phenolic compounds and phenol derivatives	NS	NS	0.69(3)	0.78(3)	NS

Fig. 4.5. Histograms showing means of bacteriological variables and total level of phenolic compounds and phenol derivatives from the Hunsworth Beck sampling sites.

Key for histograms;

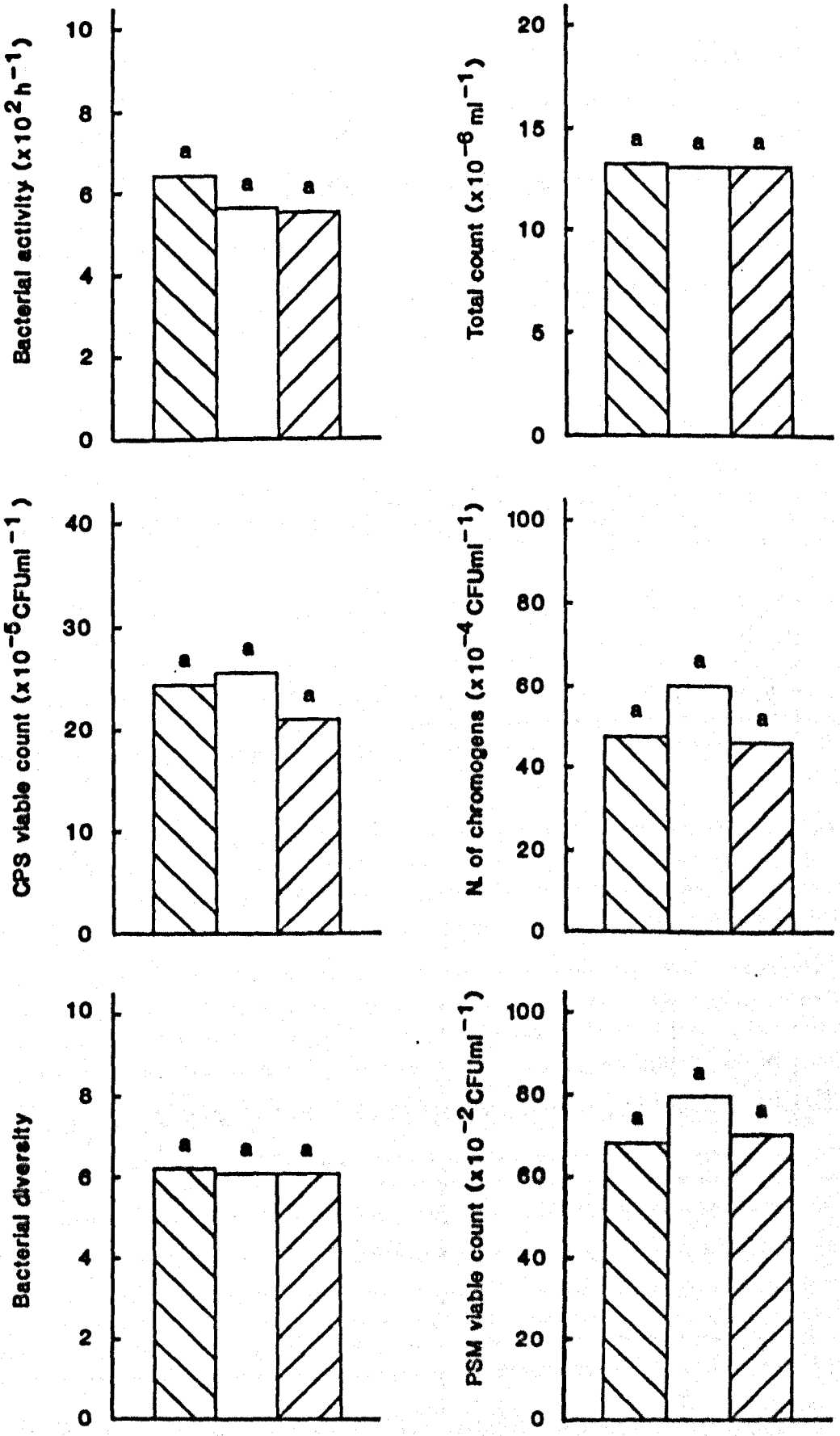
= Hunsworth Beck 17m upstream

= Hunsworth Beck 25m downstream

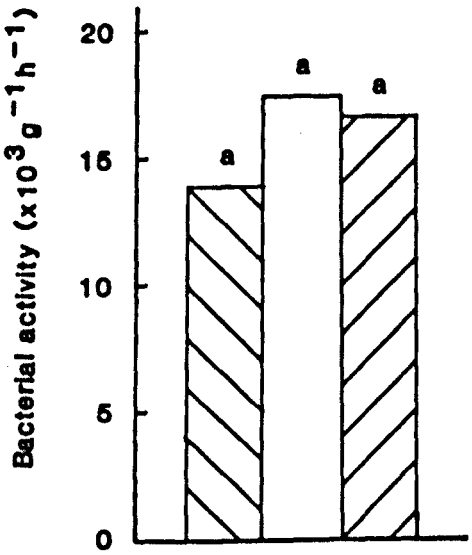
= Hunsworth Beck 110m downstream

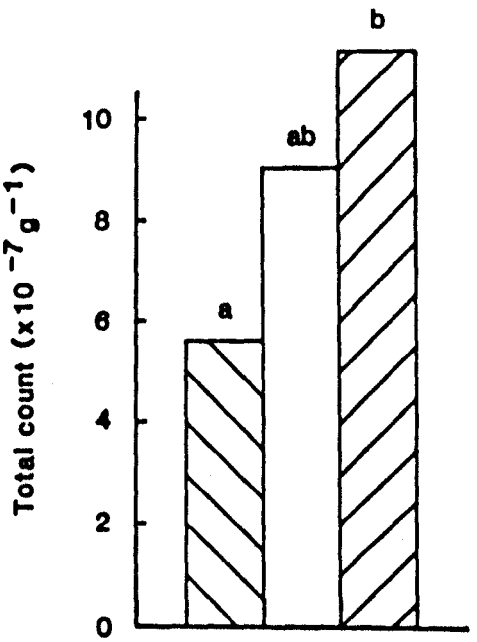
There is no significant difference ($p > 0.05$) between columns with the same superscript (anova or Kruskal-Wallis test), columns with different superscripts are significantly different ($p < 0.05$, SNK test). In the case of the concentration of phenolic compounds and phenol derivatives no statistical analysis was carried out.

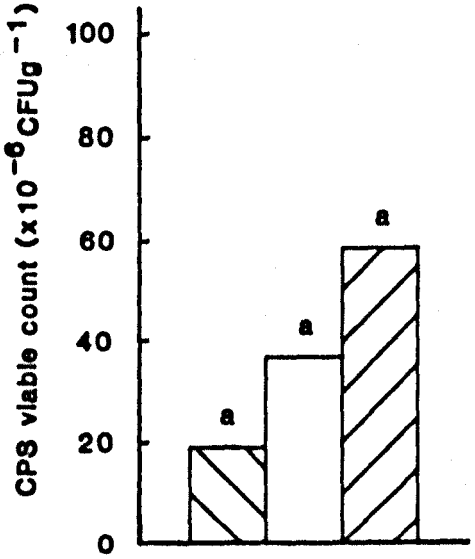
a. Water samples

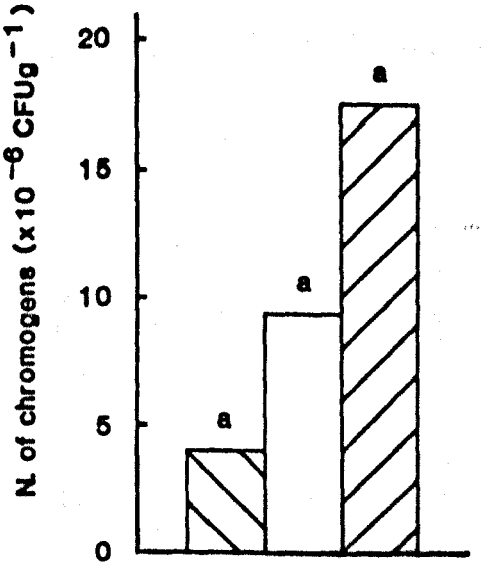


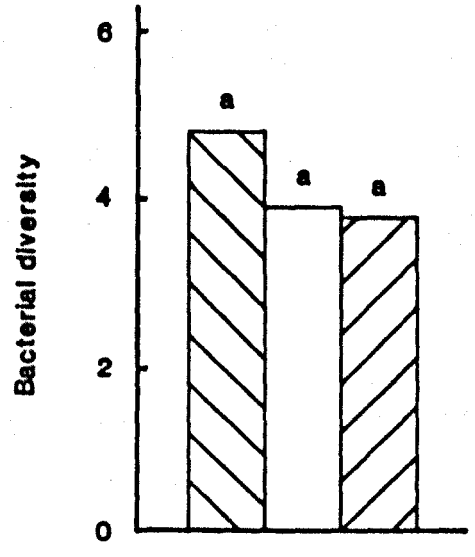
b. Gravel samples

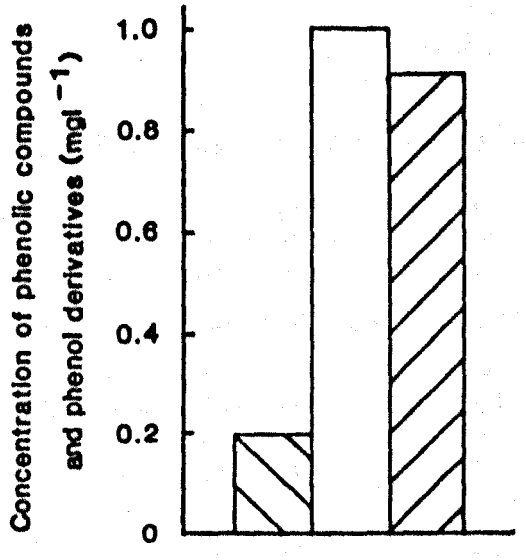












4.4. A study of the sediment bacterial community in Hunsworth Beck.

4.4.i. Introduction.

The absence of significant negative correlations between bacteriological variables with phenolic compounds and phenol derivatives (see Table 4.13.) and the increased gravel bacterial activity, total and viable counts and the number of chromogenic bacteria downstream of the confluence with Sugden Beck (see Table 4.10.a), suggested that at low concentrations these phenolic compounds and phenol derivatives may have a stimulatory effect on bacteria. A study of the natural, sediment bacterial community was undertaken to determine if there were any significant differences in viable communities upstream and downstream of the Sugden Beck confluence.

3.3.ii. Materials and methods

A total of 68 sediment samples (34 upstream and 34 downstream) were collected in sterile universal bottles, which had been calibrated to a volume of 5ml, from Hunsworth Beck on 4 May 1982. These samples were obtained by pushing, an opened, universal bottle slightly into the sediment and moving the bottle upstream until approximately 5ml of sediment had been collected. Samples were collected 10m upstream and downstream of Sugden Beck, at right angles to the South West bank. A sample was collected from the midpoint of the beck, 50cm from each bank and then two samples equally spaced between the first three samples. This was repeated every 2.5m upstream and downstream for a distance of 15m. At 25m upstream and downstream of Sugden Beck, four sediment samples, equally spaced between the banks, were collected. Sediment samples for determination of bacterial activity and viable counts were selected at random.

Bacterial activity

The method followed was similar to that of 2.1.i. Prior to determination of activity, each of the 125ml serum bottles was weighed empty and then containing 20ml of 1/4 strength Ringer solution. Excess water and sediment were removed from the universal bottles to the 5ml graduation, the contents were then scooped aseptically into the serum bottles. The universal bottles were then washed with 2 x 10ml aliquots of sterile 1/4 strength Ringer solution and added to the serum bottles, which were reweighed. The bottles were incubated as before (see 2.1.i. and 2.3.) with ^{14}C glucose and unlabelled glucose at 10^{o}C on a rotary incubator for 1 hour, at 100 r.p.m. The fraction of glucose mineralised per gram of sediment was then calculated. A total of 27 activity determinations were made (2 samples were used as blanks) for both upstream and downstream sediment samples.

Viable counts

Excess water and sediment were removed from universal bottles to the 5ml graduation and 1g of sediment aseptically removed. This was added to 1g of sterile sand, in a sterile glass vial, followed by 10ml of sterile 1/4 strength Ringer solution. After shaking the vial on a Mickle shaker (see 2.3.) for 5 minutes, a volume of 0.1ml of a 100,000 dilution was spread onto each of ten CPS agar plates. The plates were incubated at 20^{o}C for 7 days after which, the total viable count, number of chromogenic bacteria and bacterial diversity were determined (see 2.1.iii.). A total of seven sediment samples, from both the upstream and downstream sediment samples were used.

4.4.iii. Results and discussion

A summary of the means of the bacteriological variables for sediments from 10-25m upstream and downstream of the Sugden Beck confluence, and some environmental variables for water samples taken at 17m upstream

and 25m downstream of the confluence, are shown in Table 4.14. All the bacteriological variables had equal variances, and therefore, did not require transformation.

The study of the natural sediment bacterial communities showed that all of the bacteriological variables had similar values both upstream and downstream of the confluence with Sugden Beck. A t-test showed that there were no significant differences between the upstream and downstream sediment bacteriological variables (see Table 4.15.). The non-parametric Mann-Whitney U-test confirmed that there were no significant differences for any of the bacteriological variables (see also Table 4.15.).

These results showed no significant effect of Sugden Beck on the sediment bacterial community in Hunsworth Beck. This suggested that the large differences in gravel samples from the routine sampling programme (see Table 4.10.a.), were due to other environmental factors, such as local variation in current velocity, rather than to a stimulatory effect of phenolic compounds and phenol derivatives on bacterial communities.

Table 4.14. Summary of means of bacteriological variables from Hunsworth Beck sediment samples and environmental variables for water samples. Sediment samples from 10-25m upstream and downstream of Sugden Beck confluence; water samples from 17m upstream and 25m downstream of confluence.

Upstream sediment samples

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}g^{-1}h^{-1}$	20.3	12.9-32.8	25	25.5
Viable count on CPS agar x $10^{-7}CFUg^{-1}$	42.3	15.9-89.8	7	54.6
Number of chromogenic bacteria x $10^{-7}CFUg^{-1}$	9.8	3.1-23.6	7	66.3
Bacterial diversity	1.4	1.2-1.5	7	9.2
Environmental variables				
pH	7.2	-	1	-
Temperature (^{o}C)	10.0	-	1	-
Dissolved oxygen (mgl^{-1})	9.0	-	1	-
Conductivity (μScm^{-1})	1430	-	1	-

Downstream sediment samples

Variable	Mean	Range	n	c.v.(%)
Activity x $10^{3}g^{-1}h^{-1}$	22.5	15.2-34.0	25	22.9
Viable count on CPS agar x $10^{-7}CFUg^{-1}$	37.4	17.0-53.0	7	43.8
Number of chromogenic bacteria x $10^{-7}CFUg^{-1}$	8.4	3.1-14.4	7	59.7
Bacterial diversity	1.4	0.9-1.7	7	19.9
Environmental variables				
pH	7.2	-	1	-
Temperature (^{o}C)	10.0	-	1	-
Dissolved oxygen (mgl^{-1})	8.6	-	1	-
Conductivity (μScm^{-1})	1430	-	1	-

Table 4.15. Summary of t-test and Mann-Whitney U-test for significant differences in bacteriological variables between sediment samples collected upstream and downstream of the Sugden Beck confluence (NS = non-significant).

t-test

Bacteriological variable	t-value	n	p
Activity	1.48	48	0.146 NS
Viable count	0.46	12	0.653 NS
Number of chromogenic bacteria	0.47	12	0.644 NS
Bacterial diversity	0.38	12	0.711 NS

Mann-Whitney U-test

Bacteriological variable	U	n_1, n_2	p
Activity	240.0	25,25	0.160 NS
Viable count	22.0	7,7	0.805 NS
Number of chromogenic bacteria	23.0	7,7	0.902 NS
Bacterial diversity	23.0	7,7	0.902 NS

CHAPTER 5

COMPARISON OF FRESHWATER BACTERIAL COMMUNITIES FROM STUBS BECK AND SUGDEN BECK

The routine sampling programme at the Sugden Beck sites, showed that the presence of phenolic compounds and phenol derivatives caused a change in composition of the bacterial community in both water and gravel samples. Compared with Stubs Beck, the bacterial communities in Sugden Beck had a reduced bacterial diversity, a lower percentage of chromogenic bacteria and very much increased viable counts on PSM agar. This chapter further investigates the differences in bacterial communities, between Stubs Beck and Sugden Beck.

5.1. Taxonomic study of bacteria isolated from the sediment of Stubs Beck and Sugden Beck

This taxonomic study investigated the differences in composition of sediment bacterial communities, between Stubs Beck and Sugden Beck, by identifying to genus level isolates of bacteria taken from each stream.

5.1.i. Isolation of bacteria from Stubs Beck and Sugden Beck

Sediment samples were collected, in sterile universal bottles, from the Stubs Beck and Sugden Beck Upper sampling sites (see 4.1.), on 21 July 1982 and 16 August 1982. A weight of 1g of sediment was removed from these samples and added to a sterile universal bottle containing 10ml of 1/4 strength Ringer solution. The contents were thoroughly mixed on a Whirlimix for 60s, and diluted (10,000x) using sterile 1/4 strength Ringer solution. A small volume (0.1ml) was then spread over the surface of each of ten CPS agar plates, which were incubated for 7 days at 22^{0}C. After incubation, five bacterial colonies were picked-off at random from

each plate. These colonies were selected by placing the petri-dish on a 10cm by 10cm grid of 1cm squares, numbered 1 to 100, squares were then chosen by random numbers. If a square contained more than one colony, the colony nearest the centre was chosen, any colony which crossed the edge of a square was discounted. This procedure was repeated on the second sampling occasion, giving a total of 100 bacterial isolates from both Stubs Beck and Sugden Beck.

Selected colonies were streaked onto nutrient agar (NA) plates (see Appendix 2) and incubated for 4-5 days at 22°C. A single colony was then removed and placed into nutrient broth (NB), if no growth had taken place a new isolate was selected at random from the initial CPS plate. The NB was incubated at 22°C for 2 days or until turbid, and was then used to inoculate a NA slope, which was incubated at 22°C and stored at 4°C after growth had occurred. The NB culture was also stored at 4°C and was used as a stock culture throughout the identification tests.

5.1.ii. Identification scheme

The identification scheme used was based on a scheme outlined by Harrigan and McCance (1976), with additional information provided by Buchanan and Gibbons (1974). In this scheme three initial tests were used to classify the bacteria into various groups; the isolates were then identified to genus level by a further range of biochemical and physiological tests. The composition of stains and media used in this identification scheme are shown in Appendix 2.

Following initial isolation all the isolates were incubated at 22°C and 30°C, any isolates which failed to grow at the higher temperature were incubated at 22°C for the biochemical and physiological tests. Isolates capable of growth at this higher temperature, were incubated at 30°C unless otherwise stated.

5.1.ii.a. Initial tests

Gram stain

A loopful of the stock NB culture was used to inoculate a NA plate, which was incubated at 22°C or 30°C for 24 hours. A single colony was then removed and emulsified, in a drop of sterile distilled water placed on a clean glass microscope slide. The slide was allowed to air-dry and then heat-fixed, by passing three times through a bunsen flame. After allowing the slide to cool, the smear was stained with a crystal violet solution for 1-2 minutes. The crystal violet stain was removed by rinsing in water and the slide was then flooded with Gram's iodine solution for 1 minute. The iodine was poured off and the smear decolourized with 95% ethanol for 10s. A final counterstain of safranin O solution was applied for 10s, and then the slide was rinsed with water and blotted dry. The stained bacteria were viewed under oil-immersion (magnification 1000x); Gram-positive bacteria did not decolourize with ethanol and appeared purple, Gram-negative bacteria appeared pink. This test was repeated after 48 hours.

Colony pigmentation

The NA plate used for Gram's stain was reincubated at 22°C or 30°C; colony morphology and pigmentation were recorded at 3 days and finally after 7 days.

Hugh and Leifson's oxidation-fermentation test (O/F test)

In the Hugh and Leifson's oxidation-fermentation test the ability of a bacterial isolate to break down a carbohydrate (glucose), under aerobic and anaerobic conditions was determined. However, as carbohydrates may be altered when exposed to normal heat-sterilisation temperatures, particularly when the media is alkaline or contains peptone and phosphates, the basal ingredients were sterilised separately.

A 10% (w/v) glucose solution was sterilised by filtering through a Seitz filter into a sterile 150ml Buchner flask. A volume of 1ml of the sterile glucose was added to a test tube containing 10ml of freshly sterilised basal ingredients, which had been cooled to about 50°C. The basal ingredients were sterilised at 121°C for 15 minutes. After the media had cooled, duplicate tubes were stab inoculated with the stock NB culture, and anaerobic conditions created in one of the tubes by covering the surface of the medium with a 10-15mm layer of sterile liquid paraffin. Both sets of tubes were incubated, together with uninoculated control tubes for 14 days at 22°C or 30°C. A few isolates, which showed poor growth or gave an intermediate reaction, were re-incubated for a further 14 days.

The isolates were classified as oxidative or fermentative organisms on the basis of the colour change in the aerobic and anaerobic tubes. Aerobic or anaerobic breakdown of glucose produced acid, which caused a colour change of the indicator present from green to yellow. However, oxidative organisms only produce acid in the aerobic tube, whereas fermentative organisms produce acid in both tubes (see Plate 5.1.). Organisms that were unable to break down glucose, aerobically or anaerobically, produced a blue alkaline reaction in the aerobic tube and no change in the anaerobic tube.

These three initial tests were used to classify the isolates into five major divisions (see Table 5.1.). The following biochemical and physiological tests were then carried out to identify the isolates to genus level.

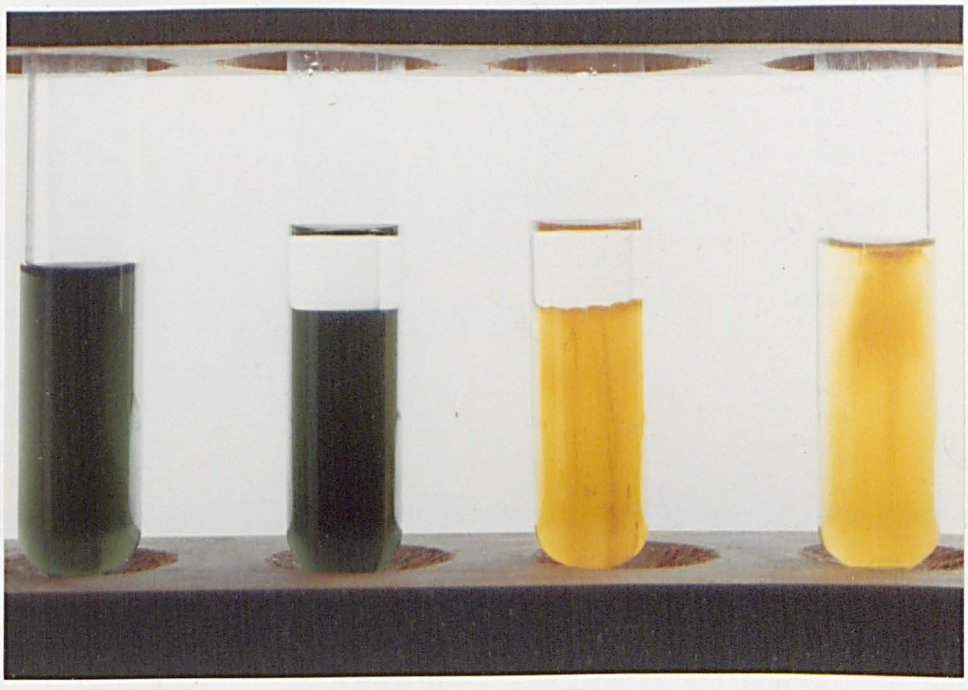
5.1.ii.b. Biochemical tests used to identify isolates

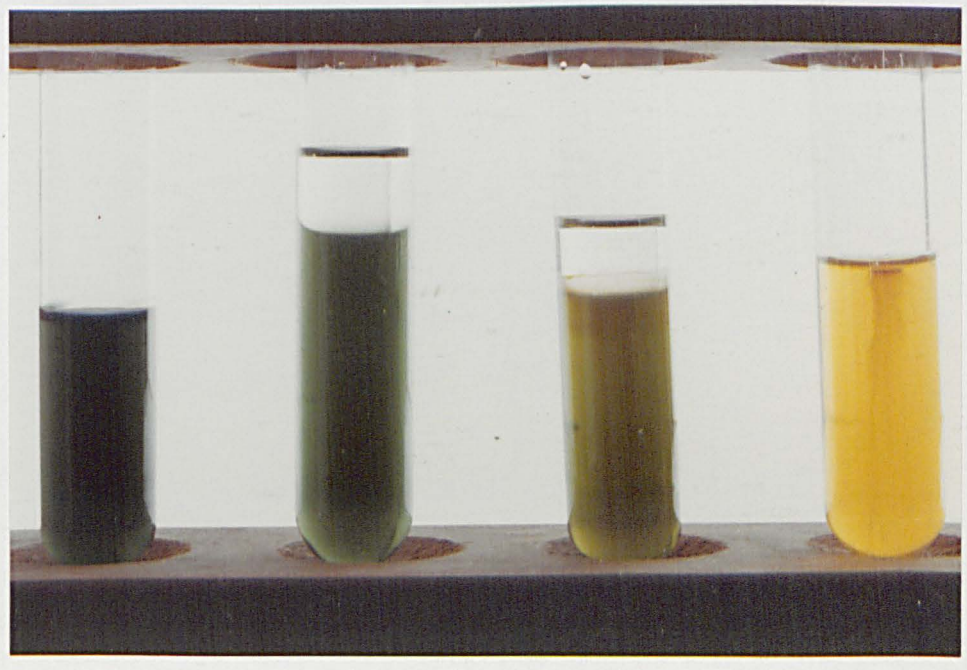
Catalase test

The presence of the catalase enzyme was determined by adding a few drops of 20 vols hydrogen peroxide, to a 7 day old colony on NA. Bubbles

Plate 5.1. Characteristic oxidative and fermentative metabolism of glucose in Hugh and Leifson's oxidation-fermentation test.

