THE IMPACTS OF LOW LEVELS OF ANTIBIOTICS ON FRESHWATER MICROBIAL COMMUNITIES

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ABSTRACT

Antibiotics are one of the most commonly used medicines in modern society. Despite this, very little work has been carried out to determine if they have any kind of environmental impact. This project looked at antibiotic use in the UK, concentrating on the fate and impact of antibiotics used in human medicine. It was found that amoxycillin was the most prescribed antibiotic in England from 1996 to 1997. GP prescriptions accounted for around 80% of prescriptions (a total of over 320 tonnes of antibiotics) for 1996. Estimates of individual antibiotics reaching the environment were in the range of 20μg/l to 0.02 μg/l. This data together with the physico-chemical properties were combined to identify the most important groups to be studied: tetracyclines and β-lactams (broad spectrum-penicillins and cephalosporins). Analysis of sewage found no antibiotics. Analysis of Thames River water found tetracyclines at μg/l concentrations. No β-lactams were detected. Two individual species assays (growth and changes in mid ultraviolet spectrum (MUVS)) were used to investigate sub-lethal effects of the antibiotics. Effects were seen in the growth assay as low as 0.08mg/l. Effects in the MUVS experiments were seen at 1 mg/l. Two types of microcosm experiments were carried out. The first used radiolabelled carbon compounds to determine the effect of antibiotics on their biodegradation. Effects were seen at 0.1mg/l of oxytetracycline and 0.5mg/l of amoxycillin. The second one used Biolog plates and changes in carbon utilisation patterns of microcosms containing antibiotics. No long-term effects were detected. Overall, it is unlikely that the levels of antibiotics currently reaching the environment from human medical use have significant impacts on the freshwater aquatic ecosystems.

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Introduction

Over the last 50 years antibiotics have been the most commonly used drugs in the world to combat bacterial disease. They have been instrumental in improving general health and life expectancy in both humans and domestic animals. However, indiscriminate use has resulted in increased resistance among pathogenic and non-pathogenic bacteria. The possible environmental impacts which extensive use of antibiotics in human medicine and agriculture may be having are also becoming of increasing concern.

Some antibiotics are degraded in the body and become inactive, but many others, particularly those excreted renally or not absorbed fully from the gut, leave the body in their active forms. In the case of humans, antibiotics then enter the sewerage system and eventually, if not degraded or removed, will reach the water environment. Antibiotics from animal use are also excreted and they will reach the water environment, either as runoff or through the use of animal waste as fertiliser. In fish farms, pellets containing antibiotics fall to the sea bed, together with antibiotics in the urine and faeces of the fish, and they are then released into the surrounding waters.

A great deal of work has been done on the spread of resistance among bacteria and some work has also been carried out on the environmental spread of resistance. Little is known about the impact of antibiotics on the aquatic environment, though there has been some research, particularly with regard to fish farms, mainly looking at the fate of a few antibiotics. This lack of relevant information was confirmed by two Water Research Centre (WRc) reports, commissioned by the Department of the Environment. These concluded that in the case of oxytetracycline (Murgatroyd *et al.*, 1994) and oxalinic acid (Gendebien *et al.*, 1994) there was not enough information available to set Environmental Quality Standards and a great deal more work needed to be carried out before this would become possible. The same could be said about all antibiotics used in the UK, and most other medical compounds.

Having taken the above into account the following aims were set out for this project:

- To determine the levels of antibiotics used in the UK and that could be reaching the aquatic environment.
- To determine any impacts on aquatic bacteria as a result of the extensive use of antibiotics
- To reach conclusions about any damaging effects that antibiotics may have, directly or indirectly, on freshwater aquatic ecosystems, particularly bacteria.

Work began with an extensive review of all the available literature, which is summarised in Chapter 1. Chapter 2 contains the results of the ongoing investigation, throughout the duration of the project, into the levels of antibiotics used in the UK for human medicine. Chapter 3 details work carried out to determine if there were detectable levels of antibiotics in sewage and river water. Work using single bacterial species, and river water and sediment microcosms, to study the impacts of sub-lethal concentrations of antibiotics on aquatic ecosystem health is described in Chapters 4 to 6. Conclusions and recommendations for further work are detailed in Chapter 7.

Chapter 1: Literature Review

1.1 Antibiotic Usage

Antibiotics are used in the United Kingdom in human medicine and animal husbandry. They are used in animal medicine for treating individual animals and whole herds and also as growth promoters, in very small quantities.

1.1.1 Human Medical Use

Worldwide sales of antibacterial for human use are second only to cardiovascular drugs (PJB publications, 1993). In 1991 total world sales were worth $15,500 million, of which European sales were worth $5,015 million. Sales are split in half between hospital and General Practitioner (GP) prescription use. The majority of injected and 20% of orally administered antibiotics are used in hospitals, with GPs prescribing mainly oral antibiotics. Prescribing patterns worldwide in 1991 were dominated by cephalosporins (nearly 50% of the market), followed by penicillins and quinolones (PJB publications, 1993).

1.1.2 Agricultural Use

In the world market for animal health and nutrition products for 1990, therapeutic antibiotics constituted 16.5% of the market or $1,683 million. In-feed antibacterials constituted 8% of the market or $800 million (Bird and Smale, 1992). In the UK, in 1990, $54 million were spent on therapeutic antibacterials. There are no figures for expenditure on feed additives. There are no records kept in this country of the quantities or kinds of antibiotics used in agriculture.

Very little work has been done on the fate of antibiotics used in agriculture, or even what levels actually reach the environment. That there is little or no concern is demonstrated by the fact that The Code of Good Agricultural Practice for the Protection of the Soil, a consultation document produced by the Ministry of Agriculture, Fisheries and Food (MAFF), in December 1992, does not mention antibiotics. Neither do the National Rivers Authority (NRA) reports of 1988 and 1989 on Water Pollution from Farm Waste in England and Wales - antibiotics are not included in any of the categories of pollutants. Neither MAFF, any other government organisation, nor the National Farmers Union (NFU) issue any guidelines on the use of antibiotics in agriculture, nor are any statistics published on amounts used.

1.2. Antibiotic Fate

The extensive use of antibiotics in modern medicine and agriculture raises questions about their fate. Both the fate and the impact of these antibiotics are areas of research that have been largely ignored, considering the quantities of antibiotics used worldwide. The most in depth work in this area is either outdated or concentrates on fish farming, which is only one of the possible routes for antibiotics entering the environment.

Once administered some antibiotics are degraded in the body and become inactive but many others, particularly those excreted renally or not absorbed fully from the gut, leave the body in their active forms (Lambert and O'Grady, 1992). Therefore, active antibiotics can be present in both faeces and urine. In the case of humans, antibiotics will then enter the sewerage system and eventually, if not degraded or removed, will reach the water environment. Antibiotics from animal use are also excreted and they will reach the water environment either as runoff or through the use of animal waste as fertiliser. In fish farms pellets containing antibiotics fall to the sea bed, together with antibiotics in the urine and faeces of the fish. These may then accumulate in the sediments or be released into the surrounding waters. The concentrations of antibiotics present in the environment are likely to vary from high, at the various points of entry into the aquatic environment, to very low once they become diluted, bound to sediments or degraded.

A considerable amount of work was carried out in the 1950's and 1960's looking at the fate of various antibiotics when combined with different kinds of clays and soils. The original aim of the work was to determine the possible fate of antibiotics that may be produced by bacteria in soil. Though antibiotics have not as yet been found to be produced in environmental soil samples, this work is very relevant to the fate of antibiotics reaching the environment from anthropogenic sources. Streptomycin was found to bind irreversibly to all kinds of soils and become inactivated (Siminoff and Gottlieb, 1951). Chloramphenicol could be recovered fully from sterile soils but was mostly degraded in non sterile soils (Gottlieb and Siminoff , 1951). Oxytetracycline and chlorotetracycline were bound and inactivated by clays (Martin and Gottlieb, 1952) .

Jeffreys (1952) found that penicillins were degraded rapidly in soils even if the pH was 5-6 at which penicillins are stable in the laboratory. Pink *et al.* (1960a) studied the ability of various kinds of soils to bind different antibiotics. Strongly basic (e.g. streptomycin) and amphoteric (e.g. oxytetracycline) antibiotics were found to bind in amounts of over 100mg of antibiotic per gram of clay. Neutral and acidic antibiotics such as penicillin and chloramphenicol are only bound in quantities of around 10mg per gram. Antibiotics from the first two groups were also adsorbed onto various soils, though in smaller quantities. Further work (Pink *et al.*, 1960b) showed that the basic antibiotics were to a great extent irreversibly bound to clays, whereas the amphoteric antibiotics could be released from the clays in their active forms under the right conditions. More recent work has concentrated on antibiotics entering the environment from fish farms. Studies have shown that antibiotics such as oxalinic acid and tetracyclines persist in the sea bed for some time. In one experiment oxytetracycline was found, by Samuelsen *et al.* (1992), to have a half life of 144 days in the sediment. Husevag *et al.* (1991) did not find residues of oxytetracycline or furazolidone in sediments of a farm abandoned in 1982, indicating that the antibiotic does not remain permanently in the sediment, though there was a tenfold increase in resistant bacteria present compared to control sites, which indicates that the impact of the antibiotic does persist. In a laboratory experiment using marine fish farm sediment from a farm that had never used antibiotics, Hansen *et al.* (1993) showed that the normal background resistance was 0.1% or less.