Fermentative bacterial isolates (top) produce acid from glucose in both aerobic and anaerobic (with liquid-paraffin layer) tubes. There is no colour change in the uninoculated control tubes (left). Oxidative bacterial isolates (bottom) produce acid from glucose in the aerobic tube only. There is no colour change in the uninoculated control tubes or inoculated anaerobic tube.





of oxygen were evolved when the reaction was positive.

Oxidase test

The presence or absence of the enzyme oxidase was established using a 1% aqueous solution of tetramethyl-4-phenylene diamine dihydrochloride. Auto-oxidation of this solution was delayed by storage in the dark at 4°C, however, the solution was discarded once it developed a deep-purple colour.

A few drops of the oxidase reagent were added to a filter paper strip which was then streaked, using a platinum loop, with a colony from a 7 day NA plate. Oxidase-positive isolates developed a deep-purple colour within 5-10s, a purple colour between 10-60s was recorded as delayed positive. Any reaction later than 60s was recorded as negative and the result checked against a known oxidase-negative Salmonella sp.

Penicillin sensitivity

The sensitivity of the isolates to penicillin was determined using Oxoid multidisks containing penicillin G (1.5 units). A lawn of bacteria was prepared by spreading 0.1-0.2ml of a 24 hour NB culture onto a NA plate. An Oxoid multidisk was placed on the plate, which was then incubated at 22°C or 30°C for 3 days or until growth occurred. Sensitive isolates had a zone of clearing 2mm or greater around the disc, whereas resistant isolates grew right up to the edge of the disc.

Sensitivity to compound 0/129

Sensitivity to the vibriostatic agent; 2,4-diamino-6,7-diisopropylpteridine (compound 0/129), was tested by sprinkling a few crystals of this compound onto a bacterial lawn. Sensitivity to the compound resulted in a zone of clearing around the crystals. In practice this test was carried out in conjunction with the penicillin sensitivity test, as both tests were easily accommodated on the same NA plate.

Production of extracellular fluorescent pigments

The production of extracellular (diffusible) fluorescent pigments is characteristic of many Pseudomonas sp. (Harrigan and McCance, 1976), although it is dependent on the composition of the medium.

A loopful of the stock NB culture was streaked onto plates of fluorescence agar, which were then incubated at 22°C or 30°C for 7 days. The presence of an extracellular fluorescent pigment was then determined by viewing the plate under ultraviolet radiation, see Plate 5.2.

Gelatin liquefaction

The ability of an isolate to liquefy gelatin was determined by transferring a loop of the NB stock culture into fresh nutrient broth, to which was aseptically added a charcoal-gelatin-disc (Oxoid, chargel). The broth was then incubated at 22°C for 7-14 days; gelatin liquefaction was shown by the presence of charcoal fragments in the bottom of the container.

Starch hydrolysis

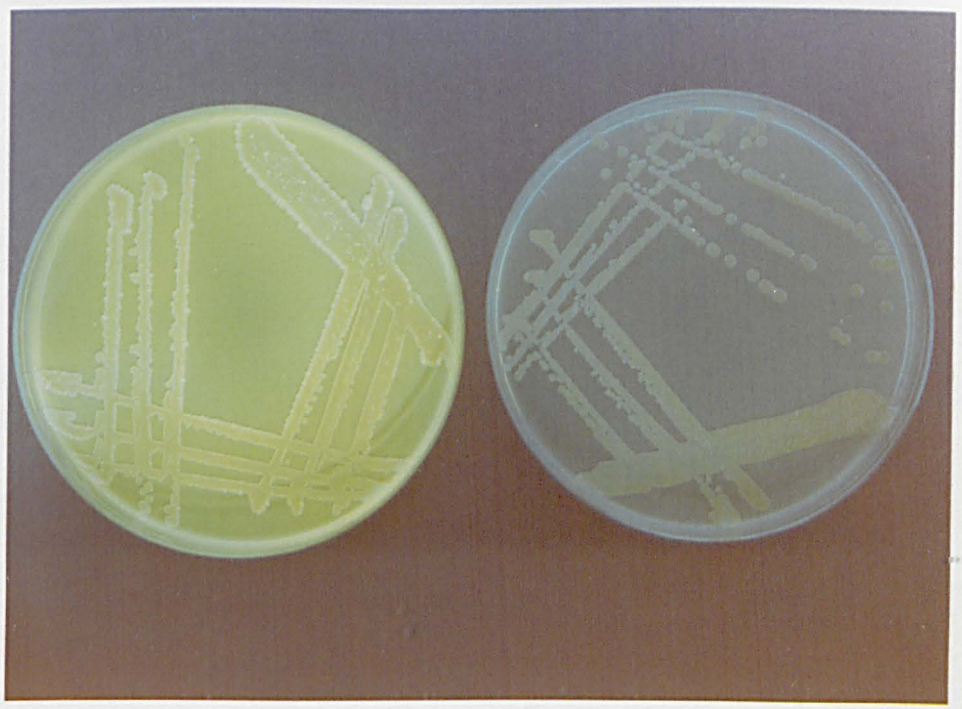
The hydrolysis of starch was tested by streaking a loopful of the stock NB culture over the surface of a NA plate containing 1% (w/v) soluble starch. The plates were incubated at 22°C for 7 days and then flooded with Gram's iodine solution. Clear or reddish-brown zones surrounding the colonies indicated starch hydrolysis.

Arginine hydrolysis

The anaerobic utilization of arginine produces ammonia, which causes a colour change of a phenol red indicator from salmon pink to red. This test was used to differentiate Gram-negative bacteria; Pseudomonas sp. and Aeromonas sp. can growanaerobically by using arginine for ATP generation whereas, members of the Enterobacteriaceae cannot (Harrigan and McCance, 1976). Thornley's semisolid arginine medium was used to

Plate 5.2. Appearance of bacterial isolates grown on fluorescence agar under ultraviolet irradiation.

The bacterial isolate grown on the agar plate on the left produces an extracellular fluorescent pigment, which causes the agar medium to fluoresce strongly under U.V. radiation. The other bacterial isolate (right) does not produce an extracellular fluorescent pigment.



detect arginine hydrolysis.

The arginine medium was dispensed in 5ml bijou bottles and sterilised at 121°C for 15 minutes. The cooled media was stab inoculated with the stock NB culture and covered with a layer of sterile liquid paraffin. Incubation, with a sterile control, was at 22°C or 30°C for 4 days; typical reactions are shown in Plate 5.3.

5.1.ii.c. Physiological tests used to identify isolates

Motility

A hanging-drop method was used to determine if the isolates were motile, this method involved the use of a glass cavity slide and coverslip. The central depression of the cavity slide was surrounded by a thin layer of vaseline, a drop of an 18 hour (22°C) NB culture was placed on the coverslip and the inverted depression of the cavity slide placed over the drop. By quickly reverting the slide a hanging drop was formed. This was transferred to a microscope and the edge of the drop viewed without oil immersion (magnification 400x).

An isolate was regarded as showing motility if the bacteria changed their relative position in the field of view. In practice, it was easy to differentiate between Brownian motion, which caused a 'jogging motion', drifting, caused by a tilted slide and true motility. Fuzzy outgrowths from the centre stab-line in Hugh and Leifson's oxidation-fermentation test and in the Arginine hydrolysis test also indicated motility.

Position of flagella

Special statining techniques are required to determine the position of flagella as they are normally below the resolution of light microscopes (Harrigan and McCance, 1976). In the method of Rhodes (1958) silver was deposited on flagella, causing an apparent increase in diameter, so allowing subsequent visualisation with a light microscope.

Plate 5.3. Characteristic reactions of the Arginine hydrolysis test.

The growth of a bacterial isolate capable of utilizing arginine anaerobically causes a colour change of the phenol red indicator in Thornley's semi-solid arginine medium (right), compared with an uninoculated control (centre). Bacterial isolates which cannot utilize arginine anaerobically, show a negative reaction with no colour change (left).

A slight modification of the Rhodes (1958) method was found to give acceptable results with a wide range of isolates.

Two reagents were used;

Ferric tannate mordant

A volume of 5ml saturated aqueous aluminium potassium sulphate was added to 10ml 10% (w/v) tannic acid in distilled water, followed by 1ml of a saturated aqueous solution of aniline. The resultant precipitate was redissolved by shaking, 1ml of 5% (w/v) ferric chloride was then added. This gave a black solution which was allowed to stand for 10 minutes prior to use.

Ammoniacal silver nitrate solution

Concentrated ammonia solution (Sp. gr. 0.880) was slowly added to 5% (w/v) silver nitrate in distilled water until the brown precipitate redissolved. Drops of fresh silver nitrate solution were added until the solution remained faintly cloudy, even after shaking. This reagent was stable for several weeks when stored in the dark at $4^{o}C$.

Isolates were incubated on yeast extract agar slopes, at $22^{o}C$, for 18-24 hours, or 48 hours if the isolate was slow growing. As motile organisms were most likely to be obtained from the liquid at the base of the slope, 1ml of sterile distilled water was added after inoculation. Two loopfuls of the liquid, at the base of the slope, were incubated in 5ml of sterile distilled water for 1 hour to remove debris and excess mucilage from the organisms. A drop was then transferred to a new glass slide and allowed to air-dry. The smear was covered with the iron-tannate mordant for 3-5 minutes and then washed thoroughly with distilled water. Meanwhile, the silver nitrate reagent was heated nearly to boiling and applied to the slide for 2.5 minutes. This was removed with distilled water and fresh silver stain added for a further 2.5 minutes.

After washing thoroughly with distilled water and blotting-dry, the slide was viewed under oil-immersion (magnification 1000x).

This method proved adequate with the majority of motile isolates, however, some isolates proved difficult to stain without the delicate flagella becoming detached. In addition the stain gave a background precipitate, which on occasion made observation of flagella difficult. To overcome these problems, the position of flagella on motile isolates, which could not be determined by silver staining, were determined using transmission electron microscopy, following a negative-staining technique (Gregory and Pirie, 1973).

Preparation of isolates for transmission electron-microscopy

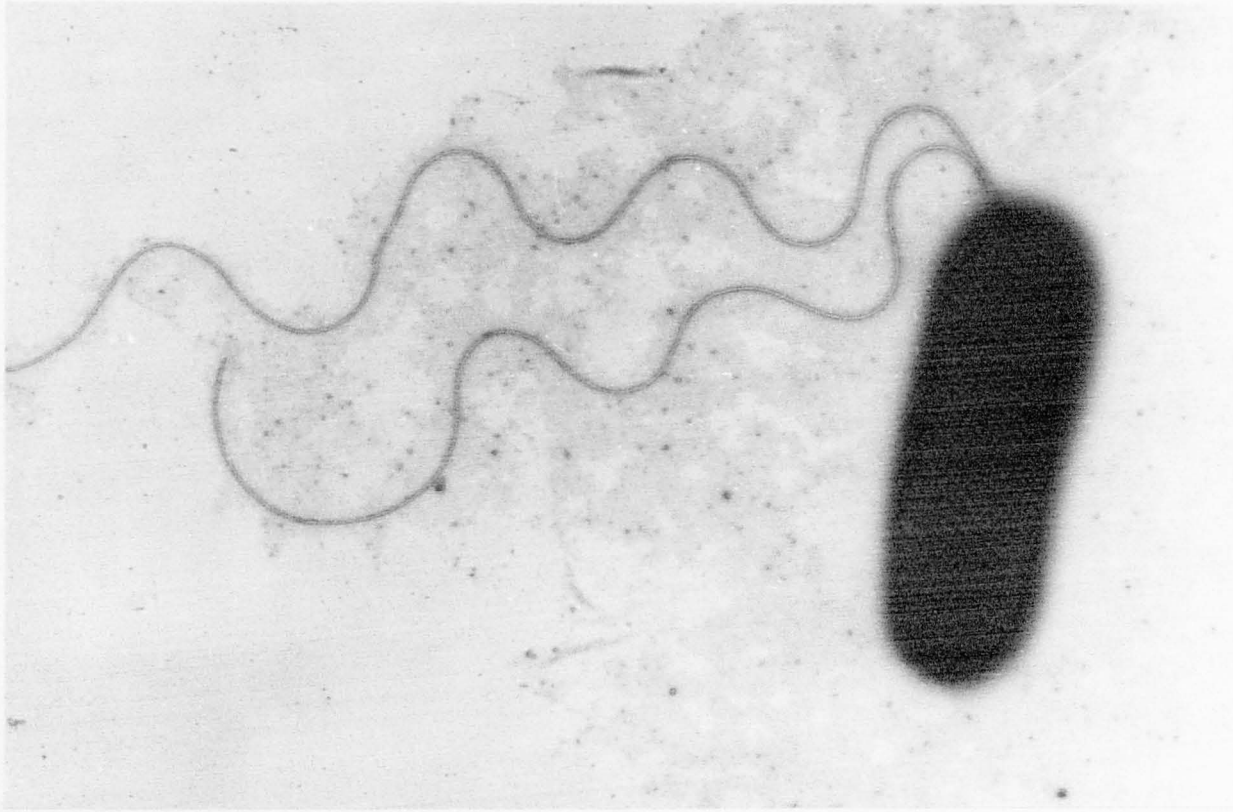
A single colony was picked off a 7 day NA plate, which had been incubated at 20°C, and inoculated into 10ml of NB and incubated at 20°C for 24 hr. A loopful of this culture was then transferred to fresh NB and reincubated for a further 24 hours at 20°C, to encourage flagella production. The cells were then precipitated by centrifugation (RCF c.500) for 10 minutes, and washed three times in 0.1M ammonium acetate in distilled water to remove nutrient broth. The cells were finally resuspended in 0.1M ammonium acetate. One drop of this suspension was then placed on a formvar coated grid for 5 minutes, after which excess liquid was removed by careful blotting with filter paper. The cells were stained by placing a drop of 0.5% (w/v) uranyl acetate in distilled water, on the grid for 1 minute. Excess stain was then removed with filter paper and the grids allowed to air-dry. The grids were examined in a Jeol 100c electron microscope operating at 80 or 100KV, with a 50μm thin foil objective aperture and using a liquid-nitrogen cooled anti-contamination device. Plate 5.3 shows a photomicrograph of a polarly flagellate organism.

Plate 5.4. Appearance of a polarly-flagellate bacterial isolate following negative staining with uranyl acetate.

Magnification 20,000x, bar represents 1μm.

Plate 5.4. Appearance of a polarly-flagellate bacterial isolate following negative staining with uranyl acetate.

Magnification 20,000x, bar represents 1μm.



5.1.iii. The importance of pure cultures in taxonomy

Characteristics used in the identification of bacteria, depend on the behaviour of pure cultures containing one strain of the organism. The use of mixed cultures or subsequent contamination of pure cultures, may lead to errant results and hence a false identification.

Isolates were initially obtained by picking-off single colonies from the original CPS agar plates and streaking onto NA plates. After incubation a single colony was selected, which had the same morphological characteristics as the original isolate, and grown as a stock culture in NB. This NB culture was assumed to contain only one strain of the bacterial isolate. Many of the biochemical and physiological tests carried out, allowed the purity of the isolate to be checked;

a. Staining techniques - particularly Gram's stain, showed any morphological differences between bacteria and allowed the detection of mixed cultures of Gram-positive and Gram-negative organisms.

b. Biochemical tests - several tests involved the use of agar plates; production of extracellular fluorescent-pigments, starch hydrolysis and colony pigmentation, these tests readily showed any differences in colony morphology or pigmentation indicating a mixed culture.

A final check on the purity of the isolate, after identification, was made by streaking a loopful of the stock culture onto a NA plate and incubating at 22°C or 30°C for 5 days. Contamination of cultures during identification was avoided by using aseptic technique throughout.

If at any stage a mixed culture was suspected, the isolate was removed from the taxonomic study.

5.1.iv. Results and discussion

Before discussing the results of this taxonomic study it must be mentioned that the isolation technique employed, only isolated those aerobic-heterotrophic bacteria capable of growth on CPS agar, under the incubation conditions used. CPS agar was used in the initial isolation as

it was recommended for counting heterotrophic bacteria in freshwater by Jones (1970), and Staples and Fry (1973). A further selection of bacteria occurred on transferring isolates from CPS agar to NA, as about 10% of the Stubs Beck CPS isolates failed to grow on NA, although all the isolates from Sugden Beck CPS plates grew on NA.

Thirteen of the initial 200 isolates were mixed cultures and were, therefore, removed from the taxonomic study. Using the identification scheme shown in Table 5.1., 97 isolates from Sugden Beck and 90 isolates from Stubs Beck, were classified in the following genera or groups (in order of divisions);

Chromobacterium

Isolates placed in the genus Chromobacterium produced an intracellular purple pigment. They were fermentative, motile and catalase positive, although the pigment interfered with the oxidase test. The purple pigment was soluble in ethanol, insoluble in water and became green in 10% ethanolic solution of H_2SO_4, which is characteristic of the pigment of Chromobacterium sp (Harrigan and McCance, 1976).

Flavobacterium

The colour genus Flavobacterium is known to be heterogenous for many characteristics (Buchanan and Gibbons, 1974). All isolates placed in this genus were; yellow or orange pigmented, unable to utilise glucose aerobically or anaerobically and resistant to penicillin. Motility and the presence of catalase varied, although the majority of isolates were non-motile, catalase positive and oxidase positive.

Aeromonas

All isolates were; non-pigmented, fermentative, motile, oxidase and catalase positive, and resistant to both penicillin and the vibriostatic agent compound 0/129. In addition they liquefied gelatin and

hydrolysed both starch and arginine.

Enterobacteriaceae group

Isolates placed in this group, on the basis of the initial tests, were tentatively identified to genus level using API 20e test strips (API Laboratory Ltd., Basingstoke).

Pseudomonas

All isolates placed in the genus Pseudomonas were; non-pigmented, catalase positive, motile, polarly flagellate and resistant to both penicillin and compound 0/129. Isolates varied in their oxidase reaction and their ability to hydrolyse arginine, oxidise glucose and produce an extracellular fluorescent pigment. A few isolates were; unable to utilise glucose aerobically or anaerobically, oxidase negative and arginine negative, none of these isolates produced a fluorescent pigment.

Gram-positive bacteria

Gram-positive bacteria were not identified to genus level. They were classified as Gram-positive rods, cocci or belonging to the Streptomyces group. The Streptomyces group were easily identified by their characteristic many-branched mycelium, formation of hard colonies, which were partially embedded in agar medium and the production of an extracellular pigment. In addition, they produced an odour characteristic of moist soil.

Unidentified Gram-negative bacteria

Any unidentified Gram-negative bacteria were placed in this group.

The distribution of these genera and groups in Stubs Beck and Sugden Beck, using combined data from both sampling occasions, are shown in Table 5.2. Table 5.3. shows the relative frequency of Gram-negative and Gram-positive organisms in Stubs Beck and Sugden Beck.

Table 5.3. shows that the sediment heterotrophic bacterial community in Stubs Beck was dominated by Gram-negative organisms. A similar percentage of Gram-negative bacteria was found by Baker and Farr (1977) in sediment from the River Frome, where 74% of isolates on CPS agar were Gram-negative. Gram-negative bacteria are also known to dominate freshwater planktonic bacterial communities. Baker and Farr (1977), and Nuttall (1982a) studied the Gram-stain reaction of bacteria, isolated on CPS agar, from the River Frome and River Dee respectively. In the River Frome 87% of bacterial isolates were Gram-negative and 93% in the River Dee.

Table 5.2. showed that of the Gram-negative organisms isolated from Stubs Beck, bacteria belonging to the genera; Pseudomonas, Flavobacterium, Aeromonas and Acinetobacter were numerically the most important. These genera have been shown by other workers, to be important in freshwater sediment and planktonic bacterial communities. Inniss and Mayfield (1979) found that of the bacteria isolated from Lake Ontario sediment, Pseudomonas and Flavobacterium were the most frequently occurring genera. Bacteria belonging to these genera usually accounted for over 70% of all sediment bacterial isolates. Other Gram-negative genera found were; Aeromonas, Chromobacterium and Alcaligenes. Bacteria belonging to the genera Pseudomonas, Flavobacterium and Acinetobacter were numerically the most important genera isolated in water samples, from the River Frome (Baker and Farr, 1977) and River Dee (Nuttall, 1982a), although Aeromonas sp and Enterobacteriaceae were also isolated. Bell et al. (1980) showed that Pseudomonas was the single most important genus isolated in water samples, from two Canadian rivers. Other Gram-negative genera isolated were; Flavobacterium, Aeromonas, Chromobacterium, Acinetobacter, Alcaligenes and various Enterobacteriaceae.

In common with Stubs Beck, Table 5.3. showed that heterotrophic bacteria, isolated from Sugden Beck sediment, were dominated by Gram-

negative organisms. However, the relative proportion of Gram-negative bacteria was much higher in Sugden Beck, which therefore had a much reduced proportion of Gram-positive bacteria, compared with Stubs Beck.

Table 5.2. showed that, unlike Stubs Beck, the heterotrophic-bacterial community in Sugden Beck was composed of relatively few different genera, with over 90% of the isolated bacteria belonging to the genus Pseudomonas. No Gram-negative bacteria belonging to the genera; Flavobacterium, Aeromonas, Acinetobacter, Chromobacterium or to the Enterobacteriaceae were detected in Sugden Beck.

This taxonomic study showed that Gram-negative bacteria formed the major portion of sediment bacterial communities in both streams. In Stubs Beck there was a wide range of different genera present, whereas in Sugden Beck, which contained phenolic compounds and phenol derivatives, the bacterialcommunity was composed of bacteria belonging to the genus Pseudomonas. These results agree well with the results of the routine sampling programme, undertaken in Sugden Beck, which showed that the bacterial community in both water and gravel samples had a low species diversity, reduced percentage chromogenic bacteria and very much increased viable counts on PSM agar compared with Stubs Beck.

Clearly, the results of this taxonomic study suggest that adaptation to the presence of phenolic compounds and phenol derivatives, in Sugden Beck, results in a shift in species composition away from a diverse bacterial community, containing a wide range of Gram-negative and Gram-positive genera, to one comprised of Gram-negative Pseudomonas spp. Therefore, bacteria belonging to the genus Pseudomonas were more tolerant to phenolic compounds and phenol derivatives (except under acid pH conditions) compared to other Gram-negative and Gram-positive genera. Thus, phenolic compounds and phenol derivatives, were thought to have a differential toxicity to freshwater bacteria.

Table 5.1. Scheme used for the identification of bacteria isolated from Stubs Beck and Sugden Beck sediment samples.

Key; + = Positive reaction

- = Negative reaction

V = Variable reaction

V(+) = Variable, most strains positive

V(-) = Variable, most strains negative

? = Test not applied or significant or result unknown.

DIVISION 1: Gram-negative bacteria producing an intracellular (non-diffusible) purple pigment.

Genus; Chromobacterium

DIVISION 2: Gram-negative bacteria producing an intracellular yellow or orange pigment.

Group or genus	O/F test	Motile	Position of flagella	Oxidase test
Flavobacterium	Oxidative or no attack	V(-)	peritrichous	?
Pseudomonas	Oxidative	+	polar	+ (occasionally -ve)
Enterobacteriaceae	Fermentative	V(+)	peritrichous	-

DIVISION 3: Gram-negative, non-pigmented fermentative bacteria.

Group or genus	Oxidase test	Motile	Position of flagella	Sensitivity to O/129
Vibrio	+	+	polar	+
Aeromonas	+	+	polar	-
Plesiomonas	+	+	polar	V(+)
Enterobacteriaceae	-	V(+)	peritrichous	-

continued

Table 5.1. (continued)

Group or genus	Arginine hydrolysis	Gelatin Liquefaction	Starch Hydrolysis
Vibrio	?	+	+
Aeromonas	+	+	+
Plesiomonas	?	-	-
Enterobacteriaceae	V(-)	V(-)	?

DIVISION 4: Gram-negative, non-pigmented bacteria that do not ferment glucose and bacteria producing a yellow, green-yellow or green extracellular (diffusible) pigment.

Genus	Motile	Position of flagella	Glucose oxidised	Penicillin sensitivity
Agrobacterium	+	peritrichous	+	-
Pseudomonas	+	polar	V	-
Acinetobacter	-	?	+	-
Moraxella	-	?	-	+
Alcaligenes	+	peritrichous	-	-

continued

Genus	Extracellular fluorescent pigment	Thornley's arginine test	Oxidase test
Agrobacterium	-	-	+
Pseudomonas	V	V(+)	V(+)
Acinetobacter	-	V	-
Moraxella	-	?	+
Alcaligenes	-	V(+)	+

DIVISION 5: Gram-positive bacteria.

Table 5.2. Relative frequency of genera or groups of bacteria in Stubs Beck and Sugden Beck. Total number of isolates identified; Stubs Beck 90 and Sugden Beck 97. Figures in brackets refer to the number of isolates in each genera or groups.

Genera or group	Stubs Beck frequency (%)	Sugden Beck frequency (%)
Pseudomonas	27.8 (25)	93.8 (91)
Flavobacterium	17.8 (16)	
Aeromonas	15.6 (14)	
Acinetobacter	6.7 (6)	
Unidentified Gram-negative rods	3.3 (3)	4.1 (4)
Chromobacterium	1.1 (1)	
Escherichia	1.1 (1)	
Yersinia	1.1 (1)	
Unidentified Enterobacteriaceae	1.1 (1)	
Gram-positive rods	18.8 (17)	2.1 (2)
Gram-positive cocci	2.2 (2)	
Streptomyces	3.3 (3)	

Table 5.3. Relative frequency of Gram-negative and Gram-positive organisms in Stubs Beck and Sugden Beck. Figures in brackets refer to the number of Gram-negative and Gram-positive isolates.

Site	Frequency of Gram-negative organisms (%)		Frequency of Gram-positive organisms (%)	
Stubs Beck	75.6	(68)	24.4	(22)
Sugden Beck	97.9	(95)	2.1	(2)

5.2. Morphological study of bacteria in Stubs Beck and Sugden Beck using transmission electron microscopy.

Differences in morphology of suspended bacteria between Stubs Beck and Sugden Beck were studied, using transmission electron-microscopy, following negative staining of bacteria using uranyl acetate. The staining method used was similar to the method for determining flagellal position with an electron microscope (see 5.1.i.c).

5.2.i. Materials and Methods

Water samples were collected from the Stubs Beck and Sugden Beck Upper sampling sites on 5 November 1982, in separate sterile 300ml glass bottles. Both samples were then pre-filtered through GF/C Whatman glass-fibre filters, into sterile Buchner flasks, to remove any suspended solids. Using sterile filtering equipment, 100ml of the filtrate was filtered through a 0.22μm cellulose acetate (Oxoid) membrane-filter. The membrane-filter was then placed in a sterile universal bottle together with 5ml of, 0.22μm membrane-filtered, 0.1M ammonium acetate in distilled water. Bacteria were removed from the membrane filter by agitating the universal bottle for 30s. A drop of the bacterial suspension was placed on a formvar-coated grid, and the grid placed in an oven for 15 minutes at 70^{o}C. This caused the drop to evaporate and the bacteria present to be deposited onto the grid. After allowing the grid to cool, the bacteria were stained for 1 minute, by placing a drop of 0.5% (w/v) aqueous solution of uranyl acetate on the grid. The excess stain was then removed by careful blotting and the grid allowed to air-dry.

The grids were examined in a Jeol 100c electron microscope operating at 80KV with a 50μm thin-foil objective aperture and using a liquid-nitrogen cooled anti-contamination device. The average length and

width of bacterial cells were measured against a scale, drawn in pencil, on the viewing screen inside the microscope. This scale consisted of two 3cm long (calibrated in mm) axes, at 90^{o} to each other, to give vertical and horizontal lines. Magnification was maintained throughout at 10,000X, as at this magnification 10mm on the sale equalled 1μm. Two 100ml samples from each site were filtered and two grids prepared from each individual sample. A total of 50 bacteria were measured on each grid, giving a total of 200 bacteria from each site. For each sample the first 50 bacteria encountered, which were either horizontal or vertical with respect to the scale, were measured.

5.2.ii. Results and discussion

The means of bacterial cell dimensions in Stubs Beck and Sugden Beck are shown in Table 5.4. Three bacterial shapes were recorded, which had the following length to width ratios (in brackets); cocci (1), short rods (>1 - <1.5) and rods (>1.5).

This transmission electron-microscopy technique clearly showed major differences in bacterial cell-morphology between the streams. In Sugden Beck only rod-shaped bacteria were found, however, Stubs Beck contained rods, short rods and cocci. These morphological differences between Stubs Beck and Sugden Beck agree well with the qualitative differences between the streams, shown by both the routine sampling programme and the taxonomic study. It is likely that the presence of only rod-shaped bacteria in Sugden Beck reflects the high incidence of Pseudomonas sp in this stream, whereas the more varied morphology in Stubs Beck was a result of the greater bacterial diversity.

Assuming that bacterial rods and short rods were cyclindrical in shape, and that cocci were spherical, estimates of the bacterial cell-volumes were calculated according to the following formulae;

$$\text{Volume of rod or short rod} = \pi r^2 l \ (\mu m^3)$$

$$\text{Volume of cocci} = 4/3 \pi r^3 \ (\mu m^3)$$

where r = radius of bacterial cell (μm)

l = length of bacterial cell (μm)

The calculated mean cell-volumes from Stubs Beck and Sugden Beck are shown in Table 5.5. The calculated cell volumes in Stubs Beck and Sugden Beck had equal variances (see 2.6.). A t-test showed that the mean bacterial cell-volume was significantly greater in Sugden Beck compared to Stubs Beck ($t = 6.68$, 398 d.f., $p<0.001$).

The estimated cell-volumes in Stubs Beck and Sugden Beck were generally larger than reported values in other aquatic environments. Krambeck et al. (1981) used scanning electron-microscopy to study bacterial cell-volumes in ten freshwater lakes, they found that the mean cell-volumes ranged from 0.015 to 0.022 μm^3. Bowden (1977) showed that the average bacterial cell-volume (as determined by scanning electron microscopy), in an American estuary, was 0.047 μm^3. Salonen (1977) studied water samples from twelve Finnish lakes using phase-contrast microscopy. The mean cell-volume was found to range from 0.041 to 0.241 μm^3.

Bacterial cell-volumes larger than those in Stubs Beck and Sugden Beck have, however, been reported. Fry and Zia (1982), studied nine freshwater habitats in South Wales. They found that mean bacterial cell-volumes, as determined by phase-contrast microscopy ranged from 0.32-0.77 μm^3 depending on the nutrient level of the water. Unpolluted freshwater sites had bacteria with smaller mean cell-volumes and most cells were in the 0-0.2 μm^3 class, whilst polluted sites had higher mean cell-volumes and few small cells. A sewage sample was found to contain the largest cells (mean cell volume 1.00 μm^3) and the lowest proportion of

of small cells. Fry and Zia (1982) suggested that the small size of freshwater bacteria, with consequent minimum maintenance requirements and turnover of cell components, was an adaptation to low nutrient conditions prevalent in many freshwater systems. This suggests that differences in mean cell-volumes between Stubs Beck and Sugden Beck may reflect differences in nutrient status between the two streams.

The taxonomic study of the sediment bacterial communities in Stubs Beck and Sugden Beck showed major differences in genera of bacteria isolated from the two streams. However, the taxonomic study only examined those heterotrophic, aerobic, bacteria capable of growth under the incubation conditions used. In comparison, this transmission electron microscopy study showed major differences between the bacterial communities in Stubs Beck and Sugden Beck, when the whole bacterial community was examined.

Table 5.4. Cell dimensions of suspended bacteria from Stubs Beck and Sugden Beck.

Stubs Beck

Bacterial cell length (μm)

Shape	Mean	Range	n	c.v.(%)
Rods	0.94	0.50 - 1.80	138	25.9
Short Rods	0.51	0.40 - 0.80	38	17.0
Cocci	0.42	0.30 - 0.60	24	19.8

Bacterial cell width (μm)

Shape	Mean	Range	n	c.v.(%)
Rods	0.30	0.10 - 0.50	138	25.1
Short Rods	0.39	0.30 - 0.60	38	18.7
Cocci	0.42	0.30 - 0.60	24	19.8

Sugden Beck

Bacterial cell length (μm)

Shape	Mean	Range	n	c.v.(%)
Rods	1.22	0.60 - 2.50	200	26.8

Bacterial cell width (μm)

Shape	Mean	Range	n	c.v.(%)
Rods	0.33	0.15 - 0.60	200	29.2

Table 5.5. Mean cell-volumes of suspended bacteria from Stubs Beck and Sugden Beck.

Site	Mean cell volume (μm^3)	Range	n	c.v.(%)
Stubs Beck	0.068	0.008-0.353	200	63.2
Sugden Beck	0.116	0.022-0.491	200	80.2

5.3. Metabolism of phenolic compounds and phenol derivatives by bacterial communities from Stubs Beck and Sugden Beck.

The metabolism of phenolic compounds and phenol derivatives by the bacterial communities of Stubs Beck and Sugden Beck was investigated by measuring the uptake of 2,4-dichlorophenoxyacetic acid. The mineralization of ^{14}C 2,4D ie. conversion to $^{14}CO_2$, was used as a measure of uptake and was determined over a range of substrate concentrations. The response of natural bacterial communities to a range of substrate concentration can be described using Michaelis-Menten enzyme kinetics. An advantage of this kinetic approach was that it allowed the determination of V_{max} (the maximum rate of mineralization of the substrate), which can be used to compare the potential heterotrophic activity of different populations (Wright and Burnison, 1979).

5.3.i. Materials and methods

Preparation of ^{14}C labelled 2,4-dichlorophenoxyacetic acid

2,4-dichlorophenoxy [2-^{14}C] acetic acid (251 $\mu Cimg^{-1}$) was supplied dissolved in 1ml of toluene solvent, this was removed by aseptically passing nitrogen over the surface of the toluene, whilst the vial was warmed to around 50°C. After the toluene had evaporated and the vial had cooled to room temperature, the ^{14}C-2,4D was redissolved by adding 0.1ml acetone followed rapidly by 0.9ml sterile distilled water. The contents of the vial were well mixed, dispensed, and stored at -20°C prior to use.

Determination of bacterial activity

The heterotrophic activity of bacterial communities in Stubs Beck and Sugden Beck was determined by measuring the response of these communities to added 2,4D, in the concentration range 5.5-18.5μgl^{-1}.

However, Sugden Beck was known to contain appreciable quantities of 2,4D i.e. the mean concentration of 2,4D in Sugden Beck during the sampling programme was 2069μgl^{-1} (see Table 4.1.b), such a high concentration of 'natural' 2,4D would obviously swamp the added 2,4D. To overcome this problem a filtering and washing procedure was employed, however, as this procedure removed the 'natural' 2,4D the turnover time of the 2,4D substrate could not be calculated.

Water samples were collected from Stubs Beck and Sugden Beck Upper sampling sites on 3 May 1983, in separate sterile 1 litre glass reagent bottles. A volume of 50ml was then filtered through a 47mm diameter, 0.22μm cellulose acetate membrane filter. This separated suspended bacteria from the water samples, which contained 2,4D. The filter was washed with 50ml of 0.22μm membrane-filtered Stubs Beck water, to remove any 2,4D remaining on bacteria or the filter. The filter was then removed and cut into five strips, which were then placed into a single, sterile, 125ml serum bottle, followed by 25ml of 0.22μm membrane-filtered, Stubs Beck water. The water samples from Stubs Beck were treated in the same way.

Twenty serum bottles from each site were prepared in this way and split into five sets of four replicates. To each serum bottle 0.1ml of ^{14}C-labelled 2,4D was added (0.05μCi per 0.1ml), followed by a volume of 1.625mg unlabelled 2,4D l^{-1} (0-0.2ml), to give five concentrations of added 2,4D in the range 5.5-18.5μgl^{-1}. To one of each group of four bottles, 1ml of 2.5M H_2SO_4 was added to provide a control. The bottles were then sealed with a rubber serum-cap to which was attached a glass rod and cup, containing a folded square of Whatman No.1. chromotography paper (see Plate 2.1.). All the bottles were then incubated in the dark at 10^{o}C; Sugden Beck samples were incubated for 30 hours and Stubs Beck samples were incubated for 100 hours.

The incubation was terminated by injecting 1ml of 2.5M H_2SO_4

through the serum cap, which also released dissolved $^{14}CO_2$. The $^{14}CO_2$ was adsorbed overnight by injecting 0.25ml of 2-phenylethylamine onto the filter paper, which was then transferred to a disposable scintillation vial, containing 10ml scintillation fluid (Appendix 2). Radioactivity, as counts per minute (c.p.m.), was assayed by liquid scintillation counting. The number of c.p.m. added initially to each serum bottle was measured by adding 0.1ml of the ^{14}C-2,4D solution to five scintillation vials and radioactivity was determined by liquid scintillation counting. The counting efficiency was determined by spiking all the vials with an internal standard of ^{14}C-hexadecane; disintegrations per minute (d.p.m.) were then calculated. The fraction of available 2,4D substrate mineralized (f) is given by the following equation;

$$f = \frac{\text{mean d.p.m. sample - blank d.p.m.}}{\text{mean d.p.m. isotope added to each bottle.}}$$

A modified Lineweaver-Burk plot (see Wright and Hobbie, 1966) of t/f against A (where t = incubation time in hours and A = added substrate concentration) allowed the determination of V_{max} (slope = $1/V_{max}$).

5.3.ii. Results and discussion

Both sites showed a significant linear relationship (Sokal and Rohlf, 1969 p.342) of t/f against A; Stubs Beck $p<0.001$ and Sugden Beck $p<0.05$. The calculated values of V_{max} for the 2,4D substrate, allowing for the initial volume (50ml) of sample filtered, are shown in Table 5.6., together with 95% confidence limits (i.e. the reciprocals of confidence intervals around the linear regression, Sokal and Rohlf, 1969 p.434-436). These calculated values represented the maximum velocity for mineralization of 2,4D under the experimental conditions employed; note, however, that some 2,4D will also be assimilated within bacterial cells. The results in Table 5.6. showed that the bacterial community in Sugden

Beck had a very much greater capacity for mineralization of 2,4D (approximately 75 times greater) compared with the bacterial community in Stubs Beck.

The calculated V_{max} values in Sugden Beck, and to a lesser extent in Stubs Beck, were similar to reported V_{max} values using other more easily metabolised substrates. Harrison et al. (1971) found that bacteria in water samples from an American lake had a V_{max} value for glucose mineralization of 0.026 $\mu g l^{-1} h^{-1}$. Goulder (1980) showed that at two sites on the River Hull, the range of V_{max} values for glucose mineralization, ranged from 0.04-2.5$\mu g l^{-1} h^{-1}$ over a two year sampling period. Albright and Wentworth (1973) determined V_{max} for uptake plus mineralization of glucose, to study entrophication in five Canadian rivers. They found that V_{max} ranged from 0.0022-0.7 $\mu g l^{-1} h^{-1}$.

V_{max} per bacterium was also calculated for Stubs Beck and Sugden Beck (see Table 5.7.). V_{max} per bacterium was the average maximal velocity for mineralization of 2,4D for each individual bacterium, and was calculated by dividing the mean V_{max} value by the total count of bacteria in the water sample (as determined by AODC, see 2.1.ii.). Table 5.7. showed that the value of V_{max} per bacterium in Sugden Beck was much greater (approximately 25 times) compared with the corresponding value in Stubs Beck. Again, these values were similar to reported values of V_{max} per bacterium using more easily metabolised substrates. Goulder (1980) showed that V_{max} per bacterium for glucose mineralization, at two sites on the River Hull, ranged from 0.07 to 5.2 fg $bacterium^{-1} h^{-1}$ over a two year sampling period. An earlier study by Goulder (1979) of V_{max} per bacterium values, in the River Hull and the Humber Estuary, showed that at the river sites V_{max} per bacterium was 0.37-0.50 fg $bacterium^{-1} h^{-1}$ (range 0.07-5.22) and in the estuary was 0.03-0.26 fg $bacterium^{-1} h^{-1}$ (range 0.005-1.07). Nuttall (1982b) determined values of V_{max} per

bacterium at three sites on the River Dee for ^{14}C acetic acid uptake, values ranged from 0.02-12.7 fg bacterium$^{-1}h^{-1}$.

The results of the study reported here show that there was a large difference in the ability of the bacterial communities, in Sugden Beck and Stubs Beck, to mineralize 2,4D. This suggests that the bacterial community in Sugden Beck was better adapted to the presence of phenolic compounds and phenol derivatives, and that adaptation resulted in an increased ability to metabolise these compounds.

Mineralization of ^{14}C-2,4D, by Sugden Beck and Stubs Beck bacterial communities, resulted in the release of $^{14}CO_2$, However as ^{14}C-2,4D was labelled in the side chain (see Fig.5.1.) release of $^{14}CO_2$ resulted from metabolism of the side chain and did not indicate complete breakdown of 2,4D. However, there is considerable evidence for the degradation of 2,4D and other phenolic compounds and phenol derivatives in the freshwater environment (see 1.3.iii.b.). In addition, considering the known nutritional versatility of the pseudomonads, which predominate in Sugden Beck, it is likely that degradation of phenolic compounds and phenol derivatives does occur in Sugden Beck. For example, Stanier et al. (1966) showed that some species of Pseudomonas are capable of utilising up to 108 different organic compounds as sole sources of carbon and energy for growth on agar plates.

Table 5.6. Calculated V_{max} values of 2,4D by bacteria from Stubs Beck and Sugden Beck, 95% confidence limits in brackets.

Site	V_{max} ($\mu g\ 2,4D l^{-1} h^{-1}$)	
Stubs Beck	0.004	(0.003 - 0.005)
Sugden Beck	0.298	(0.172 - 1.090)

Table 5.7. Calculated V_{max} per bacterium values in Stubs Beck and Sugden Beck.

Site	V_{max} ($\mu g\ 2,4D l^{-1} h^{-1}$)	Total bacterial count (x $10^{-6} ml^{-1}$)	V_{max}/bacterium ($fg\ bacterium^{-1} h^{-1}$)
Stubs Beck	0.004	1.65	0.003
Sugden Beck	0.289	4.17	0.073

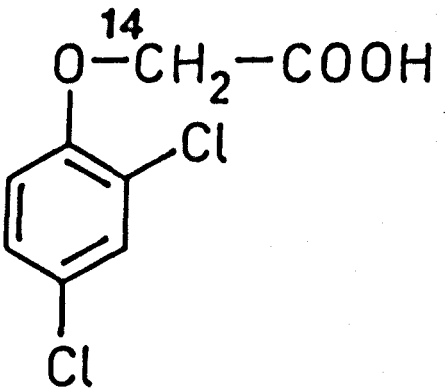


Fig. 5.1. Position of radio-labelled carbon atom in 2,4-dichlorophenoxy [2-^{14}C] acetic acid.

CHAPTER 6

TOXICITY OF PHENOLIC COMPOUNDS AND PHENOL DERIVATIVES TO BACTERIA ISOLATED FROM STUBS BECK AND SUGDEN BECK.

The routine sampling programme showed that the presence of phenolic compounds and phenol derivatives in Sugden Beck caused a change in composition of the bacterial community compared with Stubs Beck, which did not contain these compounds. The Sugden Beck bacterial community had fewer percentage chromogenic bacteria, a lower species diversity and increased counts on PSM agar. The taxonomic study of these two streams showed that the bacterial community in Sugden Beck was dominated by Gram-negative organisms belonging to the genus Pseudomonas. In contrast Stubs Beck, which did not contain any phenolic compounds or phenol derivatives, had a much more diverse Gram-negative and Gram-positive bacterial community. These results suggested that the phenolic compounds and phenol derivatives had a differential toxicity towards bacteria and that certain genera or groups of bacteria were resistant to these compounds.

This chapter investigates the toxicity of phenolic compounds and phenol derivatives to bacterial isolates from Stubs Beck and Sugden Beck.

6.1. Method of assessment of toxicity towards bacterial isolates

A modification of the method of Liu and Kwasnieska (1981) was used to determine the toxicity of phenolic compounds and phenol derivatives, by measuring the inhibition of growth of bacterial isolates on agar plates.

In the method of Liu and Kwasnieska (1981) a mixed bacterial culture, obtained from activated sludge, was grown in a liquid medium of 0.08% nutrient broth, with the addition of $0.2gl^{-1}$ glucose and $0.2gl^{-1}$ sodium acetate. After overnight incubation at $21^{o}C$, with shaking, the cell

concentration was adjusted to 0.10 O.D. (650nm) with fresh medium and a volume of 1ml spread over the surface of a pre-dried agar plate (15g agar l^{-1} growth medium). After the culture had dried, a range of solution-concentrations of toxic compounds were spotted onto the surface of the agar. Following incubation at 21°C for 18 hr the diameter of the zone of clearing was measured, which indicated the relative toxicity of the compound. An advantage of this method was that it allowed a rapid assessment of a compound's toxicity to bacteria. However, many of the isolates from the taxonomic study, particularly Stubs Beck isolates, exhibited poor growth in this liquid medium and consequently gave a poor bacterial lawn after plating. A liquid medium of 10% nutrient broth No.2. (see Appendix 2) and 0.2gl^{-1} each, of glucose and sodium acetate gave good, turbid, growth over the initial incubation period with a wide range of isolates, and was used as a basal medium throughout the toxicity-testing experiments.

6.2. Toxicity of 2,4D and picric acid to bacterial isolates from Stubs Beck and Sugden Beck

An initial study was undertaken to assess the toxicity of two commonly occurring phenolic compounds and phenol derivatives in Sugden Beck water; 2,4D and picric acid, to Stubs Beck and Sugden Beck isolates.

6.2.i. Materials and methods

A loopful of the growth of each isolate, from the taxonomic study of Stubs Beck and Sugden Beck, was aseptically removed from the stored (at 4°C) NA slopes and incubated in 10ml of sterile growth medium in 50ml, cotton-wool plugged, Erlenmeyer flasks. These flasks were then incubated for 24 hr at 20°C, in a rotary incubator at 100 r.p.m. The cell density was then adjusted to O.D. 0.1 at 650nm, measured spectrophotometrically against a blank of growth medium, with fresh sterile growth medium.

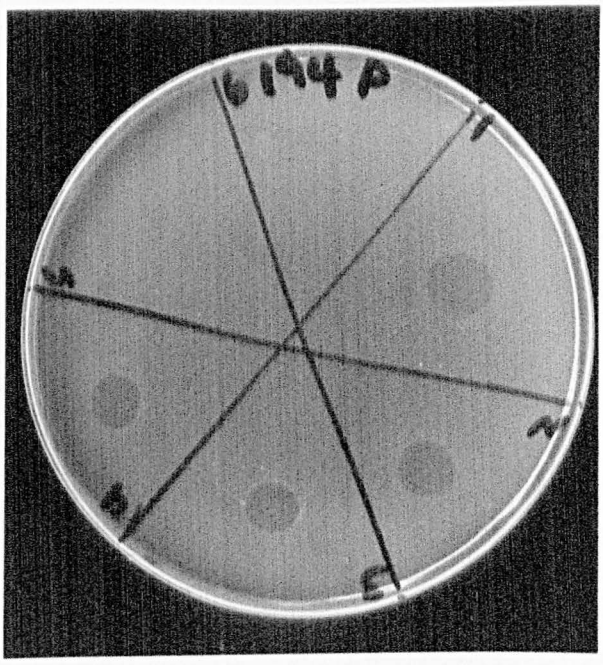
Agar plates were prepared by adding 15g agar No.3 l^{-1} of growth medium and autoclaving at 121°C for 15 minutes, these plates were allowed to dry in a 37°C incubator for 2-3h prior to use. A volume of 0.25ml of adjusted broth culture was then added aseptically to each of three agar plates and spread evenly over the surface. This volume dried rapidly onto the agar surface, usually within 15-20 minutes, and eventually yielded a good bacterial lawn. The plates were then spotted with one 10µl volume of each of various concentrations of 2,4D and picric acid; 25mM, 50mM, 100mM and 250mM, prepared by dilution, with distilled water, of a 2M stock solution of 2,4D and picric acid in acetone. Both 2,4D and picric acid were initially purified to constant melting point by recrystalisation from boiling distilled water. A control spot consisting of 10µl of the highest concentration of acetone solvent (50% v/v acetone in distilled water) was incorporated onto each plate to show any effect of the solvent on bacteria. After allowing the spots to dry, the plates were incubated at 20°C for 18 hours and the diameters of the zones of clearing (mm) were measured. Plate 6.1. shows the inhibition of growth of a bacterial isolate at the concentrations of 2,4D and picric acid employed.

6.2.ii. Statistical analysis of results

The results of this toxicity test were analyzed using Generalised Linear Interactive Modelling (GLIM; Baker and Nelder, 1978), which showed a linear relationship between the $\log_e$ (concentration + 1) of the five concentrations of both 2,4D and picric acid (0mM, 25mM, 50mM, 100mM and 250mM) and the diameter of the zone of clearing (see Fig. 6.1.). The slope of this line indicated the relative toxicity of 2,4D and picric acid to bacterial isolates i.e. a steep slope showed that the compound was particularly toxic to the isolate. Deviance, which was the residual sum of squares, was calculated before and after fitting genera or groups of

Plate 6.1. Inhibition of bacterial growth of a Pseudomonas sp , on agar plates, caused by 10µl of the following concentrations of picric acid (top) and 2,4D (bottom);

1. 250mM
2. 100mM
3. 50mM
4. 25mM
5. Blank (10µl of 50% v/v acetone in distilled water)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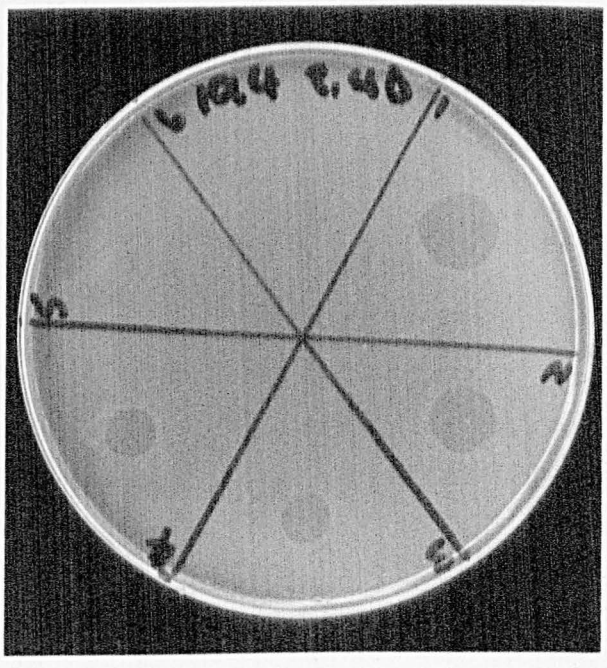
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bacteria to the linear model. The change in deviance, with associated change in the degrees of freedom, followed a chi-squared distribution. This allowed the determination of significant differences in toxicity of 2,4D and picric acid to genera or groups of bacteria, both between streams and within streams (see Fig. 6.2. for worked examples).

6.2.iii. Results and discussion

The genera or groups of bacteria used in the toxicity testing of 2,4D and picric acid are shown in Table 6.1. No zone of clearing was found with the control spot for any of the bacterial isolates, indicating that the acetone/distilled water solvent had no effect on bacterial growth, as presumably the acetone rapidly evaporated from the surface of the plate. Therefore, any inhibition of bacterial growth was due solely to the test compounds. All the isolates exhibited a zone of clearing at the lowest concentration (25mM) of both 2,4D and picric acid.

The straight lines describing the relationship between compound concentration and inhibition of bacterial growth, as measured by the zone of clearing, passed through the origin (0, 0). However, this value added nothing to the deviance, but affected the number of degrees of freedom, and therefore, was weighted out of the analysis.

Prior to determining significant differences between genera or groups of Stubs Beck bacterial isolates, the Gram-positive organisms (Gram-positive rods, Gram-positive cocci and Streptomyces, see Table 6.1.) were grouped together and the unidentified Gram-negative rods (see Table 6.1.) were excluded from the analysis. Highly significant differences ($p<0.005$) in toxicity of both 2,4D and picric acid, were found between the different genera or groups of Stubs Beck bacterial isolates. The relative sensitivities of these isolates, to both 2,4D and picric acid are shown in Table 6.2. This table showed that the Gram-positive organisms were the most sensitive isolates to 2,4D followed by bacteria

belonging to the genera or groups; Acinetobacter, Aeromonas, Chromobacterium, Enterobacteriaceae, Pseudomonas and Flavobacterium. With picric acid, bacteria belonging to the genus Flavobacterium were the most sensitive, followed by bacteria belonging to the genera or groups; Gram-positive organisms, Acinetobacter, Aeromonas, Pseudomonas, Chromobacterium and Enterobacteriaceae.