Tetracycline residues have been found in wild fish populations surrounding a farm and resistant bacteria have been found in their guts (Bjorklund *et al.*, 1990), showing that antibiotics do not only affect the benthic microflora directly beneath the fish cages. The half life of oxytetracycline in sediment was found to be doubled if covered quickly by further sedimentation from 32 days to 64 days (Samuelsen, 1989) and degradation in the water column was found to be slower at lower temperatures and light intensities. No route for the biodegradation of tetracyclines has been discovered, only photodecomposition seems to have an effect, therefore the impact of tetracycline on the sediment may be expected to be long term. Samuelsen *et al.*(1994) have found that oxytetracycline remained present in sediment in a closed system for 6 months. However, when unmodified sediment (i.e. no attempt made to extract antibiotic from sediment) was used in bioassays no antibacterial effect was found. This binding to sediment has been shown by Pink *et al.*(1960b) to be reversible and antibiotic to be released in its active form. These findings point to the possibility of large reservoirs of antibiotics, such as tetracyclines, bound to sediments. These might then be released in active forms if conditions were right, after a change in water pH for example, resulting in fluctuating levels of antibiotics in water. This would strongly affect the kind of impacts tetracyclines may have in the environment.

Addison (1984) designed a tentative model for the fate of antibiotics in sediments and run-off from feedlots. This was based on work carried out on the fate of pesticides in the environment, which is a much more studied area, though not necessarily directly applicable to the fate of antibiotics. However, the model did identify possible routes for antibiotic cycling in the environment and the various factors, such as antibiotic binding, degradation and volatilisation, that would be of importance.

Sithole and Guy (1987a and 1987b) looked at interactions between tetracycline and various kind of soils. Adsorption on to clay was very quick, with 30 minutes required to achieve equilibrium. Adsorption onto peat and humic substances required about 18 hours to achieve equilibrium. Adsorption was pH-dependent and various bonding sites were involved. These experiments were carried out within a concentration range of 1-10ppm, as these are the levels the authors expected to be found in water.

Work carried out by Galvaschin and Katz (1994) looked at the fate in soils of antibiotics used in feeds. Experiments used soil amended with chicken faeces, which would result in a higher microbiological activity than in soil alone, therefore possibly increasing the degradation of antibiotics. Rates of disappearance of antibiotics were found to be temperature-dependent. Results showed that some antibiotics such as streptomycin and penicillin were undetectable at all temperatures. This was probably because the first was irreversibly bound and the second broken down by β-lactamase-producing bacteria that can be found in soil. At the higher temperatures all antibiotics tested were lost to a certain extent, but at 4°C all of the chlorotetracycline, erytrhomycin and bambermycin were detectable. Lunestad and Goksyr (1990) found that only 5% of oxytetracycline in sea water was present in its active form, the rest was complexed with magnesium and calcium.

From the results of the above work it is apparent that different antibiotics have different fates in the environment. Their fate is dependent on their chemical structure; physical conditions such as temperature and light; chemical conditions such as pH, soil types and oxygen availability and, finally, on the presence or absence of microorganisms capable of degrading specific antibiotics.

1.3. Antibiotic impacts

Further to the determination of the concentrations and fate of antibiotics in the environment the questions arise as to what impacts the antibiotics that are present and active in the environment have on bacteria and other organisms. Little work has been done in this area. Again most is concerned with the impacts of antibiotics from fish farms.