There were significant differences between the relative sensitivities of genera or groups of Stubs Beck bacterial isolates, to both 2,4D and picric acid, which are shown in Table 6.3. This table showed that in the case of 2,4D, two groups were found; a relatively sensitive group (Gram-positive organisms, Acinetobacter and Aeromonas) and a relatively more resistant group (Chromobacterium, Enterobacteriaceae, Pseudomonas and Flavobacterium), which was significantly ($p<0.005$) more resistant to 2,4D than the sensitive group. Within each group there were no significant differences ($p>0.05$) in sensitivity to 2,4D, except that bacteria belonging to the genus Aeromonas were significantly ($p<0.05$) more resistant to 2,4D compared with the Gram positive organisms.

A more complex pattern of bacterial sensitivity, of Stubs Beck isolates, was found with picric acid (see Table 6.3.). Bacteria belonging to the genus Flavobacterium were the most sensitive to picric acid, which contrasted markedly to their sensitivity to 2,4D. The most resistant group of organisms to picric acid were bacteria belonging to the genera or groups; Pseudomonas, Chromobacterium and Enterobacteriaceae. Bacterial isolates belonging to the genera or groups; Gram-positive organisms, Acinetobacter and Aeromonas had an intermediate sensitivity to picric acid (see Table 6.3. for significant differences between these genera or groups).

These results showed that different genera or groups of bacterial isolates from Stubs Beck, had significantly differing sensitivities to 2,4D

and picric acid, although the relative sensitivity of each genera or group depended on the compound. This was illustrated especially by the genus Flavobacterium, which exhibited the most sensitive response to picric acid, but one of the most resistant responses to 2,4D. Generally speaking bacteria belonging to the genera or groups; Pseudomonas, Chromobacterium and Enterobacteriaceae were the most resistant to both 2,4D and picric acid whilst Gram-positive organisms and bacteria belonging to the genus Acinetobacter were the most sensitive. The relatively greater sensitivity of the Gram-positive organisms, compared with Gram-negative organisms, may be a result of morphological and biochemical differences between these groups of organisms. The sensitivity of Gram-positive organisms to phenolic compounds was also shown by Kamshilov and Flerov (1978). They studied the sensitivity of certain genera of aquatic bacteria to phenol at a concentration of $10mgl^{-1}$. At this concentration, there was an inhibitory effect on Gram-positive genera such as Corynebacterium and Micrococcus, but bacteria belonging to the Gram-negative genus Pseudomonas were more resistant, as $1000mgl^{-1}$ was required for inhibition.

Prior to determining significant differences in the toxicity of 2,4D and picric acid to Sugden Beck bacterial isolates, the unidentified Gram-negative organisms were excluded (see Table 6.1.). The relative sensitivities of the Sugden Beck bacterial isolates, to both 2,4D and picric acid, are shown in Table 6.2. There were highly significant differences ($p<0.005$) between the relatively sensitive Gram-positive organisms and the more resistant pseudomonads, for both 2,4D and picric acid.

The results obtained from this toxicity study of 2,4D and picric acid, on bacteria isolated from Stubs Beck and Sugden Beck, cannot be directly related to the situation in the natural environment. A reason for this, is that the concentration of 2,4D and picric acid were very much higher than their respective concentrations in Sugden Beck (e.g. 9.4μM

2,4D and 8.6μM picric acid at Sugden Beck Upper), although, in the toxicity test, detoxification may occur through adsorption into the agar medium. In addition only two compounds, from a total of fourteen identified compounds in Sugden Beck, were used. However, this study does give an indication of some of the factors bringing about the differences between bacterial communities in Stubs Beck and Sugden Beck. We have seen from the taxonomic study that the bacterial community in Sugden Beck was dominated by Gram-negative organisms belonging to the genus Pseudomonas. The results of this toxicity study, showed that Stubs Beck bacterial isolates belonging to the genus Pseudomonas were amongst the most resistant genera of bacteria towards both 2,4D and picric acid. Gram-positive organisms, in contrast, were more sensitive to both 2,4D and picric acid, which may explain the absence of these organisms from Sugden Beck. Clearly, the presence of 2,4D and picric acid may cause a selection of pseudomonads at the expense of Gram-positive and sensitive Gram-negative genera such as Flavobacterium, Acinetobacter and Aeromonas. Therefore, the diversity of the bacterial community was lower in Sugden Beck compared with Stubs Beck, due to the selection of a few resistant bacterial species by the presence of phenolic compounds and phenol derivatives.

Significant differences were found when the sensitivity of all the Stubs Beck isolates, to both 2,4D and picric acid, was compared with all the Sugden Beck isolates. Table 6.4. shows the relative toxicity of 2,4D and picric acid to Stubs Beck and Sugden Beck bacterial isolates. Both compounds were more toxic to Stubs Beck isolates; analysis of the data showed that there was a highly significant difference ($p < 0.005$) between the more sensitive Stubs Beck isolates and the more resistant Sugden Beck isolates, to both 2,4D and picric acid. An examination of the relative sensitivity of Stubs Beck and Sugden Beck pseudomonads (see Table 6.5.), showed that Sugden Beck pseudomonads were significantly ($p < 0.005$) more

resistant, to both 2,4D and picric acid, than the Stubs Beck pseudomonads. These results suggest that the bacterial community in Sugden Beck was better adapted to phenolic compounds and phenol derivatives.

Table 6.1. Genera or groups of bacterial isolates from Stubs Beck and Sugden Beck used in toxicity testing of 2,4D and picric acid. The numbers in brackets show the number of isolates used.

Stubs Beck		Sugden Beck	
Pseudomonas	(25)	Pseudomonas	(86)
Aeromonas	(14)	Unidentified Gram-negative rods	(4)
Flavobacterium	(9)	Gram-positive rods	(2)
Acinetobacter	(4)		
Enterobacteriaceae	(3)		
Unidentified Gram-negative rods	(2)		
Chromobacterium	(1)		
Gram-positive rods	(17)		
Gram-positive cocci	(2)		
Streptomyces	(1)		
Total	(78)	Total	(92)

Table 6.2. Relative toxicity of 2,4D and picric acid to Stubs Beck and Sugden Beck bacterial isolates. A steep slope indicates high toxicity.

Site; Stubs Beck Compound; 2,4D

Group or genera	Slope	Standard error	n. of isolates
Gram positive organisms	2.733	0.0217	20
Acinetobacter	2.704	0.0530	4
Aeromonas	2.656	0.0338	14
Chromobacterium	2.571	0.0992	1
Enterobacteriaceae	2.525	0.0600	3
Pseudomonas	2.501	0.0291	25
Flavobacterium	2.485	0.0389	9

Site; Stubs Beck Compound; picric acid.

Group or genera	Slope	Standard error	n. of isolates
Flavobacterium	3.351	0.0459	9
Gram positive organisms	2.290	0.0553	20
Acinetobacter	2.266	0.0828	4
Aeromonas	2.228	0.0587	14
Pseudomonas	2.056	0.0536	25
Chromobacterium	2.018	0.1452	1
Enterobacteriaceae	1.972	0.0919	3

Site; Sugden Beck Compound; picric acid

Group or genera	Slope	Standard error	n. of isolates
Gram positive organisms	2.270	0.0435	4
Pseudomonas	1.900	0.0440	86

Site; Sugden Beck Compound; 2,4D

Group or genera	Slope	Standard error	n. of isolates
Gram positive organisms	2.553	0.0456	4
Pseudomonas	2.320	0.0461	86

Table 6.3. Results of tests of significance: differences in sensitivity of genera or groups of bacterial isolates from Stubs Beck to 2,4D and picric acid. Genera in the same group showed no significant differences ($p > 0.05$) in sensitivity, a < sign indicates significant differences ($p < 0.05$) between genera or groups of bacterial isolates.

Compound; 2,4D

Most sensitive/least resistant		Most resistant/least sensitive
Gram-positive organisms Acinetobacter Aeromonas	<	Chromobacterium Enterobacteriaceae Pseudomonas Flavobacterium

Compound; picric acid

Most sensitive/least resistant				Most resistant/least sensitive		
Flavobacterium	<	Gram-positive organisms Acinetobacter	<	Aeromonas	<	Pseudomonas Chromobacterium Enterobacteriaceae

Table 6.4. Relative toxicity of 2,4D and picric acid to all bacterial isolates from Stubs Beck and Sugden Beck

Compound; 2,4D

Site	Slope	Standard error	n. of isolates
Stubs Beck	2.604	0.0094	78
Sugden Beck	2.328	0.0128	92

Compound; picric acid

Site	Slope	Standard error	n. of isolates
Stubs Beck	2.309	0.0147	78
Sugden Beck	1.918	0.0200	92

Table 6.5. Relative toxicity of 2,4D and picric acid to pseudomonads from Stubs Beck and Sugden Beck

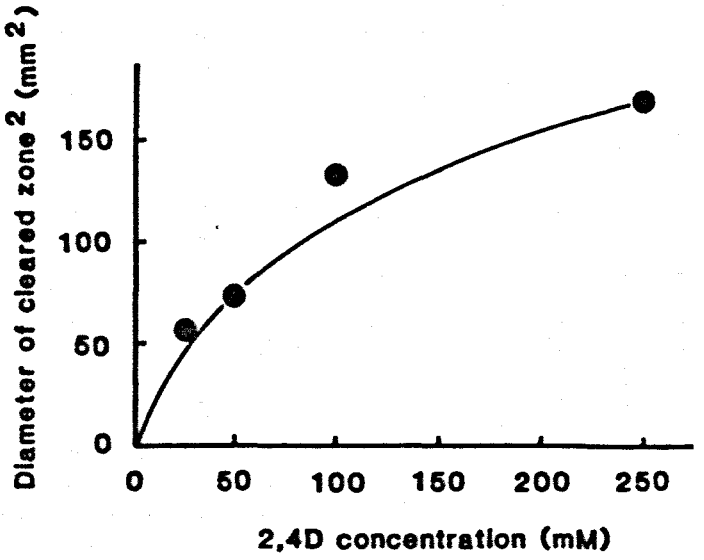
Compound; 2,4D

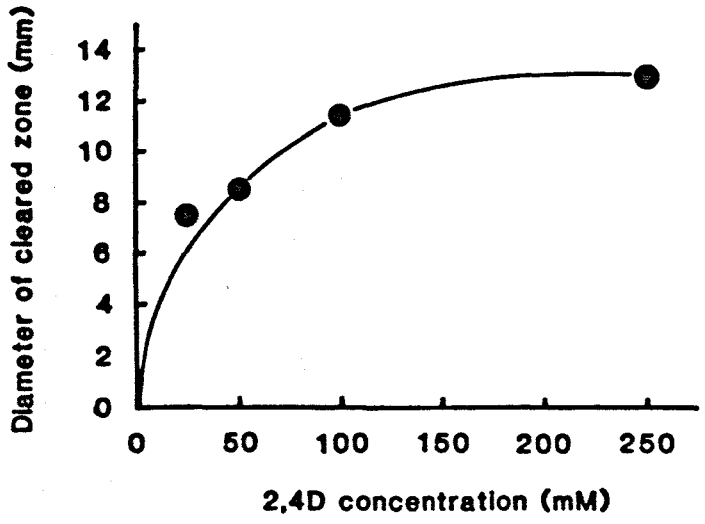
Genera	Slope	Standard error	n. of isolates
Stubs Beck Pseudomonas	2.500	0.0137	25
Sugden Beck Pseudomonas	2.320	0.0156	86

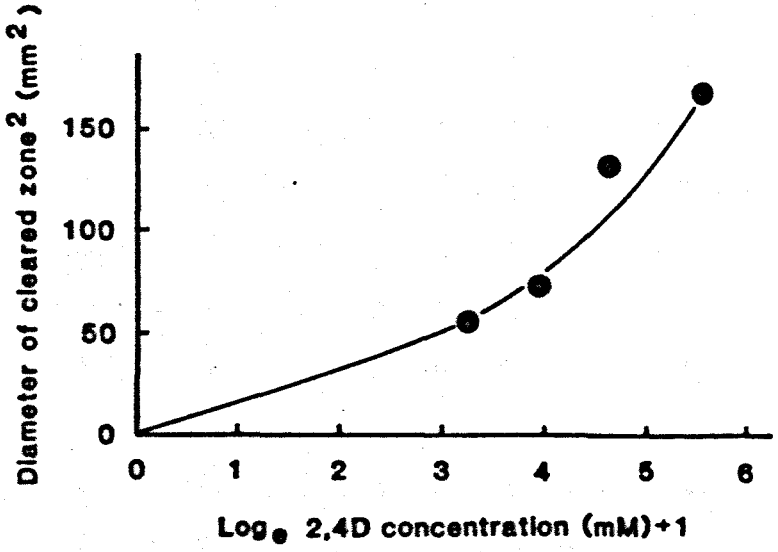
Compound; picric acid

Genera	Slope	Standard error	n. of isolates
Stubs Beck Pseudomonas	2.056	0.0126	25
Sugden Beck Pseudomonas	1.900	0.0143	86

Fig. 6.1. Plots of diameter of zone of bacterial inhibition (d) and d^2, against concentration of toxic compound (2,4D) and $\log_e$ (2,4D). The plot of d against $\log_e$ (2,4D) gave a straight line. Plots from experiments using 10 other isolates produced similar results, hence the slope of this line could be used as a measure of toxicity.


Diameter of cleared zone² (mm²)
150
100
50
0
0
50
100
150
200
250
2,4D concentration (mM)


Diameter of cleared zone (mm)
14
12
10
8
6
4
2
0
0
50
100
150
200
250
2,4D concentration (mM)


Diameter of cleared zone² (mm²)
150
100
50
0
0
1
2
3
4
5
6
Log e 2,4D concentration (mM)+1

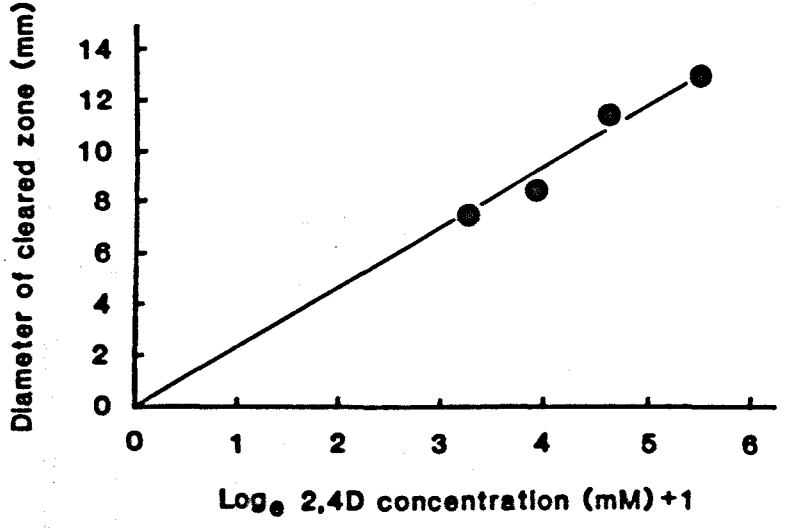

Diameter of cleared zone (mm)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
Log e 2,4D concentration (mM)+1

Figure 6.2. Worked examples of statistical procedure used in determining differences in sensitivity of bacterial isolates from Stubs Beck and Sugden Beck to 2,4D and picric acid. (see 6.2.ii. and 6.2.iii.).

Worked example 1. Differences in sensitivity of all Stubs Beck and all Sugden Beck bacterial isolates to 2,4D.

n. of Stubs Beck isolates tested = 78

n. of Sugden Beck isolates tested = 92

The deviance of the slopes of all Stubs Beck and all Sugden Beck isolates tested from the mean was = 4051 (2039 d.f.), after weighting out all the 0,0 points.

Differences in sensitivity between Stubs Beck isolates and Sugden Beck isolates were then determined by grouping all the Stubs Beck isolates together and all the Sugden Beck isolates together. Fitting these two groups to the linear model reduced the deviance to = 3299 (2038 d.f.)

		d.f.
Initial deviance	4051	2039
Final deviance after fitting Stubs Beck and Sugden Beck isolate groups	3299	2038
Change in deviance	-752	-1

The change in deviance together with the associated change in d.f. can then be compared with a chi-squared distribution. As the critical chi-square at p = 0.05 and 1 d.f. was 3.841, the two groups were significantly different in their sensitivity to 2,4D.

	Mean slope	Standard error	95% C.L.
All Stubs Beck isolates	2.604	0.0094	2.586-2.622
All Sugden Beck isolates	2.328	0.0128	2.303-2.353

Figure 6.2. (continued)

Worked example 2. Differences in sensitivity of Flavobacterium isolates and Gram-positive isolates, from Stubs Beck, to picric acid.

n. of Stubs Beck Flavobacterium isolates tested = 9

n. of Stubs Beck Gram-positive isolates tested = 20

Before determining differences in sensitivity to picric acid, between these isolates, all the Sugden Beck bacterial isolates and all the other Stubs Beck bacterial isolates were weighted out of the analysis.

The deviance of the slopes of all the stubs Beck Gram-positive organisms and Flavobacterium isolates from the mean was = 4724 (347 d.f.), after weighting out all the 0,0 points.

Differences in sensitivity between Stubs Beck Flavobacterium isolates and Stubs Beck Gram-positive organisms were then determined by grouping all the Stubs Beck Flavobacterium isolates together and all the Stubs Beck Gram-positive isolates together. Fitting these two groups to the linear model reduced the deviance to = 3093 (346 d.f.)

		d.f.
Initial deviance	4724	347
Final deviance after fitting Stubs Beck Flavobacterium and Gram-positive isolate groups	3093	346
Change in deviance	-1631	-1

The change in deviance together with the associated change in d.f. can then be compared with a chi-squared distribution. As the critical chi-square at p = 0.05 and 1 d.f. was = 3.841, the two groups were significantly different in their sensitivity to picric acid.

	Mean slope	Standard error	95% C.L.
All Stubs Beck Flavobacterium isolates	3.351	0.0652	3.221-3.478
All Stubs Beck Gram-positive isolates	2.290	0.0785	2.136-2.444

6.3. Toxicity of phenolic compounds and phenol derivatives to selected bacterial isolates from Stubs Beck and Sugden Beck

6.3.i. Materials and methods

In the work described in this section, the relative toxicity of sixteen, phenolic compounds and phenol derivatives, to selected bacterial isolates from Stubs Beck and Sugden Beck, was determined. The following phenolic compounds and phenol derivatives (see Appendix 1 for structures) were used;

Chlorophenols (abbreviations used in brackets)

2-chlorophenol (2CHLR)

3-chlorophenol (3CHLR)

4-chlorophenol (4CHLR)

2,4-dichlorophenol (2,4DCP)

4-chloro-2-methyl-phenol (PCOC)

Nitrophenols

2,4-dinitrophenol (2,4DNP)

2,4,6-trinitrophenol (picric acid)

2-methyl-4,6-dinitrophenol (DNOC)

2-sec-butyl-4,6-dinitrophenol (DNBP)

Phenoxyalkanoic acids

2,4-dichlorophenoxyacetic acid (2,4D)

2,4,5-trichlorophenoxyacetic acid (2,4,5T)

2-methyl-4-chlorophenoxyacetic acid (MCPA)

2-(2,4-dichlorophenoxy)-proprionic acid (2,4DP)

2-(2-methyl-4-chlorophenoxy)-proprionic acid (CMPP)

4-(2,4-dichlorophenoxy)-butyric acid (2,4DB)

4-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB)

All of these compounds, except for 2CHLR and 2,4,5T, were identified as present in Sugden Beck water (see Table 4.1.b.). All of the compounds are solid at room teperature, except for 2CHLR which is a liquid.

Because of the large number of phenolic compounds and phenol derivatives used, only a small number of isolates was tested. Isolates, representative of the major genera of groups, were selected at random from the stored (at $4^{o}C$) isolates obtained in the taxonomic study of Stubs Beck and Sugden Beck. The following isolates were used; Pseudomonas (1 Sugden Beck isolate, 2 Stubs Beck isolates), Aeromonas (1 isolate from Stubs Beck), Flavobacterium (1 isolate from Stubs Beck) and Gram-positive organisms (1 Stubs Beck isolate). The relative toxicity of the sixteen phenolic compounds and phenol derivatives to these isolates, was determined by measuring the degree of inhibition of bacterial growth on agar plates, at a single compound concentration.

The preparation of bacterial lawns was identical to the method used in testing the toxicity of 2,4D and picric acid to baterial isolates (see 6.2.i.). Each of the sixteen phenolic compounds and phenol derivatives were dissolved, separately, in 50% (v/v) acetone in distilled water, to give a final concentration of 250mM. A volume of 10µl of these compounds, together with a 'blank' of the acetone/distilled water solvent, was added to each of ten replicate plates. After the spots had dried, the plates were incubated for 18 hours at $20^{o}C$ and the diameter of the zones of clearing were measured. This was repeated three times, giving a total of 30 diameter measurements, for each compound, with each isolate. Plate 6.2. shows the effect of phenolic compounds and phenol derivatives on the inhibition of bacterial growth. The results were analyzed using Generalised Linear Interative Modelling (GLIM), which allowed the comparison of both compound means and isolate means.

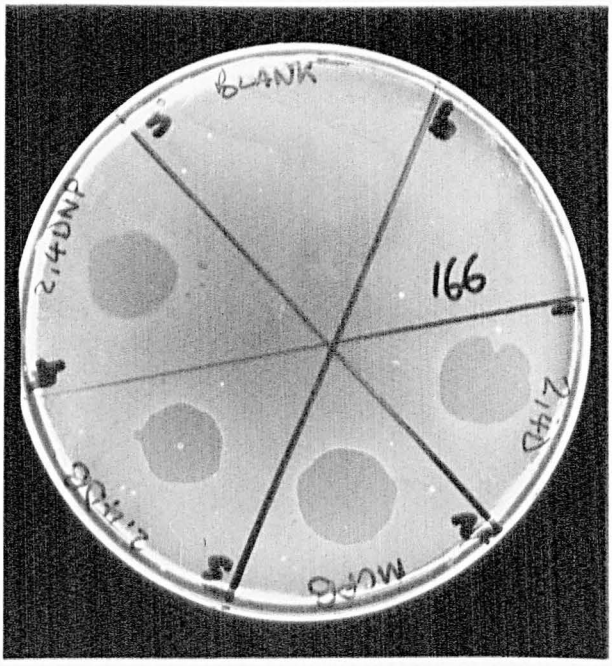
6.3.ii. Results and discussion

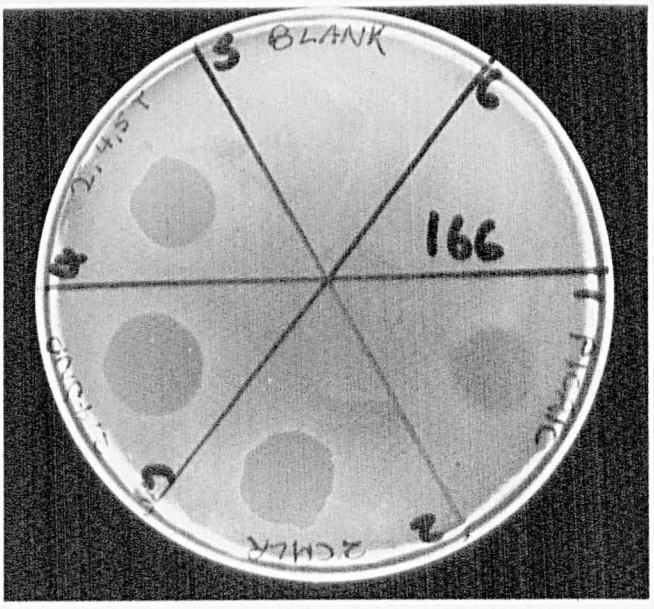
There were no zones of clearing with the control spot for any of

Plate 6.2. Inhibition of the growth of a Pseudomonas sp on agar plates, caused by 10µl of a 250mM solution of the following compounds;

Upper plate
1. 2,4D
2. MCPB
3. 2,4DB
4. 2,4DNP
5. Blank (10µl of 50% v/v acetone in distilled water)

Lower plate
1. Picric acid
2. 2CHLR
3. 2,4DNP
4. 2,4,5T
5. Blank


BLANK
166
2,4DNP
2,4DCP
MCPB
2,4D
2,4,5T


BLANK
166
2,4,5T
2,4D
MCPB
2,4DCP
PICLORAM

the bacterial isolates tested, indicating that the acetone/distilled water solvent had no effect on isolate growth and that any inhibition of growth was due to the presence of the phenolic compounds or phenol derivatives. All the isolates exhibited a zone of clearing with each individual phenolic compound or phenol derivative.

Table 6.6. shows the relative senstivities of the bacterial isolates, in order of increasing sensitivity, to all the phenolic compounds and phenol derivatives. Although the number of bacterial isolates used in this study was limited, the general trends found in the previous toxicity experiment were followed. The Gram-positive organism was the most sensitive isolate to all the phenolic compounds and phenol derivatives, followed by Flavobacterium sp and Aeromonas sp, the pseudomonads were the most resistant organisms. The results obtained showed that the pseudomonads were more resistant to a wide range of phenolic compounds and phenol derivatives compared to other Gram-negative and Gram-positive organisms, therefore, the dominance of the bacterial community in Sugden Beck by pseudomonads may be a consequence of their increased resistance to phenolic compounds and phenol derivatives. Conversely, the absence of other Gram-negative, and Gram-positive organisms, may be explained by the increased sensitivity of these organisms to phenolic compounds and phenol derivatives.

Table 6.7. shows the relative toxicity of the individual phenolic compounds and phenol derivatives, in order of increasing toxicity, to all of the bacterial isolates combined (see Appendix 6 for the relative toxicity of phenolic compounds and phenol derivatives to each isolate). Significant differences, ($p < 0.05$) were found between the toxicity of phenolic compounds; chlorophenols were the most toxic to all bacterial isolates, followed by nitrophenols and finally phenoxyalkanoic acids. This agrees with the results of the routine sampling programme at Sugden Beck, which showed fewer negative correlations between bacteriological

variables and phenoxyalkanoic acids compared with chlorophenols and nitrophenols (see Table 4.4.a.). Phenoxyalkanoic acids were present in larger quantities in Sugden Beck than either chlorophenols or nitrophenols (see Table 4.1.b.), emphasizing the relative lower toxicity of phenoxyalkanoic acids to bacteria.

Table 6.8. shows the differences in toxicity of the individual phenolic compounds, and phenol derivatives. These results showed that the chemical structure of the phenolic compound and phenol derivative played an important role in determining toxicity. In general, the relatively more complex phenoxyalkanoic acids were less toxic to bacterial isolates than were the structurally, simpler chlorophenols and nitrophenols. Alteration of chemical structure within these groups also affected the compound's toxicity, for example chloro substitution of methyl on the ring structure of phenoxyalkanoic acids (MCPA → 2,4D and MCPB → 2,4DB) and increase in side chain length (CMPP → MCPB, 2,4DP → 2,4DB, MCPA → MCPB and 2,4D → 2,4DB) both caused a reduction in toxicity. Chlorophenols showed differences in toxicity depending on the isomer (2CHLR, 3CHLR and 4CHLR) and on the degree of chlorination, as 2,4DCP was more toxic to bacteria than were the monochlorophenols.

The dependence of chlorophenol toxicity on the degree of chlorination, has also been noted by other workers. Liu and Kwasniewska (1981) studied the effects of various chlorophenols on the growth of a mixed bacterial culture, obtained from activated sludge (for method see 6.1.). With increasing chlorination of the test compounds; 2-chlorophenol, 2,6-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol there was an increased toxicity to the mixed culture. Liu et al. (1982) assessed the toxicity of halogenated phenols to bacteria, by measuring the inhibition of dehydrogenase activity. The ability of a Bacillus sp, isolated from activated sludge, to reduce resazurin in a liquid growth medium containing

the toxicant, was determined. It was found that an increase in chlorination caused an increase in toxicity, as 2,4-dichlorophenol had a greater toxicity than the monochlorophenols (2CHLR, 3CHLR and 4CHLR). An isomer effect was also found, as 4-chlorophenol was the most toxic monochlorophenol followed by 3-chlorophenol and finally 2-chlorophenol. Similar results were found in this study.

The toxicity of the nitrophenols was also related to their structure. In this study an increase in nitro-substitution caused a decrease in toxicity, as 2,4-dinitrophenol was more toxic to bacterial isolates than 2,4,6-trinitrophenol (picric acid). Apparently, substitution in C#6 caused an amelioration of the compound's toxicity. Similar results were obtained by Liu et al. (1982, see above), who showed that 2,4-dichlorophenol was approximately three times more toxic than 2,4,6-trichlorophenol. In addition dichlorosubstitution in C#2 and C#6 caused a reduction in toxicity as 2,3-dichlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 3,4-dichlorophenol and 3,5-dichlorophenol were more toxic to bacteria than 2,6-dichlorophenol.

The phenoxyalkanoic acid, 2,4D, was significantly ($p<0.05$) more toxic to all bacterial isolates, except for the Flavobacterium sp, than was picric acid. In the case of the Flavobacterium sp picric acid was significantly ($p<0.05$) more toxic than 2,4D. These results agreed with the findings of the initial toxicity test (see 6.2.).

Table 6.6. Relative sensitivity of tested bacterial isolates to all phenolic compounds and phenol derivatives.

Group or genera	Mean diameter of zone of clearing (mm)	Standard error
Pseudomonas*	12.99	0.225
Pseudomonas	13.85	0.318
Pseudomonas	17.28	0.318
Aeromonas	17.74	0.318
Flavobacterium	20.98	0.318
Gram positive organism	21.81	0.318

*isolate obtained from Sugden Beck, all other isolates were from Stubs Beck.

Table 6.7. Relative toxicity of phenolic compounds and phenol derivatives to bacterial isolates.

Phenolic compound or phenol derivative	Mean diameter of zone of clearing (mm)	Standard error
2CHLR	13.56	0.543
2,4DB	13.71	0.543
Picric acid	15.34	0.543
MCPB	13.56	0.543
2,4D	15.87	0.384
DNBP	16.22	0.543
2,4,5T	16.38	0.543
CMPP	16.98	0.543
2,4DP	17.13	0.543
MCPA	17.28	0.543
3CHLR	17.29	0.543
DNOC	17.70	0.543
4CHLR	18.62	0.543
PCOC	20.15	0.543
2,4DNP	21.70	0.543
2,4DCP	25.66	0.543

Table 6.8. Results of significance tests: differences in toxicity of phenolic compounds and phenol derivatives to bacterial isolates. Phenolic compounds or phenol derivatives in the same group showed no significant differences ($p>0.05$) in toxicity, a < sign indicates a significant difference ($p<0.05$) in toxicity of the phenolic compound or phenol derivative.

Least toxic												Most toxic
2CHLR 2,4DB	<	Picric acid MCPB	<	2,4D	<	DNBP 2,4,5T	<	CMPP 2,4DP MCPA 3CHLR	<	DNOC < 4CHLR < PCOC < 2,4DNP < 2,4DCP		

6.4. Effect of pH on toxicity of phenolic compounds and phenol derivatives

The routine sampling programme at the Sugden Beck sites showed that pH was a major environmental factor determining the toxicity of phenolic compounds and phenol derivatives to bacterial communities (see 4.3.ii.). In general, there were fewer negative correlations between phenolic compounds and phenol derivatives with bacteriological variables under neutral pH conditions, compared to all pH conditions in Sugden Beck.

The effect of pH on the toxicity of phenolic compounds and phenol derivatives, was investigated by studying the toxicity of 2,4D to bacterial isolates, from Stubs Beck and Sugden Beck, under varying pH conditions.

6.4.i. Materials and methods

The inhibitory action of 2,4D on bacterial growth, at various pH values, was determined using a broth culture technique. A loopful of the growth of selected (at random) bacterial isolates, from the taxonomic study of Stubs Beck and Sugden Beck, was incubated in 10ml of the sterile liquid growth medium (see 6.2.) in 50ml, cotton-wool plugged, Erlenmeyer flasks. These flasks were incubated for 24h, at 20^{o}C, in a rotary incubator at 100r.p.m. and the cell density adjusted spectrophotometrically, as before, to O.D. 0.1 at 650nm, with fresh sterile growth medium. These adjusted broth cultures provided the inoculum for subsequent experiments. The following bacterial isolates were used; five isolates belonging to the genus Pseudomonas (1 Stubs Beck isolate and 4 Sugden Beck isolates), three isolates were Aeromonas sp (all from Stubs Beck), two isolates were Gram-positive organisms (both from Stubs Beck) and one isolate was a Flavobacterium sp (from Stubs Beck).

In order to study the effect of pH on toxicity of 2,4D to these bacterial isolates, the basal growth medium used in earlier toxicity experiments (see 6.1. and 6.3.) was modified. As bacterial growth causes

a change in pH of the medium a buffer was incorporated. A mixture of disodium hydrogen phosphate and potassium dihydrogen phosphate was used as a buffer and was found to adequately maintain pH throughout the incubation period. However, as heat sterilization of a medium containing glucose and phosphate, particularly at alkaline pH, may result in the production of inhibitory substances, glucose was excluded from the modified growth medium. In addition the quantity of nutrient broth No. 2 was reduced in an attempt to limit growth of the isolates and so help maintain pH over the incubation period. The modified growth medium was prepared by dissolving 1.75g nutrient broth No.2 , 1.0g disodium hydrogen phosphate, 1.0g potassium dihydrogen phosphate and 0.2g sodium acetate in approximately 980ml distilled water, followed by 50mg 2,4D. The medium was stirred continuously until the 2,4D had dissolved, usually within 2 hours. The pH of the medium was then adjusted, to the desired pH, by using 3.0M HCl or 3.0M NaOH and the volume made-up to 1 litre with distilled water. The modified growth medium was then dispensed, in 20ml aliquots, to each of six 50ml Erlenmeyer flasks, which were then stoppered with cotton-wool bungs and autoclaved at 121^{o}C for 15 minutes. The following pH's were used; 6.0, 6.5, 7.0 and 7.5. A further series of flasks containing modified growth-medium without added 2,4D were also prepared.

A volume of 0.1ml of the adjusted culture was then added to each of five flasks of both the 2,4D containing growth medium and the ordinary growth medium, at each pH value. A sixth flask from each group, did not have any bacterial culture added and so acted as a control. All the flasks were incubated for 24h, at 20^{o}C in a rotary incubator, at 100 r.p.m. Following incubation, the optical density of the culture was measured spectrophotometrically at 650nm, against a blank of the control flask medium. The pH of the modified growth medium was measured after autoclaving and then remeasured following the incubation period.

6.4. ii. Results and discussion

Heat sterilization of the modified growth medium did not alter the initial pH of the medium, although the growth of bacterial isolates caused an alkaline shift in pH. However, the maximum change in pH of the 2,4D containing medium and the ordinary growth medium was 0.3 pH units.

Data for each individual isolate belonging to the same genera or group were combined. Table 6.9. shows the optical density at each pH value for both the medium containing 2,4D and the ordinary growth medium, together with the percentage inhibition of bacterial growth caused by 2,4D. This table showed that the pH affected the growth of bacterial isolates, with most isolates favouring neutral to slightly alkaline pH conditions. All the bacterial isolates grew well at pH 7 and 7.5, although the pseudomonads exhibited good growth at pH 6.5. Generally, isolates showed relatively poor growth under slightly acid pH conditions, indeed, the Gram-positive isolates tested failed to grow below pH 7.0. However, the presence of $50mgl^{-1}$ 2,4D in the medium caused an inhibition of bacterial growth with all the isolates tested, which was greater at lower pH values. Figure 6.3. shows the percentage inhibition of bacterial growth caused by the added 2,4D. Clearly, the toxicity of 2,4D to bacteria was affected by pH.

Other workers have shown that the toxicity of 2,4D to bacteria depends on pH. Zetterberg et al. (1977) studied the effect of pH on the toxicity of 2,4D to cell suspensions of Salmonella typhimurium. Survival of this organism was determined by plating onto nutrient agar following treatment with buffered solutions of 2,4D (500mg 2,4D l^{-1}). The survival of S. typhimurium depended on the pH of the 2,4D solution; survival was high at high pH values, for example at pH 6.8 the 2,4D had no effect on bacteria, however, survival of S. typhimurium decreased with increasing acidity. The dependence of toxicity of phenolic compounds on pH has also

been shown with higher organisms. Saarikoski and Viluksela (1981) determined the LC_{50} (lethal concentration producing 50% mortality) of substituted phenols to fish (guppy, Poecilia reticulata Peters) under varying pH conditions. The LC_{50} values were estimated after exposure to 4-chlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, 2,4-dinitro-6-sec-butyl phenol and 2,6-dibromo-4-nitrophenol for 96 hours at pH 5,6,7 and 8. The toxicity of all these phenolic compounds increased with increasing acidity. Woodward (1976) measured the acute toxicity of DNBP (2-sec-butyl-4,6-dinitrophenol, dinoseb) to cut throat trout (Salmo clarki) and lake trout (Salvelinus namaycush), by determining 96hr LC_{50}'s. A decrease in water pH, from 8.5 to 6.5, was found to increase the toxicity of DNBP to both fish species.

As phenolic compounds and phenol derivatives are weak acids the degree of dissociation of these compounds is determined by pH, according to the Henderson-Hasselbach equation (Conn and Stumpf, 1976 p.12);

$$pH = pK_a + \log_{10} \frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$

where pK_a = the negative log of the dissociation constant of the phenolic compound or phenol derivative

[conjugate base] = concentration of the dissociated form of the phenolic compounds or phenol derivatives

[conjugate acid] = concentration of the non-ionised form of the phenolic compounds or phenol derivatives.

Hence, the difference between the pH and pK_a determined the degree of ionisation of the phenolic compound or phenol derivatives i.e. when $pH = pK_a$ the compound will be 50% ionised. Therefore, the differences in toxicity of 2,4D and substituted phenols to Salmonella typhimurium

(Zetterberg et al., 1977) and fish (Woodward, 1976, and Saarikoski and Viluksela, 1981) may be explained by increased uptake of the unionised form of these compounds, which predominates at acid pH; although the ionised form may also be toxic.

Clearly, pH seems to influence the toxicity of phenolic compounds and phenol derivatives, probably through its effect on ionisation of the compound. Table 6.10. shows the percentage dissociation of 2,4D, under the experimental pH conditions employed in this study, calculated from the Henderson-Hasselbach equation. This table showed that throughout the pH range used the overwhelming proportion of 2,4D was the dissociated form; even at pH 6.0 2,4D was over 99.9% dissociated. The results of this toxicity test showed that the toxicity of 2,4D, to bacterial isolates, increased with increasing acidity, however the proportion of dissociated 2,4D remained relatively constant over the experimental pH range (pH 6.0-7.5). This suggests that (in contrast to the conclusions of Zetterberg et al., 1977) the ionic form of 2,4D was toxic to bacterial isolates, and that the effect of decreasing pH was to increase the sensitivity of bacterial isolates rather than substantially alter the ionisation of 2,4D. As the optimum pH range for aquatic bacteria is pH 6.5-8.5 (Rheinheimer, 1974), the increased sensitivity of bacterial isolates to 2,4D may have resulted from the increased stress placed on the bacteria by growth outside the optimum pH range. However, pH may enhance the toxicity to bacteria, of phenolic compounds and phenol derivatives, which have pK_a values near to 7, as small changes in pH may then cause major alterations in ionisation of the compounds and hence, toxicity.

In the routine sampling programme (see Table 4.1.b.) pH values were recorded in Sugden Beck, which were outside the optimum pH range (6.5-8.5) given by Rheinheimer (1974) i.e. lowest recorded pH at Sugden Beck Upper was 2.7. An attempt was made to grow the above isolates under low pH conditions; none of these isolates grew at pH 5.0 in the growth medium without added 2,4D. This suggests that under acidic

pH conditions in Sugden Beck, pH was the major factor controlling growth of bacteria, including those genera resistant to phenolic compounds and phenol derivatives.

The results of this experiment also showed a clear difference in the toxicity of 2,4D to different genera or groups of bacteria, which agreed with earlier toxicity tests, as pseudomonads were generally more resistant to 2,4D compared with bacterial isolates belonging to the genera Aeromonas, Flavobacterium or the Gram-positive organisms. The pseudomonads showed little inhibition of growth over the entire pH range tested (pH 6.0-7.5), whereas the isolates belonging to the genera Flavobacterium and Aeromonas showed pronounced growth inhibition below pH 7.0. Therefore, the growth of pseudomonads in Sugden Beck, compared with other Gram-negative and Gram-positive genera, would be favoured as the pseudomonads were resistant to phenolic compounds and phenol derivatives over a wider pH range.

Table 6.9. Influence of pH on the toxicity of 2,4D to bacterial isolates. All values are means of five replicates x n. of isolates.

Genus; Pseudomonas n. of isolates = 5

pH	Mean optical density (650nm) with added 2,4D	Mean optical density without 2,4D	% inhibition of growth
7.5	0.217	0.223	-2.7
7.0	0.216	0.225	-4.0
6.5	0.219	0.230	-4.8
6.0	0.189	0.204	-7.4

Genus; Aeromonas n. of isolates = 3

pH	Mean optical density (650nm) with added 2,4D	Mean optical density without 2,4D	% inhibition of growth
7.5	0.200	0.221	-9.5
7.0	0.177	0.221	-19.9
6.5	0.075	0.185	-59.5
6.0	0.029	0.086	-66.3

Genus; Flavobacterium n. of isolates = 1

pH	Mean optical density (650nm) with added 2,4D	Mean optical density without 2,4D	% inhibition of growth
7.5	0.144	0.148	-2.7
7.0	0.136	0.146	-6.9
6.5	0.051	0.065	-21.5
6.0	0.005	0.013	-61.5

Table 6.9 (continued)

Group; Gram-positive organisms n. of isolates = 2

pH	Mean optical density (650nm) with added 2,4D	Mean optical density without 2,4D	% inhibition of growth
7.5	0.154	0.168	-8.3
7.0	0.115	0.136	-15.4
6.5	*	*	-
6.0	*	*	-

*no growth detected.

Table 6.10. Percentage dissociation of 2,4D under the experimental pH conditions, calculated from Henderson-Hasselbach equation;

$$pH = pK_a + \log_{10} \frac{[\text{conjugate base}]}{[\text{conjugate acid}]}$$

where pK_a of 2,4D = 2.73 (Que Hee and Sutherland, 1981)

pH	percentage dissociation of 2,4D
7.5	99.998
7.0	99.995
6.5	99.983
6.0	99.946

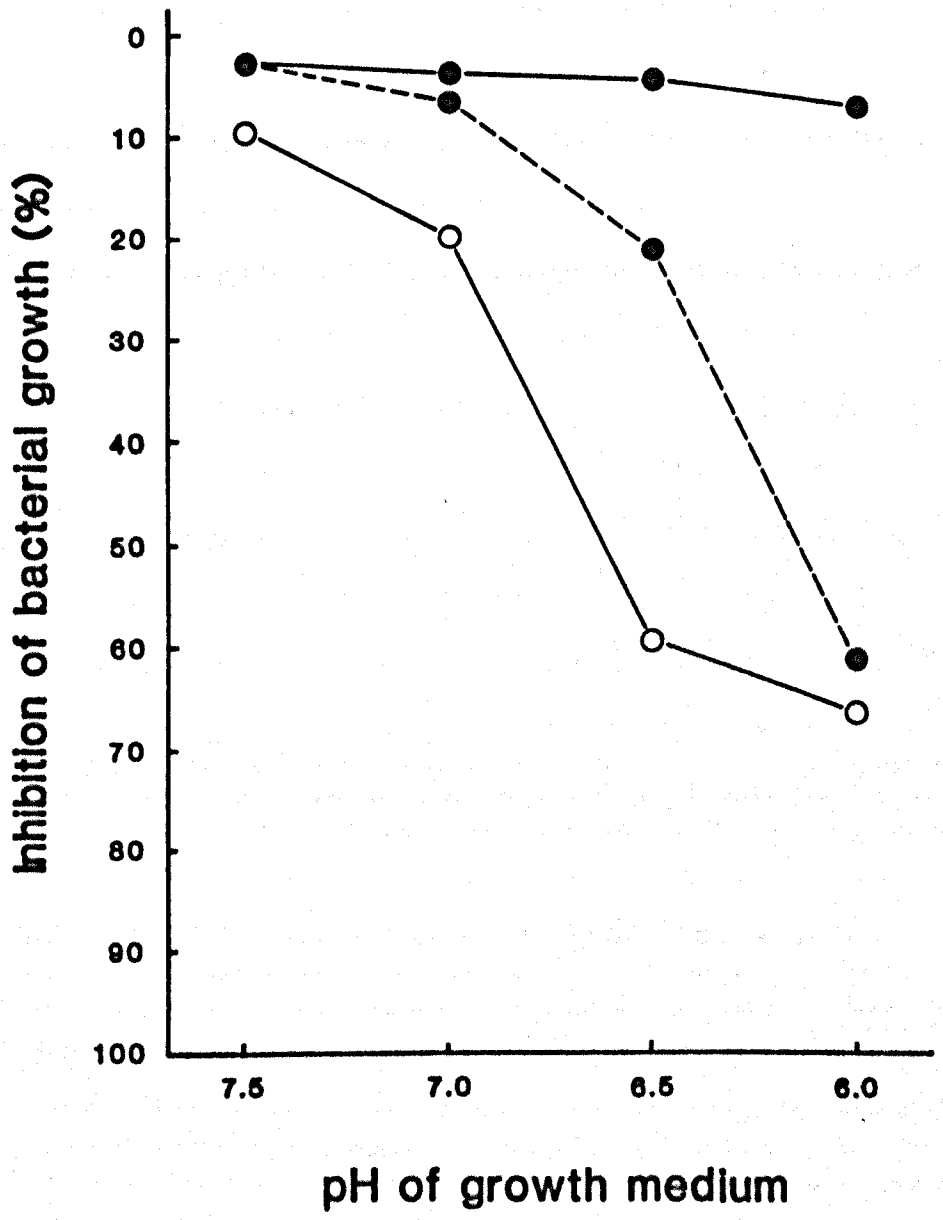
Fig. 6.3. Influence of pH on toxicity of 2,4D to selected bacterial isolates;

●———● Pseudomonas isolates (n=5)

●- - - -● Aeromonas isolates (n=3)

○———○ Flavobacterium isolate (n=1)

each value is mean of 5 replicates x n. of isolates.


Inhibition of bacterial growth (%)
0
10
20
30
40
50
60
70
80
90
100
7.5
7.0
6.5
6.0
pH of growth medium

6.5. Summary

This chapter has illustrated the interaction of some of the factors determining the toxicity of phenolic compounds and phenol derivatives to bacterial communities. Genera or groups of bacterial isolates differed in their sensitivities to phenolic compounds and phenol derivatives, which depended upon pH. The phenolic compounds and phenol derivatives had differing toxicities towards bacteria, which depended on the compound's structure.

The interaction of these factors determined the situation in the natural environment; where the growth and development of a bacterial community, in Sugden Beck, dominated by the genus Pseudomonas was favoured, due to the increased resistance of these organisms to a wide range of phenolic compounds and phenol derivatives over a relatively wide pH range, compared to other more sensitive Gram-negative and Gram-positive organisms. Therefore, the presence of phenolic compounds and phenol derivatives in Sugden Beck, under neutral pH conditions, suppressed the growth of sensitive bacteria allowing the more resistant Pseudomonas spp to flourish.

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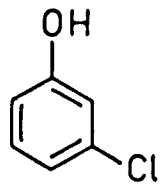
APPENDICES

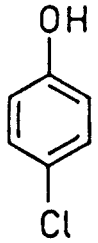
Appendix 1

Chemical structure of identified phenolic compounds and phenol derivatives in Sugden Beck.

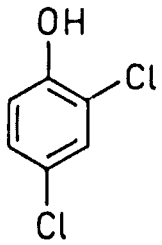
Chlorophenols



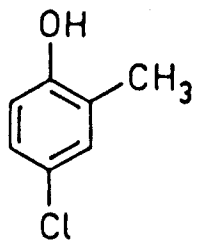
3-chlorophenol
(3CHLR)



4-chlorophenol
(4CHLR)

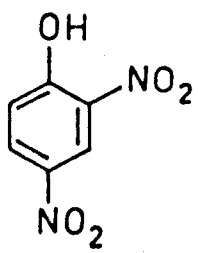


2,4-dichlorophenol
(2,4DCP)

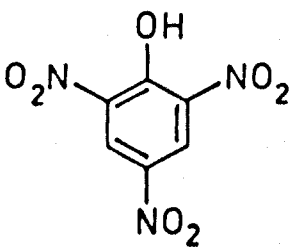


4-chloro-2-methylphenol
(PCOC)

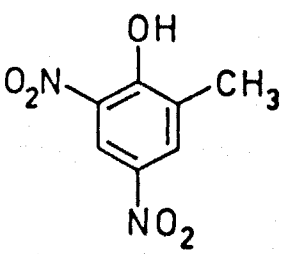
Nitrophenols



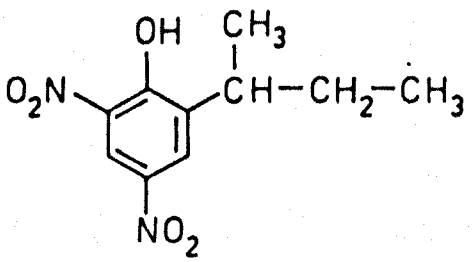
2,4-dinitrophenol
(2,4DNP)



2,4,6-trinitrophenol
(picric acid)

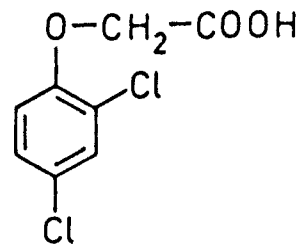


2-methyl-4,6-dinitrophenol
(DNOC)

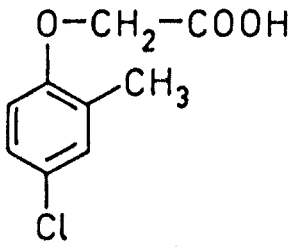


2-sec-butyl-4,6-dinitrophenol
(DNBP)

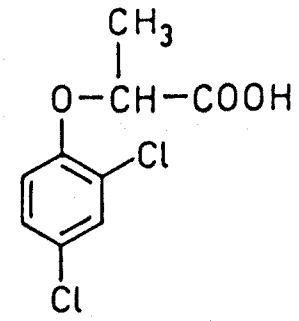
Phenoxyalkanoic acids



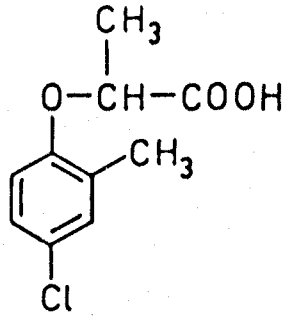
2,4-dichlorophenoxy-acetic acid (2,4D)



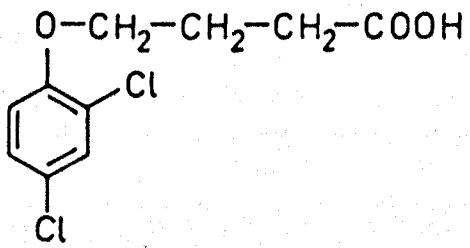
2-methyl-4-chlorophenoxy-acetic acid (MCPA)



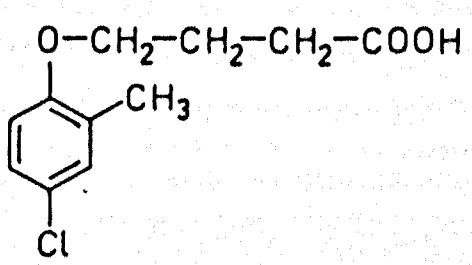
2-(2,4-dichlorophenoxy)-proprionic acid (2,4DP)



2-(2-methyl-4-chlorophenoxy)-proprionic acid (CMPP)



4-(2,4-dichlorophenoxy)-butyric acid (2,4DB)

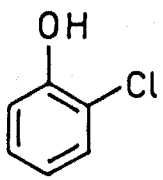


4-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB)

Appendix 1 (continued)

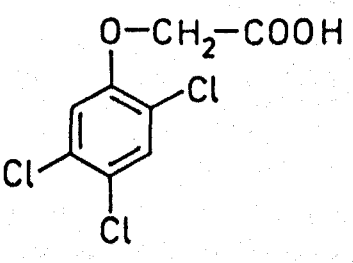
Chemical structure of additional compounds used in toxicity experiments (see Chapter 6).