A long standing interest in possible environmental impacts of antibiotics does exist, but this has not been reflected in the levels of actual scientific research carried out. In 1979 the Food and Drug Administration, in the US, produced a draft environmental impact statement (Feinman and Mathesson, 1979) looking at the impacts of antibiotics contained in animal feeds. Conclusions were limited and based on very little actual data. Some antibiotics, such as penicillin, were expected to be fully degraded within weeks of entering the environment, whereas others such as tetracyclines would be expected to remain active, being adsorbed and released from soil with changing conditions. Two WRc reports confirm that this lack of information on the environmental behaviour of antibiotics still exists. The reports, which were commissioned by the UK Department of the Environment, concluded that in the case of oxytetracycline (Murgatroyd *et al.*, 1994) and oxalinic acid (Gendebien *et al.*, 1994) there was not enough information available to set Environmental Quality Standards and a great deal more work needed to be carried out before this would become possible. These conclusions could apply to all antibiotics used in the UK.

1.3.1 Microinvertebrates and Other Eukaryotic Organisms

Some work has been carried out to determine the toxicity of antibiotics towards organisms other than bacteria. Most of it involves toxicity or bioaccumulation tests on aquatic organisms. One study (Bris *et al.*, 1995) looked at the accumulation of oxytetracycline in three shellfish species exposed to artificial fish farm effluent. Concentrations of up to 1.42mg/kg wet weight were found after 14 days exposure.

Macri and Sbadella (1984) tested the toxicity of Nitrofurazone and Furaltadone on the alga *Selenastrum capricrnutum*, on an aquatic invertebrate *Daphnia magna,* and on the common fly, *Musca domestica.* Nitrofurans are used in Italy as feed additives. Results found nitrofurazone to have an EC_{50} (concentration at which an effect was seen in half of the test organisms) after 96 hours of 1.45 mg/l, which brings it close to being classified as very toxic. EC_{50} for *Daphnia* was 28.67 mg/l after 48 hours.

Macri *et al.* (1988) studied the toxicity of Furazolidone to the larvae of three freshwater invertebrates: *Artemia salina, D. magna* and *Culex pipiens molestus*. LC_{50} (concentration at which a lethal effect was seen in half of the test organisms) after 24 hours for these organisms was 0.25, 0.06 and 0.04 mg/l respectively. These are very low levels. All three organisms are important in the aquatic food chain and large reductions in numbers could seriously affect ecosystem equilibrium.

Djomi *et al.* (1992) looked at the effect of various antibiotics on *D. magna* using both acute high dose and low dose toxicity tests. The antibiotics tested were bacitracin, erythromycin. aminosidine and linomycin and they were found to decrease in toxicity in this order, bacitracin being the most toxic with an EC_{50} of 30 mg/l after 48 hours. Linomycin, the least toxic, did not show any effects until after 72 hours and had an EC_{50} of 379.39 mg/l. Effects of the four antibiotics on phototactic behaviour were examined in the low dose test. Linomycin and bacitracin decreased phototactic behaviour, aminosidine increased the positive reaction to light and erythromycin had no effect. This experiment highlights the necessity of considering non-lethal effects of antibiotics as these may have serious repercussions on the aquatic environment. In the case of *D. magna* changes in phototactic behaviour would affect feeding efficiency and increase vulnerability to predators in the water column.

D. magna was also used in a study by Vine *et al.* (1993). They found a 21 day EC_{50} for reproduction of 30.2 mg/l for oxytetracycline. Growth inhibition of an alga, *Selenastrum capricornatum*, was also observed with a 72 hour EC_{50} of 0.585 mg/l.

Migliore *et al.* (1997) carried out work using *Artemia* cysts. This is a commonly used live food in aquaculture. Not only were cysts killed and emergence reduced by various antibiotics but alterations of development were caused, resulting in decreased fitness of individuals.

Blue-green algae have been shown to be particularly sensitive to the presence of streptomycin (Harrass *et al.*, 1985). Growth of six species was inhibited at concentrations below 1mg/l. Sensitivity of green algae varied but was generally at least an order of magnitude less. Results also showed that in mixed cultures, algal species did not behave the same as in single cultures, with effects of the antibiotics being reduced.

Plants have also been shown to be able to take up antibiotics through their roots and accumulate them (Crowdy and Palmer, 1955). More recent experiments (Batchelder, 1981) have shown that both chlorotetracycline and oxytetracycline are toxic to plants, significantly impairing development at concentrations of 10mg/l, when grown in liquid medium. When plants were grown in soils (Batchelder, 1982) effects were greatly reduced, probably due to absorption of antibiotics onto the soil. Effects varied from one plant species to another, with low concentrations increasing weight and higher concentrations causing a decrease in weight. Ares (1991) found that streptomycin caused a decrease in pea plant weight and nodulation at concentrations of 20mg/l.