Chlorophenol



2-chlorophenol (2CHLR)

Phenoxyalkanoic acid



2,4,5-trichlorophenoxyacetic acid

(2,4,5T)

Appendix 2

Media composition

Basal medium for Hugh and Leifson's oxidation-fermentation test (Difco)

Tryptone	2·0g
Sodium chloride	5·0g
Dipotassium phosphate	0·3g
Bromothymol blue	0·08g
Agar	2·0g
Distilled water	1 litre

pH 6·8

Casein-peptone-starch agar (CPS agar)

Soluble casein	0·5g
Peptone	0·5g
Soluble starch	0·5g
Dipotassium hydrogen phosphate	0·2g
Magnesium sulphate, hydrated ($MgSO_4.7H_2O$)	0·05g
Ferric choloride, 0·01% solution	4 drops
Glycerol	1 ml
Agar	15·0g
Distilled water	1 litre

pH 6·9 - 7·0

Fluorescence agar (Lab-m)

Peptone	20·0g
Dipotassium phosphate	1·5g
Magnesium sulphate	1·5g
Glycerol	10 ml
Agar	12·0g
Distilled water	990 ml

pH 7·2

Nutrient agar (Oxoid)

'Lab-Lemco' powder	1·0g
Yeast extract	2·0g
Peptone	5·0g
Sodium chloride	5·0g
Agar No.3	15·0g
Distilled water	1 litre

pH 7·4

Nutrient broth (Oxoid)

'Lab-Lemco' powder	1·0g
Yeast extract powder	2·0g
Peptone	5·0g
Sodium chloride	5·0g
Distilled water	1 litre

pH 7·4

Nutrient broth No. 2 (Oxoid)

'Lab-Lemco' powder	10·0g
Peptone	10·0g
Sodium chloride	5·0g
Distilled water	1 litre

pH 7·5

Pseudomonas-selective-medium (PSM agar, Oxoid)

Gelatin peptone	16·0g
Casein hydrolysate	10·0g
Potassium sulphate	10·0g
Magnesium chloride	1·4g
Cetrimide	0·2g
Nalidixic acid	0·015g
Agar No. 1	10·0g
Distilled water	1 litre

pH 7·1

Quarter-strength Ringer solution (Oxoid)

Sodium chloride	2·25g
Potassium chloride	0·105g
Calcium chloride	0·12g
Sodium bicarbonate	0·05g
Distilled water	1 litre

pH 7·0

Thornley's semi-solid arginine medium

Peptone	1·0g
Dipotassium hydrogen phosphate	0·3g
Sodium chloride	5·0g
L-Arginine monohydrochloride	10·0g
Phenol red, 0·2% solution	5 ml
Agar	3·0g
Distilled water	1 litre

pH 7·2

Yeast extract agar (Oxoid)

Yeast extract powder	3·0g
Peptone	5·0g
Agar No. 3	15·0g
Distilled water	1 litre

pH 7·2

Composition of stains

Gram's stain

Crystal violet stain

Crystal violet	0·5g
Distilled water	100 ml

Gram's iodine solution

Iodine	1·0g
Potassium iodide	2·0g
Distilled water	300 ml

Safranin O solution

Safranin O (2·5% solution in 95% ethanol)	10 ml
Distilled water	100 ml

Flagella stain (Rhodes, 1958)

Ferric tannate mordant

Tannic acid (10% aqueous solution)	10 ml
Aluminium potassium sulphate, saturated aqueous solution	5 ml
Aniline (saturated aqueous solution of aniline)	1 ml
Ferric chloride (5% aqueous solution)	1 ml

Ammonical silver nitrate solution

Silver nitrate	5·0g
Distilled water	100 ml
Ammonia solution (sp.gr. 0·880)	

Negative stain for transmission electron microscopy of flagella

Uranyl acetate	0·5g
Distilled water	100 ml

Composition of scintillation fluid

2, 5-diphenyloxazole (PPO)	6·0g
1, 4-di[2-(5-phenyloxazolyl)] - benzene (POPOP)	0·3g
Naphthalene	100g
2-ethoxyethanol	300 ml
Toluene	1 litre

Raw data from routine sampling programmes

Appendix 3. Data from River Aire sampling sites.

Appendix 4. Data from Stubs Beck and Sugden Beck sampling sites.

Appendix 5. Data from Hunsworth Beck sampling sites.

Note 1. Bacteriological variables.

The distribution shown by counts of total bacteria, viable bacteria on CPS agar, chromogenic bacteria and viable bacteria on PSM agar were tested for agreement with Poisson distribution, using the variance (s^2) to mean ($\bar{x}$) ratio (Elliott, 1971 P40). Usually the variance was not significantly different from the mean ($p>0{\cdot}05$: X^2 test) and a Poisson distribution was accepted and 95% confidence limits were calculated around the arithmetic mean. If the variance was significantly greater than the mean ($p<0{\cdot}05$: X^2 test) 95% confidence limits were calculated around the geometric mean, using a log (x) transformation or a log (x + 1) transformation if the data contained zero values. All means shown are arithmetic means, but if the confidence intervals given apply strictly to the geometric mean this is indicated by an asterisk.

Note 2. A dash (-) in any of the appendices indicates that a variable was not measured.

APPENDIX 3

River Aire data, January to December 1981; Bacteriological variables, figures in brackets are 95% confidence limits of the mean, except for bacterial activity where the range is given.

Date	Activity $x10^{3}h^{-1}$	Total count $x10^{-6}ml^{-1}$	CPS viable count $x10^{-5}CFUml^{-1}$	No.of chromogenic bacteria $x10^{-4}CFUml^{-1}$	PSM viable count $x10^{-2}CFUml^{-1}$
Site: 30m upstream					
24.01.81	16.7 (15.3 - 17.9)	2.0 (1.8 - 2.3)	1.4 (1.2 - 1.6)	2.8 (1.5 - 4.1)	2.1 (1.2 - 3.0)
23.02.81	10.5 (8.7 - 11.7)	3.1 (2.8 - 3.3)*	0.7 (0.5 - 1.0)	0.6 (0.1 - 1.1)	1.8 (0.6 - 3.0)
25.03.81	47.3 (43.2 - 50.5)	2.4 (2.2 - 2.6)	3.1 (2.7 - 3.5)*	5.5 (4.0 - 6.7)*	4.3 (2.9 - 5.4)*
22.04.81	10.2 (8.4 - 11.8)	6.5 (6.1 - 6.8)	7.8 (6.4 - 9.1)*	34.9 (24.4 - 43.9)*	3.6 (2.8 - 3.6)*
21.05.81	18.3 (17.5 - 19.9)	8.0 (7.6 - 8.4)	5.5 (3.6 - 7.0)*	10.0 (5.2 - 13.3)*	6.2 (5.4 - 7.1)
24.06.81	17.5 (15.2 - 18.3)	6.9 (6.5 - 7.4)	5.2 (4.0 - 6.3)*	7.4 (3.6 - 10.1)*	1.5 (1.3 - 1.6)
23.07.81	16.9 (15.5 - 17.6)	6.4 (5.8 - 7.0)	3.9 (3.5 - 4.3)	7.7 (6.0 - 9.4)	3.9 (3.4 - 4.4)*
19.08.81	14.9 (13.9 - 16.4)	5.8 (5.4 - 6.3)	3.9 (2.9 - 4.8)*	4.2 (1.9 - 6.6)	1.1 (0.8 - 1.4)
22.09.81	19.9 (18.3 - 22.3)	6.8 (5.8 - 7.3)*	12.1 (10.1 - 14.0)*	29.6 (20.9 - 37.0)*	5.1 (4.6 - 5.7)
23.10.81	25.3 (20.7 - 32.6)	3.8 (3.5 - 4.0)	-	-	6.0 (4.8 - 7.1)*
27.11.81	88.8 (77.2 - 96.1)	4.9 (4.5 - 5.3)	10.6 (8.9 - 12.2)*	23.7 (18.2 - 29.1)	10.8 (9.6 - 11.9)*
17.12.81	62.2 (58.9 - 64.2)	14.7 (13.4 - 15.6)	8.8 (7.4 - 10.1)*	19.7 (13.1 - 25.0)*	15.6 (14.8 - 16.4)
Site: 5m downstream					
24.01.81	11.4 (10.7 - 12.6)	2.1 (1.8 - 2.3)	2.3 (1.8 - 2.7)	5.8 (4.2 - 7.5)	1.8 (0.6 - 2.9)
23.02.81	0.5 (0.4 - 0.7)	2.7 (2.5 - 3.0)	0.3 (0.2 - 0.4)	0.6 (0.1 - 1.1)	0.6 (0.1 - 1.1)
25.03.81	41.8 (40.7 - 43.1)	2.9 (2.5 - 3.1)*	2.6 (2.2 - 2.9)*	6.0 (4.3 - 7.4)*	3.4 (3.0 - 3.8)
22.04.81	1.1 (1.0 - 1.2)	5.4 (5.1 - 5.7)	3.2 (2.6 - 3.8)*	11.0 (7.1 - 14.5)*	1.0 (0.7 - 1.4)
21.05.81	4.7 (4.2 - 5.4)	8.3 (7.9 - 8.7)	6.8 (5.5 - 8.0)*	15.0 (11.0 - 18.3)*	2.3 (1.8 - 2.7)
24.06.81	7.9 (6.6 - 9.4)	7.4 (7.2 - 7.7)	3.5 (3.0 - 4.0)*	5.4 (4.0 - 6.8)	3.0 (2.5 - 3.6)
23.07.81	5.9 (4.5 - 7.4)	6.9 (6.4 - 7.5)	7.9 (7.0 - 8.6)*	10.9 (9.3 - 12.6)	3.9 (3.1 - 4.5)*
19.08.81	4.9 (4.0 - 6.4)	3.5 (3.2 - 3.8)	10.6 (9.3 - 11.8)*	26.9 (22.0 - 32.0)	2.6 (2.0 - 3.1)*
22.09.81	9.5 (8.2 - 10.3)	7.8 (7.3 - 8.2)	13.2 (11.1 - 15.1)*	29.3 (20.6 - 36.9)*	1.5 (1.2 - 1.8)
23.10.81	20.0 (18.2 - 22.6)	3.3 (3.1 - 3.5)	-	-	1.9 (1.5 - 2.2)
27.11.81	63.0 (51.3 - 73.9)	5.4 (5.0 - 5.7)	13.0 (10.4 - 15.2)*	26.5 (20.1 - 31.4)*	10.1 (7.0 - 13.0)*
17.12.81	0.5 (0.2 - 1.1)	13.4 (12.7 - 14.3)	3.8 (2.9 - 4.4)*	7.7 (4.6 - 9.9)*	6.1 (5.5 - 6.8)
Site: 25m downstream					
24.01.81	16.1 (14.4 - 17.4)	2.0 (1.7 - 2.1)	1.2 (0.9 - 1.5)	0.6 (0.5 - 0.8)*	2.4 (1.0 - 3.8)
23.02.81	12.2 (11.3 - 12.9)	2.4 (2.2 - 2.6)	0.7 (0.5 - 1.0)	0.6 (0.2 - 1.0)	1.3 (0.2 - 2.4)
25.03.81	30.8 (30.3 - 31.2)	2.6 (2.4 - 2.8)	2.5 (2.1 - 2.9)*	6.8 (5.7 - 7.8)*	2.9 (2.3 - 3.4)
22.04.81	13.5 (13.0 - 14.0)	6.3 (6.0 - 6.7)	2.1 (1.8 - 2.4)	3.4 (2.1 - 4.6)	2.6 (2.3 - 2.9)
21.05.81	56.4 (54.9 - 57.7)	7.5 (7.2 - 7.7)	13.0 (10.3 - 15.6)*	45.9 (31.3 - 59.0)*	6.5 (5.8 - 7.3)
24.06.81	21.5 (18.9 - 24.7)	6.4 (6.0 - 6.7)	2.0 (1.4 - 2.5)*	2.0 (0.7 - 3.3)	2.3 (1.9 - 2.6)
23.07.81	30.2 (28.2 - 32.5)	7.0 (5.7 - 7.5)*	4.5 (4.1 - 5.0)	7.5 (4.7 - 10.2)	2.3 (2.0 - 2.6)
19.08.81	11.5 (9.2 - 14.5)	5.7 (5.2 - 6.2)	5.4 (4.6 - 6.2)*	11.1 (8.1 - 14.1)	1.2 (1.0 - 1.5)
22.09.81	16.8 (15.4 - 18.6)	7.8 (7.3 - 8.3)	11.5 (10.0 - 13.0)*	26.3 (19.3 - 32.4)*	5.3 (4.8 - 5.9)
23.10.81	1.0 (0.7 - 1.2)	2.9 (2.7 - 3.1)	-	-	1.8 (1.4 - 2.3)
27.11.81	80.6 (72.2 - 86.4)	5.2 (4.9 - 5.6)	11.0 (9.5 - 12.4)*	25.7 (21.0 - 30.6)	8.9 (7.9 - 9.8)
17.12.81	51.6 (41.9 - 60.3)	12.1 (11.2 - 13.0)	7.3 (6.6 - 8.0)	16.9 (12.9 - 20.9)	12.6 (10.7 - 14.2)*

Date	Activity $x10^{3}h^{-1}$	Total count $x10^{-6}ml^{-1}$	CPS viable count $x10^{-5}CFUml^{-1}$	No.of chromogenic bacteria $x10^{-4}CFUml^{-1}$	PSM viable count $x10^{-2}CFUml^{-1}$
Site: 50m downstream					
24.01.81	18.0 (15.2 - 21.7)	2.0 (1.8 - 2.2)	2.0 (1.1 - 2.7)*	3.8 (2.7 - 4.9)	4.1 (3.2 - 5.0)
23.02.81	11.5 (9.8 - 14.8)	2.6 (2.3 - 2.8)	0.9 (0.4 - 1.2)*	1.4 (0.1 - 2.1)*	0.9 (0.3 - 1.5)
25.03.81	38.5 (35.6 - 40.3)	2.5 (2.2 - 2.7)	2.4 (2.0 - 2.4)*	5.7 (4.9 - 6.5)	2.8 (2.3 - 3.4)
22.04.81	15.0 (13.2 - 16.5)	4.9 (4.7 - 5.2)	7.5 (5.7 - 9.0)*	31.8 (21.9 - 39.8)*	1.9 (1.6 - 2.3)
21.05.81	51.7 (42.8 - 57.2)	7.6 (7.3 - 7.9)	15.9 (14.2 - 17.5)*	64.2 (52.8 - 74.4)*	6.0 (4.9 - 7.0)*
24.06.81	13.2 (11.6 - 14.4)	6.3 (6.0 - 6.6)	2.4 (2.1 - 2.8)	2.8 (1.2 - 3.7)	2.1 (1.8 - 2.5)
23.07.81	47.2 (41.0 - 51.7)	6.9 (5.8 - 7.4)*	3.0 (2.3 - 3.5)*	5.0 (3.6 - 6.4)	3.5 (3.1 - 3.8)
19.08.81	6.2 (4.1 - 8.2)	7.4 (6.9 - 7.9)	1.2 (0.6 - 1.7)*	1.8 (0.8 - 2.9)	0.9 (0.6 - 1.1)*
22.09.81	7.3 (5.4 - 11.5)	7.8 (7.3 - 8.3)	16.9 (14.3 - 19.2)*	45.7 (37.8 - 53.6)	4.4 (3.5 - 5.3)*
23.10.81	0.9 (0.3 - 2.5)	2.7 (2.5 - 2.9)	-	-	0.5 (0.4 - 0.7)
27.11.81	87.0 (86.0 - 96.0)	5.8 (5.4 - 6.3)	8.7 (7.2 - 10.1)*	20.9 (17.1 - 24.7)	11.5 (10.9 - 12.2)
17.12.81	59.5 (58.6 - 60.2)	11.2 (10.6 - 11.8)	7.8 (7.1 - 8.6)	16.8 (13.9 - 19.7)	15.9 (14.7 - 17.1)
Site: 100m downstream					
24.01.81	15.2 (14.1 - 16.2)	2.1 (1.5 - 2.3)*	2.6 (1.8 - 3.3)*	6.5 (4.1 - 8.9)	1.8 (1.1 - 2.5)
23.02.81	12.4 (11.6 - 13.1)	2.6 (2.4 - 2.7)	0.4 (0.3 - 0.5)	0.4 (0.1 - 0.7)	1.3 (0.6 - 2.0)
25.03.81	22.9 (19.4 - 25.9)	2.8 (2.6 - 3.0)	2.2 (1.9 - 2.5)*	6.4 (4.9 - 7.7)*	2.5 (2.0 - 3.0)
22.04.81	16.3 (13.2 - 19.0)	5.2 (5.0 - 5.4)	9.5 (6.8 - 11.8)*	40.0 (23.4 - 52.6)*	2.2 (1.7 - 2.7)*
21.05.81	47.4 (44.1 - 52.2)	7.7 (7.3 - 8.0)	17.7 (16.1 - 19.3)*	59.3 (49.5 - 68.4)*	5.5 (5.1 - 6.0)
24.06.81	17.6 (14.5 - 20.2)	6.2 (5.9 - 6.5)	4.8 (4.2 - 5.5)	5.4 (3.8 - 7.1)	2.8 (2.4 - 3.1)
23.07.81	44.4 (41.7 - 46.6)	6.5 (6.0 - 6.9)	2.9 (2.6 - 3.3)	5.7 (3.8 - 7.6)	2.9 (2.6 - 3.3)
19.08.81	12.0 (7.0 - 24.2)	5.2 (4.6 - 5.7)	3.5 (2.0 - 4.7)*	5.2 (2.1 - 7.1)*	0.8 (0.7 - 0.9)
22.09.81	19.3 (16.3 - 24.4)	7.7 (7.3 - 8.2)	9.5 (7.5 - 11.1)*	24.3 (16.4 - 30.2)*	4.9 (4.1 - 5.6)
23.10.81	0.3 (0.2 - 0.4)	3.0 (2.7 - 3.2)	-	-	0.2 (0.1 - 0.3)
27.11.81	70.1 (62.9 - 76.3)	5.6 (5.4 - 5.9)	11.3 (8.6 - 13.6)*	28.3 (22.2 - 34.0)	9.6 (8.9 - 10.4)
17.12.81	48.2 (46.4 - 50.2)	12.6 (11.9 - 13.3)	9.7 (8.4 - 10.9)*	23.3 (19.9 - 26.7)	19.6 (18.3 - 20.9)
Site: 500m downstream					
24.01.81	18.3 (17.4 - 19.0)	1.9 (1.7 - 2.1)	1.3 (0.8 - 1.7)*	2.2 (1.2 - 4.3)*	2.7 (1.6 - 3.8)
23.02.81	12.2 (11.3 - 13.9)	2.6 (2.4 - 2.8)	1.0 (0.7 - 1.2)	0.8 (0.1 - 1.5)	1.2 (0.8 - 1.6)
25.03.81	23.8 (22.1 - 25.7)	2.4 (2.2 - 2.6)*	2.3 (2.1 - 2.6)*	6.0 (5.1 - 6.9)*	2.1(1.8 - 2.4)
22.04.81	16.3 (15.2 - 17.3)	6.9 (6.6 - 7.2)	3.0 (2.5 - 3.5)	8.0 (6.2 - 9.9)	2.0 (1.7 - 2.3)
21.05.81	72.9 (71.2 - 75.7)	8.3 (7.8 - 8.7)	21.2 (17.4 - 24.8)*	61.7 (47.3 - 75.0)*	6.9 (6.4 - 7.4)
24.06.81	14.3 (13.7 - 14.8)	7.1 (6.6 - 7.7)	4.5 (3.4 - 5.3)*	5.3 (1.0 - 7.7)*	1.9 (1.6 - 2.2)
23.07.81	49.9 (47.4 - 52.6)	7.7 (7.2 - 8.2)	4.4 (3.9 - 4.9)	7.6 (4.9 - 10.3)	4.2 (3.6 - 4.7)*
19.08.81	12.8 (10.7 - 15.2)	5.6 (5.1 - 6.1)	2.6 (1.7 - 3.4)*	2.4 (1.1 - 3.8)	0.5 (0.4 - 0.7)
22.09.81	14.2 (12.8 - 16.4)	6.9 (6.4 - 7.5)	9.1 (7.1 - 11.0)*	26.9 (17.0 - 35.3)*	-
23.10.81	1.1 (0.3 - 2.8)	3.0 (2.7 - 3.3)	-	-	0.3 (0.2 - 0.4)
27.11.81	77.6 (59.7 - 92.6)	6.0 (5.6 - 6.4)	9.4 (8.2 - 10.5)*	22.4 (18.5 - 26.3)	10.2 (9.4 - 12.3)*
17.12.81	57.8 (57.0 - 58.4)	12.3 (11.4 - 13.2)	9.1 (8.4 - 10.1)	21.6 (17.6 - 25.6)	19.2 (18.1 - 20.3)

APPENDIX 3 (continued)

River Aire data, January to December 1981; Environmental variables.

Date	pH	Temperature (^{o}C)	Dissolved oxygen concentration (mgl^{-1})	BOD (mg oxygen l^{-1})	Conductivity (μScm^{-1})	Turbidity (Arbitrary units)	Flow rate* ($m^{3}s^{-1}$)
Site: 30m upstream							
24.01.81	7.2	8.0	12.4	4.3	-	8	14.8
23.02.81	7.6	9.0	11.7	-	529	11	7.9
25.03.81	7.6	9.0	10.5	-	350	13	62.8
22.04.81	7.1	18.5	10.8	-	601	4	5.5
21.05.81	7.5	23.0	10.7	-	658	8	8.0
24.06.81	8.0	25.0	7.8	12.5	701	8	5.3
23.07.81	7.8	22.0	8.0	11.0	715	11	10.1
19.08.81	7.3	22.0	9.0	9.0	615	4	5.4
22.09.81	7.5	14.5	9.4	9.0	372	6	9.4
23.10.81	8.1	13.0	8.8	24.0	415	4	10.2
27.11.81	7.4	9.0	10.6	28.5	272	35	97.3
17.12.81	7.8	8.0	9.3	21.0	558	11	7.3
Site: 5m downstream							
24.01.81	7.1	8.0	11.5	5.9	-	6	
23.02.81	7.1	9.0	10.8	-	658	14	
25.03.81	6.4	9.0	10.2	-	358	19	
22.04.81	7.0	15.0	10.4	-	772	12	
21.05.81	7.5	22.0	9.2	19.5	844	34	
24.06.81	7.8	22.0	6.3	18.5	872	26	
23.07.81	7.8	22.0	6.6	18.5	1144	25	
19.08.81	7.2	22.0	7.4	26.5	829	16	
22.09.81	7.3	16.0	8.6	10.5	572	19	
23.10.81	7.8	15.0	7.5	10.5	729	19	
27.11.81	7.2	9.0	10.7	21.0	486	38	
17.12.81	7.7	8.0	8.5	12.6	644	15	
Site: 25m downstream							
24.01.81	7.3	8.5	12.2	6.5	-	4	
23.02.81	7.5	9.0	11.4	-	558	9	
25.03.81	7.5	10.0	10.3	-	358	14	
22.04.81	7.2	20.0	10.4	-	672	6	
21.05.31	7.6	23.0	11.0	-	644	14	
24.06.81	7.9	23.0	8.9	9.3	644	13	
23.07.81	7.8	21.0	8.0	7.0	658	13	
19.08.81	7.4	23.0	8.4	11.5	644	6	
22.09.81	7.4	15.0	9.6	12.5	400	11	
23.10.81	7.9	13.0	8.6	10.5	443	6	
27.11.81	7.6	8.5	10.4	18.0	257	36	
17.12.81	7.7	8.9	8.9	25.8	701	13	

*Flow rate measured at Armley, Leeds, data provided by Y.W.A.

Date	pH	Temperature (^{o}C)	Dissolved oxygen concentration (mgl^{-1})	BOD (mg oxygen l^{-1})	Conductivity (μScm^{-1})	Turbidity (Arbitrary units)
Site: 50m downstream						
24.01.81	7.3	8.0	12.6	6.4	-	6
23.02.81	7.4	9.0	11.7	-	558	9
25.03.81	7.7	10.0	10.3	-	350	14
22.04.81	7.3	21.0	10.5	-	658	9
21.05.81	7.9	23.0	10.9	15.5	644	12
24.06.81	8.0	23.0	8.2	5.5	644	18
23.07.81	7.8	21.0	8.8	8.5	615	8
19.08.81	7.3	22.0	8.4	17.5	644	5
22.09.81	7.2	15.0	9.3	10.5	386	7
23.10.81	7.8	13.5	7.8	15.0	443	5
27.11.81	7.7	9.0	10.5	18.3	243	35
17.12.81	7.5	8.0	8.7	22.5	615	11
Site: 100m downstream						
24.01.81	7.2	8.0	12.2	7.4	-	5
23.02.81	7.4	9.0	11.6	-	558	9
25.03.81	7.7	10.0	10.6	-	350	15
22.04.81	7.4	21.5	10.4	-	645	13
21.05.81	8.0	23.0	11.0	-	629	9
24.06.81	8.0	23.0	8.0	8.5	629	13
23.07.81	7.8	21.0	8.8	8.5	601	8
19.08.81	7.2	22.0	8.5	17.5	629	5
22.09.81	7.3	15.0	9.5	6.0	386	7
23.10.81	7.8	13.5	8.3	7.5	429	6
27.11.81	7.6	9.0	10.4	22.8	243	34
17.12.81	7.8	8.0	8.9	23.1	586	12
Site: 500m downstream						
24.01.81	7.2	8.0	12.2	5.4	-	6
23.02.81	7.5	9.5	11.4	-	558	7
25.03.81	7.7	10.0	10.5	-	350	13
22.04.81	7.2	21.0	10.6	-	644	11
21.05.81	8.0	22.0	11.1	10.5	629	10
24.06.81	8.0	23.0	8.0	9.5	629	7
23.07.81	7.8	21.0	8.0	10.5	586	9
19.08.81	7.4	23.0	8.4	24.5	644	3
22.09.81	7.3	15.0	9.4	8.5	372	6
23.10.81	7.6	13.0	9.2	12.0	443	5
27.11.81	7.7	9.0	10.4	21.9	243	40
17.12.81	7.9	8.0	9.3	25.5	558	12

APPENDIX 3 (continued)

River Aire data, January to December 1981; Metal variables ($\mu g l^{-1}$).

Date	Total copper	Total zinc	Total cadmium	Total lead	Total metals
Site: 30m upstream					
24.01.81	25.8	34.2	0.7	7.7	68.4
23.02.81	12.3	31.7	0.3	4.8	49.1
25.03.81	28.9	87.4	0.5	24.8	142
22.04.81	16.5	60.0	1.1	20.1	97.7
21.05.81	63.9	62.5	1.1	23.6	151
24.06.81	20.9	75.6	1.4	8.9	107
23.07.81	10.7	19.5	1.5	11.5	43.2
19.08.81	36.7	15.5	0.4	10.7	63.3
22.09.81	8.3	27.1	4.0	13.8	53.2
23.10.81	16.6	17.8	1.4	11.8	47.6
27.11.81	30.5	119	1.2	32.1	183
17.12.81	15.3	34.4	0.9	12.3	62.9
Site: 5m downstream					
24.01.81	145	65.1	5.5	38.9	225
23.02.81	143	158	1.8	70.9	373
25.03.81	54.4	46.7	4.6	38.0	144
22.04.81	339	118	4.9	39.8	502
21.05.81	163	147	8.4	53.2	371
24.06.81	243	139	7.5	22.5	412
23.07.81	430	172	15.9	87.7	706
19.08.81	546	147	6.0	79.8	779
22.09.81	122	132	7.7	74.8	337
23.10.81	749	107	2.0	19.9	877
27.11.81	36.2	99.4	7.1	159	302
17.12.81	921	219	5.1	117	1261
Site: 25m downstream					
24.01.81	27.9	30.0	0.4	8.4	66.7
23.02.81	13.4	39.8	0.2	9.8	63.2
25.03.81	21.4	37.1	1.9	23.9	84.3
22.04.81	45.3	50.9	0.8	16.1	113
21.05.81	39.1	46.5	0.7	12.7	99.0
24.06.81	16.5	33.1	2.4	8.1	60.1
23.07.81	175	65.4	4.0	28.5	272
19.08.81	121	59.0	2.2	73.1	255
22.09.81	15.9	31.4	5.3	18.5	71.1
23.10.81	92.2	36.2	1.4	13.9	144
27.11.81	39.9	93.3	4.5	69.9	208
17.12.81	56.3	82.0	4.0	44.9	187

Date	Total copper	Total zinc	Total cadmium	Total lead	Total metals
Site: 50m downstream					
24.01.81	13.6	14.9	0.5	5.3	34.3
23.02.81	22.1	52.1	0.3	10.1	84.6
25.03.81	24.4	57.0	0.3	28.5	110
22.04.81	35.0	40.7	0.8	15.3	91.8
21.05.81	20.4	48.3	0.9	15.7	85.3
24.06.81	38.7	65.5	3.2	12.5	120
23.07.81	118	66.2	0.6	23.3	208
19.08.81	58.5	72.3	0.8	12.8	144
22.09.81	31.4	18.0	0.3	15.4	65.1
23.10.81	60.2	33.0	0.7	16.0	110
27.11.81	48.8	85.5	2.3	70.3	207
17.12.81	28.5	15.9	0.9	6.1	51.4
Site: 100m downstream					
24.01.81	12.5	24.9	0.7	8.5	46.6
23.02.81	13.8	32.7	0.2	6.5	53.2
25.03.81	42.0	34.5	0.4	18.9	95.8
22.04.81	19.2	21.5	1.5	9.4	51.6
21.05.81	35.4	35.4	1.3	18.3	90.4
24.06.81	28.1	79.2	0.9	17.9	126
23.07.81	78.5	43.2	0.6	16.4	139
19.08.81	78.4	50.2	1.0	21.3	151
22.09.81	28.5	22.3	0.6	13.2	64.6
23.10.81	29.5	38.0	0.8	12.4	80.7
27.11.81	15.3	92.3	1.6	38.7	148
17.12.81	37.5	28.5	0.4	8.3	74.7
Site: 500m downstream					
24.01.81	13.1	19.7	0.1	11.3	44.2
23.02.81	12.8	32.0	0.2	5.3	50.3
25.03.81	17.7	74.3	0.4	25.9	117
22.04.81	15.4	10.4	0.9	7.5	34.2
21.05.81	18.5	51.8	0.7	21.0	92.0
24.06.81	11.5	43.1	0.3	9.0	63.9
23.07.81	38.7	37.3	0.7	13.6	90.3
19.08.81	22.7	84.7	0.4	19.5	127
22.09.81	8.5	33.0	0.9	9.8	52.2
23.10.81	73.2	30.7	0.8	15.8	121
27.11.81	16.0	75.6	1.1	15.3	108
17.12.81	38.5	42.7	0.1	8.0	89.3

APPENDIX 3 (continued)

River Aire data, January to December 1981; Environmental and metal variables in effluent (concentration of metal variables μgl^{-1}).

Date	pH	Temperature (^{o}C)	Dissolved oxygen concentration (mgl^{-1})	BOD (mg oxygen l^{-1})	Conductivity (μScm^{-1})	Turbidity (Arbitrary units)
24.01.81	6.9	10.0	9.7	-	-	-
23.02.81	7.0	6.5	9.6	-	901	27
25.03.81	6.0	11.5	8.7	-	801	37
22.04.81	6.8	12.0	9.5	-	1030	22
21.05.81	7.4	19.0	6.7	14.0	1090	53
24.06.81	7.6	21.0	5.2	-	1090	33
23.07.81	7.0	21.0	5.4	-	1430	37
19.08.81	7.4	21.0	7.1	23.5	1140	25
22.09.81	7.3	19.5	7.9	13.0	1040	9
23.10.81	7.6	17.0	6.8	9.5	944	24
27.11.81	7.3	13.5	8.5	10.2	686	85
17.12.81	7.8	8.0	8.5	28.2	800	32

Date	Total copper	Total zinc	Total cadmium	Total lead	Total metals
24.01.81	428	120	16.6	89.5	655
23.02.81	431	410	5.4	146	992
25.03.81	169	98.1	13.8	57.3	338
22.04.81	370	268	20.0	54.7	713
21.05.81	212	192	8.9	69.6	482
24.06.81	880	192	8.0	58.1	1140
23.07.81	1310	259	8.9	100	1680
19.08.81	1270	382	7.3	152	1820
22.09.81	261	268	18.3	81.4	628
23.10.81	1980	248	3.3	60.0	2290
27.11.81	1300	595	9.2	281	2180
17.12.81	3240	309	10.0	181	3740

APPENDIX 4

Stubs Beck and Sugden Beck data, April 1981 to April 1982; Bacteriological variables, figures in brackets are 95% confidence limits of the mean, except for bacterial activity where the range is given.

Site: Stubs Beck, water samples

Date	Activity $x10^{4}h^{-1}$	Total count $x10^{-5}ml^{-1}$	CPS viable count $x10^{-4}CFUml^{-1}$	Number of chromogenic bacteria $x10^{-3}CFUml^{-1}$	Bacterial diversity	PSM viable count $x10^{-1}CFUml^{-1}$
06.04.81	4.2 (2.9 - 6.6)	5.4 (4.9 - 6.0)	1.4 (0.8 - 1.8)*	3.7 (1.5 - 5.9)	-	0
12.05.81	7.4 (6.3 - 8.2)	6.7 (6.3 - 7.1)	1.2 (1.0 - 1.5)*	2.5 (1.5 - 3.7)*	-	2.4 (1.8 - 3.0)
11.06.81	28.9 (26.1 - 30.4)	6.5 (5.8 - 7.1)	3.1 (2.7 - 3.4)*	7.3 (5.8 - 8.3)*	-	14.7 (12.5 - 16.9)
09.07.81	-	-	-	-	-	-
14.08.81	47.8 (40.9 - 60.5)	15.0 (13.3 - 16.7)	6.8 (6.0 - 7.5)*	12.9 (10.5 - 15.2)	48.9	13.3 (11.2 - 15.3)
09.09.81	-	-	-	-	-	-
07.10.81	74.2 (51.9 - 130)	48.1 (44.0 - 52.2)	14.8 (13.9 - 15.6)	28.1 (23.0 - 33.0)	26.5	41.7 (35.8 - 47.0)*
09.11.81	35.7 (34.2 - 37.1)	41.9 (38.4 - 45.4)	7.3 (4.3 - 9.1)*	20.0 (11.9 - 28.1)	17.1	4.1 (2.6 - 5.6)
07.12.81	15.5 (12.5 - 19.6)	15.1 (14.0 - 16.2)	9.0 (7.2 - 10.8)	23.3 (15.4 - 31.3)	17.7	10.7 (9.1 - 12.3)
07.01.82	8.0 (6.5 - 9.4)	8.5 (7.5 - 9.0)	4.7 (3.6 - 5.6)*	15.2 (10.9 - 18.6)*	39.8	4.5 (3.4 - 5.6)
08.02.82	8.1 (4.6 - 14.9)	10.1 (9.1 - 10.7)	2.8 (2.4 - 3.3)	9.3 (7.0 - 11.6)	44.7	3.0 (1.9 - 4.0)
08.03.82	7.5 (6.6 - 8.8)	12.3 (11.6 - 13.0)	8.2 (7.1 - 9.2)*	29.7 (24.3 - 35.1)	33.2	5.4 (3.8 - 7.0)
06.04.82	11.7 (10.3 - 13.7)	20.5 (18.9 - 22.1)	6.8 (6.2 - 7.5)	20.0 (16.4 - 23.7)	35.6	7.2 (5.6 - 8.8)

Site: Stubs Beck, gravel samples

Date	Activity $x10^{3}g^{-1}h^{-1}$	Total count $x10^{-7}g^{-1}$	CPS viable count $x10^{-6}CFUg^{-1}$	Number of chromogenic bacteria $x10^{-5}CFUg^{-1}$	Bacterial diversity
06.04.81	-	-	-	-	-
12.05.81	21.7 (19.4 - 25.1)	1.6 (1.4 - 1.7)	8.1 (6.9 - 9.2)*	23.7 (20.1 - 27.5)	-
11.06.81	-	-	-	-	-
09.07.81	-	-	-	-	-
14.08.81	-	-	-	-	-
09.09.81	-	-	-	-	-
07.10.81	-	-	-	-	-
09.11.81	-	-	-	-	-
07.12.81	9.8 (9.4 - 10.0)	2.7 (2.4 - 3.1)	12.6 (10.4 - 14.5)*	24.4 (19.3 - 29.3)	8.0
07.01.82	16.4 (15.2 - 18.8)	2.6 (2.4 - 2.7)	8.4 (7.2 - 9.6)*	28.4 (20.1 - 34.6)*	9.6
08.02.82	15.1 (13.7 - 16.5)	2.3 (2.2 - 2.5)	1.3 (1.0 - 1.5)	5.6 (3.5 - 7.7)	15.8
08.03.82	13.5 (12.0 - 14.3)	2.8 (2.6 - 3.0)	6.0 (5.3 - 6.7)*	17.1 (14.2 - 19.9)	11.2
06.04.82	12.8 (11.0 - 15.2)	2.9 (2.7 - 3.2)	17.7 (14.1 - 20.9)*	77.1 (60.3 - 91.6)*	7.4

Site: Sugden Beck Upper, water samples

Date	Activity $x10^{4}h^{-1}$	Total count $x10^{-5}ml^{-1}$	CPS viable count $x10^{-4}CFUml^{-1}$	Number of chromogenic bacteria $x10^{-3}CFUml^{-1}$	Bacterial diversity	PSM viable count $x10^{-1}CFUml^{-1}$
06.04.81	0.3 (0.2-0.3)	4.8 (3.9-5.2)*	0.004 (0.003-0.006)	0.004 (0.001-0.007)	-	0
12.05.81	0.3 (0.03-0.7)	6.6 (6.1-7.1)	0.05 (0.04-0.06)	0.02 (0.005-0.04)	-	0
11.06.81	0.5 (0-2.3)	10.0 (9.2-10.8)	0.01 (0.008-0.02)	0.009 (0.003-0.02)	-	0
09.07.81	0.3 (0.2-0.4)	3.4 (2.9-3.8)	0.003 (0.002-0.004)	0.002 (0-0.003)	-	0
14.08.81	0.4 (0.2-0.7)	2.4 (2.2-2.6)	0.004 (0.003-0.005)	0.002 (0-0.006)	5.2	0
09.09.81	1.0 (0.8-1.2)	8.1 (7.4-8.7)	0.005 (0.003-0.006)	0.007 (0-0.02)	4.4	0
07.10.81	18.5 (14.3-20.7)	49.2 (46.7-51.7)	46.7 (42.7-50.7)	109 (88.2-130)	13.5	70.0 (58.8-79.9)*
09.11.81	1.2 (0.2-2.3)	36.4 (32.1-40.7)	12.7 (9.3-15.4)*	3.0 (0.5-5.5)	12.6	39.2 (30.2-47.2)*
07.12.81	3.6 (0-11.2)	12.6 (12.0-13.2)	9.1 (8.2-10.0)	11.1 (5.4-16.8)	12.3	33.8 (29.8-37.8)
07.01.82	1.8 (1.4-2.1)	13.5 (11.4-15.6)	7.6 (7.1-8.1)	6.0 (4.7-7.3)	28.6	47.6 (41.2-53.4)
08.02.82	0.4 (0-1.6)	25.5 (23.5-27.5)	0.04 (0.03-0.05)	0.1 (0.08-0.2)	2.4	0
08.03.82	5.9 (4.4-6.8)	18.0 (16.8-20.7)	20.9 (19.9-21.9)	25.8 (23.3-28.3)	14.0	1800 (1640-1960)*
06.04.82	1.2 (0.8-1.7)	23.9 (22.0-25.8)	2.3 (1.6-3.0)	2.5 (0.6-4.4)	19.8	6.0 (2.3-9.7)

Site: Sugden Beck Upper, gravel samples

Date	Activity $x10^{3}g^{-1}h^{-1}$	Total count $x10^{-7}g^{-1}$	CPS viable count $x10^{-6}CFUg^{-1}$	Number of chromogenic bacteria $x10^{-5}CFUg^{-1}$	Bacterial diversity
06.04.81	-	-	-	-	-
12.05.81	0.006 (0.002 - 0.01)	0.9 (0.8 - 1.0)	0.0003 (0 - 0.0007)	0.0005 (0 - 0.002)	-
11.06.81	-	-	-	-	-
09.07.81	0.006 (0.001 - 0.01)	0.2 (0.1 - 0.3)	0.0003 (0.0002 - 0.0005)	0.0005 (0 - 0.001)	-
14.08.81	0.007 (0.006 - 0.008)	1.1 (0.9 - 1.2)	0.06 (0.05 - 0.07)*	0.09 (0.06 - 0.1)*	0.2
09.09.81	0.006 (0 - 0.01)	0.7 (0.6 - 0.8)	0.0004 (0 - 0.0008)	0.003 (0 - 0.006)	1.5
07.10.81	8.2 (5.7 - 11.8)	2.6 (2.3 - 3.0)	15.6 (14.4 - 16.7)*	7.1 (5.8 - 8.3)	2.9
09.11.81	6.8 (5.2 - 7.6)	11.0 (10.2 - 11.9)	15.5 (14.2 - 16.7)	5.0 (3.7 - 6.3)	4.7
07.12.81	7.0 (5.8 - 7.8)	7.9 (7.4 - 8.4)	54.2 (48.0 - 60.4)*	4.9 (3.5 - 6.3)	2.1
07.01.82	6.0 (5.0 - 7.3)	1.4 (1.3 - 1.5)	12.5 (10.5 - 14.5)	5.5 (2.0 - 9.1)	4.5
08.02.82	0.07 (0.06 - 0.08)	2.3 (2.1 - 2.5)	0.07 (0.06 - 0.08)*	0.06 (0.04 - 0.08)*	0.1
08.03.82	2.4 (1.5 - 3.2)	1.6 (1.4 - 1.9)	5.6 (4.1 - 7.1)	4.0 (1.0 - 6.8)	3.7
06.04.82	0.3 (0.2 - 0.4)	3.1 (2.9 - 3.3)	3.3 (2.9 - 3.8)	0.4 (0.1 - 0.7)	9.0

Site: Sugden Beck Lower, water samples

Date	Activity $x10^{4}h^{-1}$	Total count $x10^{-5}ml^{-1}$	CPS viable count $x10^{-4}CFUml^{-1}$	Number of chromogenic bacteria $x10^{-3}CFUml^{-1}$	Bacterial diversity	PSM viable count $x10^{-1}CFUml^{-1}$
06.04.81	1.2 (1.0–1.5)	8.1 (6.9–9.2)	2.2 (2.1–2.4)*	–	–	7.7 (6.1 – 8.6)*
12.05.81	20.3 (15.3–24.9)	15.2 (14.0–16.4)	5.8 (5.2–6.3)*	3.7 (2.6–4.8)	–	15.6 (13.4–17.5)*
11.06.81	1.1 (0–3.9)	32.3 (29.8–34.8)	0.05 (0.04–0.06)*	0.05 (0.03–0.06)	–	0
09.07.81	0.2 (0–0.6)	3.1 (2.7–3.6)	0.002 (0.0009–0.003)	0.002 (0–0.004)	–	0
14.08.81	19.4 (11.6–23.9)	39.9 (36.7–43.2)	28.3 (27.5–29.0)	1.4 (0.9–2.0)	13.2	50.8 (46.3–55.0)*
09.09.81	0.9 (0.3–1.6)	7.4 (6.8–7.9)	0.005 (0.004–0.007)	0.02 (0.002–0.03)	7.8	0
07.10.81	74.8 (72.2–76.6)	45.8 (40.2–51.4)	79.4 (71.8–86.6)*	118 (78.6–150)*	11.3	176 (156–195)*
09.11.81	39.6 (32.4–45.0)	73.7 (67.1–80.3)	52.6 (44.4–59.8)*	54.0 (42.7–65.3)	8.7	843 (764–918)
07.12.81	20.7 (14.7–30.0)	18.7 (14.6–22.8)	66.6 (53.2–78.8)*	94.5 (64.7–124)	9.3	339 (300–759)
07.01.82	5.2 (5.0–5.6)	14.6 (13.4–15.8)	16.5 (13.3–19.7)	16.0 (2.8–29.2)	12.8	187 (141–233)
08.02.82	–	26.9 (23.9–29.9)	–	–	–	–
08.03.82	9.4 (8.8–13.5)	13.9 (12.0–15.8)	29.3 (19.7–37.3)*	27.8 (17.8–37.8)	7.4	331 (301–361)
06.04.82	8.5 (4.8–14.5)	30.2 (28.2–32.2)	19.6 (16.8–22.5)	63.0 (52.2–73.8)	18.3	36.0 (27.0–45.1)

Site: Sugden Beck Lower, gravel samples

Date	Activity $x10^{3}g^{-1}h^{-1}$	Total count $x10^{-7}g^{-1}$	CPS viable count $x10^{-6}CFUg^{-1}$	Number of chromogenic bacteria $x10^{-5}CFUg^{-1}$	Bacterial diversity
06.04.81	–	–	–	–	–
12.05.81	25.1 (23.4 – 27.7)	3.3 (2.6 – 3.6)*	33.7 (31.6 – 35.9)	–	–
11.06.81	14.7 (14.2 – 16.6)	7.6 (7.0 – 8.2)	6.0 (5.4 – 6.6)*	–	–
09.07.81	0.0004 (0 – 0.006)	1.0 (0.9 – 1.2)	–	–	–
14.08.81	17.9 (17.0 – 18.9)	4.7 (4.1 – 5.2)	7.8 (7.4 – 8.1)*	13.9 (11.9 – 15.7)*	9.5
09.09.81	0.02 (0.01 – 0.03)	1.0 (0.9 – 1.1)	0.004 (0.001 – 0.007)	0.02 (0.002 – 0.04)	–
07.10.81	21.2 (18.9 – 23.5)	4.4 (4.1 – 4.7)	45.0 (40.6 – 49.2)*	84.9 (65.5 – 102)*	3.4
09.11.81	19.0 (15.3 – 24.0)	13.2 (12.5 – 13.9)	50.6 (43.5 – 57.7)	182 (143 – 221)	3.3
07.12.81	16.9 (16.1 – 17.8)	3.7 (3.3 – 4.0)	50.1 (44.9 – 55.3)	130 (104 – 156)	2.8
07.01.82	–	–	–	–	–
08.02.82	12.7 (11.7 – 13.6)	2.4 (2.3 – 2.6)	15.5 (13.5 – 17.3)*	19.0 (10.3 – 24.6)*	5.5
08.03.82	24.6 (23.6 – 25.7)	3.8 (3.6 – 3.9)	90.9 (82.8 – 98.6)*	182 (162 – 202)	2.3
06.04.82	–	–	–	–	–

APPENDIX 4 (continued)

Stubs Beck and Sugden Beck data, April 1981 to April 1982; Environmental variables.

Date	pH	Temperature (^{o}C)	Dissolved oxygen concentration (mgl^{-1})	BOD (mg oxygen l^{-1})	Conductivity (μScm^{-1})	Turbidity (Arbitrary units)	Estimated flow rate ($m^{3}s^{-1}$)
Site: Stubs Beck							
06.04.81	7.9	6.0	12.0	4.4	429	3	
12.05.81	8.0	11.0	10.8	1.7	501	4	
11.06.81	7.0	12.0	9.2	3.6	629	7	
09.07.81	-	-	-	-	-	-	
14.08.81	7.1	17.0	7.7	8.0	715	18	
09.09.81	-	-	-	-	-	-	
07.10.81	7.7	9.0	9.4	8.0	329	11	
09.11.81	7.8	6.0	9.7	3.3	458	2	
07.12.81	7.3	5.0	10.0	9.6	1001	8	
07.01.82	7.5	1.0	8.8	10.5	343	3	
08.02.82	7.6	3.0	11.6	5.4	415	2	
08.03.81	7.1	5.0	11.8	10.8	386	4	
06.04.82	7.4	8.0	9.2	7.8	429	3	
Site: Sugden Beck Upper							
06.04.81	2.7	9.0	10.7	-	3575	36	
12.05.81	3.9	11.0	12.1	-	2860	21	
11.06.81	2.8	13.0	8.2	-	4004	11	
09.07.81	3.4	15.0	11.6	-	3146	6	
14.08.81	3.4	19.0	9.1	520	4433	15	
09.09.81	2.8	16.0	9.6	400	6292	21	
07.10.81	7.4	9.0	10.4	215	529	19	
09.11.81	6.3	9.0	9.3	230	1230	17	
07.12.81	7.1	5.5	10.2	225	787	15	
07.01.82	6.5	1.0	10.4	180	787	15	
08.02.82	4.8	6.0	12.1	265	1573	12	
08.03.82	6.8	6.5	11.4	190	543	3	
06.04.82	6.5	9.0	9.3	70	1073	17	
Site: Sugden Beck Lower							
06.04.81	5.7	7.0	12.6	-	1073	32	-
12.05.81	7.4	10.0	11.1	-	987	21	-
11.06.81	4.3	12.0	11.0	-	1859	10	-
09.07.81	3.3	14.0	9.0	-	4004	7	-
14.08.81	7.1	19.0	9.1	510	1859	41	-
09.09.81	3.5	13.0	10.7	490	4576	19	6
07.10.81	7.4	10.0	10.7	100	429	26	135
09.11.81	7.6	8.0	10.4	70	729	6	21
07.12.81	7.2	6.0	10.5	165	1430	31	-
07.01.82	7.5	2.0	8.6	70	472	4	82
08.02.82	7.4	4.0	11.4	-	758	2	21
08.03.82	7.6	5.5	11.7	115	644	4	54
06.04.82	7.2	8.5	8.5	60	758	9	18

APPENDIX 4 (continued)

Stubs Beck and Sugden Beck data, April 1981 to April 1982; Concentration of chlorophenols ($\mu g l^{-1}$) at Sugden Beck sites.

Note. No phenolic compounds or phenol derivatives were detected in Stubs Beck.

Site: Sugden Beck Upper

Date	3CHLR	4CHLR	2,4-DCP	PCOC	Total chlorophenols
06.04.81	620	540	4300	3800	9260
12.05.81	880	320	0	0	1200
11.06.81	1050	1310	3200	2600	8160
09.07.81	1900	190	2100	7800	12000
14.08.81	0	1900	0	0	1900
09.09.81	2900	470	5100	6500	15000
07.10.81	0	390	0	0	390
09.11.81	750	270	2700	0	3720
07.12.81	0	1030	4400	0	5430
07.01.82	0	740	370	0	1100
08.02.81	570	230	19000	14800	34600
08.03.82	0	0	0	0	0
06.04.82	0	0	3800	2300	6100

Site: Sugden Beck Lower

06.04.81	310	0	2500	1600	4410
12.05.81	0	250	0	0	250
11.06.81	670	580	2700	2200	6150
09.07.81	2200	310	1400	3600	7510
14.08.81	0	2000	0	0	2000
09.09.81	1800	490	8600	10600	21500
07.10.81	0	0	0	0	0
09.11.81	380	300	1400	0	2080
07.12.81	0	0	710	0	710
07.01.82	0	0	0	0	0
08.02.82	0	0	1700	410	2110
08.03.82	0	0	0	0	0
06.04.82	0	0	2300	1500	3800

APPENDIX 4 (continued)

Stubs Beck and Sugden Beck data, April 1981 to April 1982; Concentration of nitrophenols (μgl^{-1}) at Sugden Beck sites.

Date	2,4-DNP	picric acid	DNOC	DNBP	Total nitrophenols
Site: Sugden Beck Upper					
06.04.81	90	1400	50	0	1540
12.05.81	40	2000	0	50	2090
11.06.81	240	4100	0	0	4340
09.07.81	50	2700	40	0	2790
14.08.81	80	2100	40	20	2240
09.09.81	140	2100	80	0	2320
07.10.81	0	1000	0	0	1000
09.11.81	40	830	70	0	940
07.12.81	0	1300	20	0	1320
07.01.82	50	1700	0	30	1780
08.02.82	200	4400	90	0	4690
08.03.82	0	360	30	0	390
06.04.82	0	1500	0	0	1500
Site: Sugden Beck Lower					
06.04.81	30	500	20	0	550
12.05.81	20	400	0	0	420
11.06.81	290	1900	50	0	2240
09.07.81	100	1900	40	0	2040
14.08.81	40	1400	20	20	1480
09.09.81	60	960	90	0	1110
07.10.81	0	170	0	0	170
09.11.81	80	790	0	0	870
07.12.81	0	390	0	0	390
07.01.82	0	460	20	20	480
08.02.82	30	1100	20	0	1150
08.03.82	0	160	0	0	160
06.04.82	0	620	0	0	620

APPENDIX 4 (continued)

Stubs Beck and Sugden Beck data, April 1981 to April 1982; Concentration of phenoxyalkanoic acids (μgl^{-1}) at Sugden Beck sites.

Date	2,4D	MCPA	2,4DP	CMPP	2,4DB	MCPB	Total of phenoxy-alkanoic acids	Total of phenolic compounds and phenol derivatives
Site: Sugden Beck Upper								
06.04.81	600	0	7800	8000	1200	300	17900	28700
12.05.81	3100	9000	11900	13800	1300	0	39100	42400
11.06.81	300	420	7100	5600	300	0	13700	26200
09.07.81	0	400	4800	5700	360	0	11300	26000
14.08.81	3500	4100	2200	4500	0	0	14300	18400
09.09.81	2000	320	5000	9600	450	0	17400	34700
07.10.81	400	880	650	3300	380	0	5610	7000
09.11.81	2900	0	12600	11100	0	1200	27800	32500
07.12.81	2500	2700	2100	7600	940	0	15800	22600
07.01.82	6400	6400	17300	21600	1200	520	53400	56300
08.02.82	3800	0	17100	9500	0	0	30400	69700
08.03.82	1400	1300	1700	0	0	0	4400	4790
06.04.82	0	1500	6200	5400	0	0	13100	20700
Site: Sugden Beck Lower								
06.04.81	300	300	4100	4700	1100	500	11000	16000
12.05.81	1500	2800	2400	4300	400	0	11400	12100
11.06.81	300	0	5000	5100	0	300	10700	19100
09.07.81	0	0	3800	3400	0	0	7200	16800
14.08.81	2800	3700	3300	6800	0	0	16600	20100
09.09.81	320	0	7600	17000	780	0	25700	48300
07.10.81	0	0	0	770	0	0	770	940
09.11.81	3700	0	6600	6000	0	600	16900	19900
07.12.81	630	880	320	2400	0	0	4230	5330
07.01.82	930	1300	5500	3300	0	0	11000	11500
08.02.82	770	1800	3600	3500	0	0	9670	12900
08.03.82	920	520	470	0	0	0	1910	2070
06.04.82	0	480	3900	3000	0	0	7380	11800

APPENDIX 5

Hunsworth Beck data, April 1981 to April 1982; Bacteriological variables, figures in brackets are 95% confidence limits of the mean, except for bacterial activity where the range is given.

Date	Activity $\times 10^{2} h^{-1}$	Total count $\times 10^{-6} ml^{-1}$	CPS viable count $\times 10^{-5} CFUml^{-1}$	Number of chromogenic bacteria $\times 10^{-4} CFUml^{-1}$	Bacterial diversity	PSM viable count $\times 10^{-2} CFUml^{-1}$
Site: Hunsworth Beck 17m upstream, water sample						
06.04.81	2.9 (2.3-3.1)	5.4 (5.1-5.8)	8.4 (5.3-11.1)*	17.4 (8.1-24.3)*	-	9.6 (7.8-11.2)*
12.05.81	3.6 (3.1-4.0)	5.6 (5.3-5.9)	5.6 (3.7-7.2)*	6.8 (3.7-9.9)	-	32.0 (29.5-34.5)
11.06.81	5.5 (4.2-6.8)	15.3 (14.3-16.3)	21.0 (18.3-23.4)*	34.1 (28.2-40.0)*	-	87.8 (71.8-101)*
09.07.81	12.6 (11.7-13.8)	13.8 (13.0-14.6)	25.0 (19.5-29.7)*	24.6 (8.4-34.3)*	-	15.5 (14.3-33.5)*
14.08.81	4.0 (3.3-4.6)	14.4 (13.2-15.6)	56.6 (49.9-62.8)*	81.7 (5.1-107)*	3.0	17.9 (16.5-19.3)
09.09.81	5.8 (4.7-6.9)	18.9 (17.5-20.3)	99.8 (91.2-108)	293 (231-346)*	2.3	50.5 (43.2-57.8)
07.10.81	15.9 (13.2-17.8)	17.3 (15.6-19.0)	38.3 (28.1-47.4)*	61.1 (24.5-91.0)*	3.7	222 (197-246)*
09.11.81	4.4 (3.3-4.8)	29.9 (28.7-31.1)	9.6 (7.6-11.6)	10.5 (5.6-15.4)	5.4	31.1 (25.4-36.8)
07.12.81	4.3 (3.9-4.8)	12.3 (11.5-13.1)	10.2 (7.7-12.7)	17.5 (11.2-23.8)	5.4	51.0 (38.4-63.6)
07.01.82	1.3 (1.1-1.6)	7.9 (7.3-8.4)	5.7 (5.0-6.4)	13.4 (11.1-15.7)	11.4	36.3 (27.9-43.2)
08.02.82	5.1 (5.0-5.2)	6.5 (5.4-6.9)*	7.6 (7.0-8.2)	14.2 (10.7-17.7)	9.9	85.4 (60.8-107)*
08.03.82	4.0 (3.8-4.2)	5.8 (5.0-6.5)	6.6 (5.3-7.7)*	13.4 (10.4-16.4)	7.9	66.1 (58.0-74.2)
06.04.82	14.2 (13.6-14.7)	18.0 (16.5-19.5)	21.1 (17.2-24.5)*	28.7 (21.1-34.8)*	6.4	173 (130-211)*
Site: Hunsworth Beck 25m downstream, water sample						
06.04.81	2.4 (2.0-3.0)	4.1 (3.8-4.5)	10.6 (8.3-12.7)*	18.1 (10.4-23.7)*	-	16.3 (15.1-17.5)
12.05.81	2.7 (2.2-3.1)	4.8 (4.1-5.1)*	5.7 (4.4-6.9)*	8.9 (5.6-12.1)	-	32.6 (28.1-37.1)
11.06.81	5.7 (5.1-6.0)	19.6 (17.7-20.8)	15.3 (13.5-16.9)*	27.7 (22.8-32.6)	-	64.8 (55.3-73.3)*
09.07.81	11.1 (10.5-11.6)	15.1 (13.4-16.1)*	20.4 (15.5-24.8)*	16.7 (7.6-25.7)	-	21.3 (19.3-23.1)*
14.08.81	7.5 (4.2-9.3)	13.1 (12.1-14.4)	46.1 (38.0-53.3)*	47.0 (31.4-62.7)	3.1	68.0 (58.1-76.9)*
09.09.81	7.2 (6.0-8.2)	21.9 (20.5-23.3)	134 (120-147)*	516 (442-590)	2.5	92.2 (85.1-99.3)
07.10.81	10.4 (9.1-12.0)	12.8 (11.8-13.8)	42.7 (31.9-52.2)*	50.0 (26.9-73.1)	3.5	221 (193-247)*
09.11.81	4.3 (3.6-4.8)	29.5 (28.3-30.7)	5.9 (4.4-7.4)	6.5 (2.0-11.0)	5.6	75.5 (55.7-95.3)
07.12.81	4.0 (3.7-4.5)	10.5 (9.7-11.3)	8.5 (6.9-10.1)	15.6 (11.5 - 19.6)	5.0	69.5 (54.9-84.2)
07.01.82	1.4 (1.2-1.5)	6.7 (6.3-7.2)	6.6 (6.0-7.2)	14.5 (13.6-15.4)	11.5	43.3 (37.4-49.2)
08.02.82	4.6 (4.4-4.9)	8.3 (7.5-9.1)	9.9 (8.4-11.3)*	16.0 (11.7-20.3)	9.7	91.1 (77.4-104)*
08.03.82	3.7 (3.2-4.1)	6.1 (5.6-6.7)	5.6 (4.5-6.5)*	10.3 (7.9-12.7)	8.0	86.5 (75.1-97.9)
06.04.82	7.5 (7.3-7.8)	17.0 (16.0-18.0)	20.9 (17.6-23.8)*	29.4 (25.6-33.1)	6.3	151 (129-172)
Site: Hunsworth Beck 110m downstream, water sample						
06.04.81	1.4 (1.3-1.5)	4.5 (4.2-4.7)	9.9 (8.4-11.3)*	18.8 (15.3-22.3)	-	12.6 (11.5-13.6)
12.05.81	3.2 (2.8-3.7)	4.6 (4.3-4.9)	5.8 (5.1-6.5)	9.0 (7.2-10.8)	-	45.5 (34.6-54.8)*
11.06.81	4.6 (3.9-5.5)	16.8 (15.8-17.9)	22.2 (20.5-23.7)*	44.0 (36.7-51.3)	-	67.9 (60.6-75.2)
09.07.81	11.9 (11.7-12.2)	13.8 (12.9-14.7)	12.3 (8.1-15.5)*	33.1 (8.2-42.9)*	-	23.4 (15.9-30.8)
14.08.81	9.9 (7.5-13.8)	14.4 (13.6-15.2)	50.8 (47.4-53.9)	97.2 (72.4-122)	3.2	30.3 (26.9-33.7)
09.09.81	6.2 (5.4-6.8)	20.2 (19.0-21.5)	73.5 (56.7-87.6)*	225 (134-293)*	2.7	85.8 (77.0-94.7)
07.10.81	7.2 (5.8-9.2)	11.9 (11.1-12.7)	36.6 (32.8-40.3)*	60.1 (47.3-72.8)	3.7	164 (139-187)*
09.11.81	4.9 (4.6-5.3)	31.8 (29.6-34.0)	10.6 (7.3-13.3)*	14.0 (9.7-18.1)	5.1	59.5 (42.9-76.1)
07.12.81	3.2 (2.7-3.7)	10.0 (9.3-10.7)	6.8 (5.2-8.4)	10.0 (5.5-14.5)	5.6	51.0 (38.4-63.6)
07.01.82	1.4 (1.3-1.5)	6.8 (6.2-7.4)	5.6 (5.0-6.3)	12.7 (10.0-15.9)	11.6	43.4 (37.3-49.5)
08.02.82	6.1 (4.9-7.5)	7.5 (6.8-8.3)	10.7 (9.5-11.9)*	22.2 (18.2-26.4)	9.2	114 (92.8-133)*
08.03.82	3.8 (3.5-4.1)	6.7 (5.9-7.5)	7.6 (6.3-8.8)*	15.8 (12.6-19.0)	7.2	65.7 (59.7-71.7)
06.04.82	8.0 (7.6-8.4)	20.1 (18.8-21.4)	19.6 (16.9-22.0)*	34.0 (22.6-42.7)	6.8	148 (125-171)

Date	Activity $x10^{3}g^{-1}h^{-1}$	Total count $x10^{-7}g^{-1}$	CPS viable count $x10^{-6}CFUg^{-1}$	Number of chromogenic bacteria $x10^{-6}CFUg^{-1}$	Bacterial diversity
Site: Hunsworth Beck 17m upstream, gravel samples					
06.04.81	-	-	-	-	-
12.05.81	20.3 (17.1 - 21.9)	3.2 (2.9 - 3.6)	6.2 (4.4 - 8.1)	1.4 (0.8 - 2.0)	-
11.06.81	17.0 (15.4 - 18.4)	9.0 (8.3 - 9.7)	17.6 (16.1 - 19.1)	3.2 (2.7 - 3.7)	-
09.07.81	8.9 (7.7 - 10.7)	3.6 (3.1 - 4.0)	5.8 (4.6 - 6.9)	1.0 (0.4 - 1.5)	-
14.08.81	-	-	-	-	-
09.09.81	4.1 (3.0 - 4.9)	7.3 (6.5 - 8.1)	4.3 (3.1 - 5.6)	1.1 (0.4 - 1.8)	6.2
07.10.81	15.0 (12.8 - 17.1)	5.9 (5.1 - 6.8)	24.7 (20.1 - 28.8)*	6.4 (4.6 - 8.2)*	3.8
09.11.81	-	-	-	-	-
07.12.81	10.5 (7.5 - 14.7)	8.7 (8.1 - 9.2)	41.7 (35.1 - 47.8)*	6.8 (5.1 - 8.6)	3.6
07.01.82	16.7 (15.4 - 19.0)	3.8 (3.4 - 4.2)	18.2 (14.1 - 21.7)*	3.6 (2.7 - 4.5)	4.7
08.02.82	-	-	-	-	-
08.03.82	14.2 (12.3 - 16.5)	4.0 (3.6 - 4.4)	27.3 (23.0 - 31.2)*	4.1 (3.5 - 4.8)	5.5
06.04.82	18.1 (17.5 - 18.7)	5.3 (4.9 - 5.7)	23.6 (17.8 - 28.8)*	8.0 (1.0 - 13.4)	4.8
Site: Hunsworth Beck 25m downstream, gravel samples					
06.04.81	-	-	-	-	-
12.05.81	23.6 (21.4 - 27.1)	11.0 (10.1 - 11.9)	41.8 (34.4 - 48.7)*	10.1 (7.8 - 12.5)	-
11.06.81	16.7 (15.9 - 18.5)	19.9 (19.1 - 20.8)	38.3 (33.0 - 43.4)*	8.5 (6.6 - 9.5)	-
09.07.81	13.2 (8.4 - 17.3)	4.6 (4.1 - 5.2)	8.4 (5.4 - 10.5)*	1.6 (0.4 - 2.3)	-
14.08.81	19.9 (18.6 - 21.3)	7.1 (6.1 - 7.5)	48.3 (43.4 - 53.1)	11.0 (8.7 - 13.2)	3.0
09.09.81	17.4 (14.5 - 19.3)	8.5 (7.7 - 9.2)	63.2 (55.2 - 70.8)*	21.6 (19.0 - 24.2)	3.7
07.10.81	14.6 (13.8 - 15.2)	13.3 (12.5 - 13.4)	84.4 (62.1 - 103)*	22.4 (15.3 - 27.9)*	3.4
09.11.81	17.7 (16.0 - 20.8)	18.9 (17.4 - 20.5)	34.6 (29.9 - 39.3)	8.8 (6.1 - 11.5)	3.6
07.12.81	12.8 (12.3 - 13.0)	4.7 (4.4 - 5.1)	11.2 (6.8 - 14.4)*	0.8 (0.5 - 1.2)	2.8
07.01.82	25.4 (25.0 - 25.8)	2.2 (2.0 - 2.4)	42.5 (38.4 - 46.6)	12.7 (10.7 - 14.6)	4.8
08.02.82	13.4 (12.8 - 14.0)	3.6 (3.2 - 3.9)	1.4 (0.7 - 2.0)	0.5 (0.3 - 0.7)	5.7
08.03.82	16.9 (13.4 - 19.0)	5.4 (5.0 - 5.8)	28.5 (23.2 - 33.1)*	4.9 (3.7 - 5.8)*	4.3
06.04.82	-	-	-	-	-
Site: Hunsworth Beck 110m downstream, gravel samples					
06.04.81	-	-	-	-	-
12.05.81	18.8 (17.5 - 19.9)	9.0 (8.4 - 9.6)	18.3 (13.1 - 22.8)*	4.1 (2.1 - 5.6)*	-
11.06.81	15.9 (15.2 - 16.5)	19.8 (17.7 - 21.0)*	49.1 (41.8 - 55.8)*	12.2 (10.1 - 14.2)	-
09.07.81	20.2 (17.9 - 21.4)	10.2 (9.5 - 10.8)	78.6 (71.2 - 85.4)*	22.3 (19.3 - 25.2)	-
14.08.81	17.3 (16.5 - 18.2)	8.1 (7.2 - 9.0)	69.7 (63.8 - 75.6)	18.2 (15.2 - 20.9)	3.3
09.09.81	15.4 (14.7 - 15.9)	17.3 (16.3 - 18.3)	188 (178 - 198)	72.0 (68.7 - 75.3)	2.5
07.10.81	15.2 (12.9 - 18.0)	8.4 (7.7 - 9.2)	65.2 (55.5 - 74.1)*	22.3 (18.5 - 25.4)*	3.7
09.11.81	14.5 (13.2 - 17.4)	21.7 (20.5 - 22.9)	19.8 (16.4 - 23.2)	4.2 (2.7 - 5.6)	4.7
07.12.81	15.1 (14.7 - 15.6)	8.4 (7.8 - 9.1)	51.3 (34.5 - 65.4)*	8.9 (5.1 - 11.9)*	3.1
07.01.82	-	-	-	-	-
08.02.82	15.5 (15.0 - 16.0)	5.3 (4.9 - 5.6)	0.8 (0.2 - 1.3)	0.1 (0.02 - 0.2)	4.8
08.03.82	18.5 (17.8 - 19.1)	7.3 (6.6 - 8.0)	39.7 (35.1 - 44.0)*	8.4 (7.4 - 9.3)*	4.4
06.04.82	16.1 (15.8 - 16.5)	8.4 (7.8 - 9.0)	59.2 (40.3 - 75.2)*	19.9 (10.1 - 27.4)*	3.5

APPENDIX 5 (continued)

Hunsworth Beck data, April 1981 to April 1982; Environmental variables.

Date	pH	Temperature (^{o}C)	Dissolved oxygen concentration (mgl^{-1})	BOD (mg oxygen l^{-1})	Conductivity (μScm^{-1})	Turbidity (Arbitrary units)	Level of Hunsworth Beck at gauging point (cm)
Site: Hunsworth Beck 17m upstream							
06.04.81	7.3	9.0	10.8	-	815	5	-
12.05.81	7.4	13.0	8.4	9.5	801	5	-
11.06.81	6.9	15.0	10.4	19.0	1020	15	-
09.07.81	6.8	18.0	7.4	-	1290	6	-
14.08.81	6.7	19.0	8.0	7.5	1000	8	-
09.09.81	7.1	18.0	6.3	19.5	1130	11	37
07.10.81	7.6	11.0	9.7	22.3	400	22	52
09.11.81	7.2	9.5	6.7	6.5	1120	5	39
07.12.81	7.0	7.5	9.3	21.3	1100	12	59
07.01.82	7.1	4.0	9.8	21.6	501	6	46
08.02.82	7.1	7.5	9.1	21.3	958	6	39
08.03.82	7.0	7.5	9.2	18.9	958	4	39
06.04.82	7.1	11.5	6.5	41.7	1190	33	45
Site: Hunsworth Beck 25m downstream							
06.04.81	7.0	9.0	9.0	-	844	8	
12.05.81	7.3	13.0	8.2	7.8	815	6	
11.06.81	6.7	14.0	11.0	-	1060	10	
09.07.81	6.8	18.0	7.2	-	1240	8	
14.08.81	7.0	19.0	8.3	21.0	858	8	
09.09.81	7.0	17.0	5.6	-	1240	11	
07.10.81	7.6	11.0	9.4	19.5	429	20	
09.11.81	7.3	10.0	6.9	12.8	1170	6	
07.12.81	7.1	7.5	9.2	27.9	1390	15	
07.01.82	7.3	4.0	9.6	22.8	558	4	
08.02.82	7.2	7.5	9.4	24.3	1040	5	
08.03.82	7.2	7.5	9.1	17.4	987	6	
06.04.82	7.2	11.5	7.0	42.3	1220	15	
Site: Hunsworth Beck 110m downstream							
06.04.81	7.2	9.0	10.3	-	829	7	
12.05.81	7.4	13.0	9.2	20.8	815	7	
11.06.81	6.6	15.0	10.0	22.5	1030	12	
09.07.81	6.8	19.0	7.2	-	1340	7	
14.08.81	7.2	19.0	8.2	20.0	1000	8	
09.09.81	6.9	17.0	5.8	29.5	1220	6	
07.10.81	7.5	11.0	8.7	25.0	472	26	
09.11.81	7.3	10.5	7.1	15.0	1170	6	
07.12.81	7.2	7.0	9.2	19.8	958	15	
07.01.82	7.4	4.0	9.8	21.0	615	5	
08.02.82	7.3	7.5	9.9	21.9	1060	6	
08.03.82	7.0	7.5	9.4	21.0	1000	6	
06.04.82	7.1	11.5	6.0	43.8	1190	17	

APPENDIX 5 (continued)

Hunsworth Beck data, April 1981 to April 1982; Concentration of chlorophenols and nitrophenols ($\mu g l^{-1}$) at Hunsworth Beck sites.

Date	2,4DCP	PCOC	Total of chlorophenols	picric acid	DNBP	Total of nitrophenols
Site: Hunsworth Beck 17m upstream						
06.04.81	0	0	0	0	0	0
12.05.81	0	0	0	0	0	0
11.06.81	0	0	0	0	0	0
09.07.81	0	0	0	0	0	0
14.08.81	-	-	-	-	-	-
09.09.81	0	0	0	40	0	40
07.10.81	0	0	0	170	0	170
09.11.81	0	0	0	20	0	20
07.12.81	0	0	0	0	0	0
07.01.82	0	0	0	0	0	0
08.02.82	0	0	0	0	0	0
08.03.82	0	0	0	70	0	70
06.04.82	0	0	0	60	0	60
Site: Hunsworth Beck 25m downstream						
06.04.81	0	0	0	20	0	20
12.05.81	0	0	0	0	0	0
11.06.81	310	210	520	140	0	140
09.07.81	0	0	0	100	0	100
14.08.81	0	0	0	60	30	90
09.09.81	1400	1100	2500	180	30	210
07.10.81	0	0	0	160	0	160
09.11.81	0	0	0	140	0	140
07.12.81	0	0	0	0	0	0
07.01.82	0	0	0	130	0	130
08.02.82	0	0	0	100	0	100
08.03.82	0	0	0	70	0	70
06.04.82	0	0	0	110	0	110
Site: Hunsworth Beck 110m downstream						
06.04.81	0	0	0	0	0	0
12.05.81	0	0	0	0	0	0
11.06.81	0	0	0	160	0	160
09.07.81	0	0	0	40	0	40
14.08.81	0	0	0	60	0	60
09.09.81	2000	1500	3500	160	0	160
07.10.81	0	0	0	0	0	0
09.11.81	0	0	0	30	0	30
07.12.81	0	0	0	0	0	0
07.01.82	0	0	0	50	0	50
08.02.82	0	220	220	60	0	60
08.03.82	0	0	0	70	0	70
06.04.82	0	0	0	130	0	130

APPENDIX 5 (continued)

Hunsworth Beck data, April 1981 to April 1982; Concentration of phenoxyalkanoic acids (μgl^{-1}) at Hunsworth Beck sites.

Date	2,4DP	CMPP	2,4DB	Total of phenoxyalkanoic acids	Total of phenolic compounds and phenol derivatives
Site: Hunsworth Beck 17m upstream					
06.04.81	0	0	700	700	700
12.05.81	0	0	0	0	0
11.06.81	0	0	0	0	0
09.07.81	0	0	0	0	0
17.08.81	-	-	-	-	-
09.09.81	0	340	0	340	380
07.10.81	0	0	0	0	170
09.11.81	0	0	0	0	20
07.12.81	0	0	0	0	0
07.01.82	0	0	0	0	0
08.02.82	0	0	0	0	0
08.03.82	0	0	0	0	70
06.04.82	500	430	0	930	990
Site: Hunsworth Beck 25m downstream					
06.04.81	0	0	0	0	20
12.05.81	0	0	0	0	0
11.06.81	0	730	0	730	1390
09.07.11	0	490	0	490	590
14.08.81	0	360	0	360	450
09.09.81	890	2500	0	3390	6100
07.10.81	300	0	0	300	460
09.11.81	1030	890	0	1920	2060
07.12.81	0	0	0	0	0
07.01.82	0	0	0	0	130
08.02.82	0	0	0	0	100
08.03.82	0	0	0	0	70
06.04.82	690	890	0	1580	1690
Site: Hunsworth Beck 110m downstream					
06.04.81	0	0	0	0	0
12.05.81	0	0	0	0	0
11.06.81	0	610	0	610	770
09.07.81	0	0	0	0	40
14.08.81	0	0	0	0	60
09.09.81	960	3100	0	4060	7720
07.10.81	0	330	0	330	330
09.11.81	0	0	0	0	30
07.12.81	0	0	0	0	0
07.01.82	0	480	0	480	530
08.02.82	0	0	0	0	280
08.03.82	0	0	0	0	70
06.04.82	690	1200	0	1890	2020

APPENDIX 6

Toxicity of phenolic compounds and phenol derivatives to selected bacterial isolates from Stubs Beck and Sugden Beck. Mean diameter of cleared zone (n=30) for each compound (16 compounds) and isolate (6 isolates).

Phenolic compound or phenol derivative[1]	Bacterial isolates					
	Pseudomonas sp.[2]	*Pseudomonas* sp.	*Pseudomonas* sp.	*Aeromonas* sp.	*Flavobacterium* sp.	Gram positive organism
Chlorophenols						
2CHLR	13.45	13.43	13.85	13.76	13.17	13.73
3CHLR	13.00	14.88	17.92	14.31	24.27	19.40
4CHLR	13.75	14.16	21.50	14.10	27.90	20.35
2,4DCP	14.72	16.30	22.93	26.50	45.33	28.23
PCOC	13.88	14.05	19.48	18.93	32.35	22.20
Nitrophenols						
2,4DNP	13.75	14.53	19.70	25.96	24.87	31.40
Picric acid	11.17	11.63	14.90	15.28	24.35	14.73
DNOC	11.40	12.21	12.88	21.71	20.18	27.85
DNBP	10.03	10.85	12.65	16.88	16.30	30.63
Phenoxyalkanoic acids						
2,4D	13.00	14.18	17.50	17.48	14.60	18.48
MCPA	13.90	15.16	18.60	17.61	15.65	22.80
2,4,5T	13.28	13.53	17.48	16.75	16.02	21.26
2,4DP	14.40	15.15	18.38	18.68	15.57	20.60
CMPP	13.33	15.11	18.80	18.35	15.67	20.65
2,4DB	11.62	12.73	14.35	13.36	13.90	16.31
MCPB	13.13	13.65	15.55	14.01	15.48	20.33

1. For structure of phenolic compounds or phenol derivatives see Appendix 1.

2. *Pseudomonas* sp isolated from Sugden Beck, all other bacterial isolates were from Stubs Beck.