There is, therefore, evidence that antibiotics do not only affect bacteria. They accumulate in and are toxic to other organisms, though usually to a lesser extent. This could be of significance, depending on the levels of antibiotics reaching the environment.

1.3.2 Bacteria

Bacteria are present in large numbers in river sediments, but for a long time the importance of bacteria in the water column was underestimated as they were not thought to be present in sufficient numbers to have a significant impact. The reasons for this were twofold. Firstly, many aquatic bacteria are non-culturable, and their real numbers were not realised until techniques for direct counting of bacteria (e.g. staining) were developed. Secondly, the turnover of bacteria in water is very high, so simply looking at bacterial numbers in water at any given time gives a false impression of their activity.

Bacteria in the freshwater aquatic environment are now recognised as a vital component of the ecosystem. They function as decomposers, as a nutritional source for insect consumers of decaying leaves, as consumers of dissolved organic matter (DOM) and as part of a pool of fine particulate organic matter that is consumed by insect larvae (Meyer, 1994). They are important food sources for protozoa as part of the microbial loop, which is itself important in supporting the metazoan food web, which includes microinvertebrates, larvae and fish (Sherr and Sherr, 1988). As part of the microbial loop bacteria also act as a major route for the uptake of nutrients such, as P and N, into the aquatic ecosystem (Kirchman, 1994). Bacteria also produce extracellular hydrolytic enzymes (Turley, 1994) that break down particulate organic matter (POM) which can then be utilised and becomes part of the food web. This is in addition to their importance as consumers of DOM.

A knowledge of the role of bacteria in the aquatic environment indicates that any compound that has an effect on the ability of bacteria to function efficiently may have a detrimental effect by affecting higher trophic levels that rely on them as a food source. There may also be effects on the efficiency of the system in the uptake and cycling of nutrients, which is a vital role of bacteria.

1.3.2.1. Bacterial resistance and possible environmental impacts

If antibiotic concentrations in the aquatic environment are high they will cause bacterial death and an eventual shift in the population towards resistance. This resistance can arise directly, due to exposure to antibiotics or indirectly, by horizontal transmission of resistance genes from resistant bacteria arriving in the water environment.

The most extensive studies of the increase in resistance after exposure to antibiotics in the aquatic environment has again been carried out in relation to fish farming. Nygaard *et al* (1992) studied the bacteria that were present in sediments treated with the antibiotics oxytetracycline and oxalinic acid, and then placed in the seabed for a year. Levels of the antibiotics remained stable in the sediment layers below 5 cm throughout the sampling period, but levels on the surface were reduced to 10% of original concentration. Background resistance before treatment was 5% to oxytetracycline and 7% to oxalinic acid. After 10 months resistance levels were 10% and 14% respectively, and increased further after 12 months to 16% and 20%, respectively. No changes were found in control sediments.

Samulesen *et al* (1992) found that after medication with oxytetracycline resistance of the sediment bacteria was 100%, this fell to around 20% after 72 days and eventually stabilised at between 10-50%. This shows that the presence of antibiotics had an effect on the resistance patterns of the bacteria in the benthos beneath the cages and in the surrounding areas.

The spread of resistance could have a serious effect on the ecology of the microbial communities, especially if resistance plasmids confer other advantages in addition to resistance and therefore have a permanent impact on the stability of the communities.. This possibility was reviewed by Bennet and Linton (1986). They found that some cell strains containing a particular plasmid can remain present unchanged for up to years in a bacterial population without any direct selective pressure being present, e.g. antibiotic application. Repeated isolation of what appears to be the same strain over an extended period argues for enhanced survival characteristics, which could well be due to unknown plasmid characteristics. This would explain the maintenance, unchanged of the plasmid over such a long period of time. Bouma and Lenski (1988) found that though initially plasmid carriage decreased bacterial host fitness, after culturing 500 generations in the presence of antibiotic, the host genome had adapted to plasmid carriage and outcompeted the original plasmid-free counterpart, even in the absence of the antibiotic.

Amstrong *et al* (1982) found that water treatment enhanced the resistance to streptomycin of the aquatic bacteria, and managed to duplicate these results in the laboratory by chlorinating river water. Increased survival of chlorination due to resistance was also found by Murray *et al.* (1984), this time in treated wastewater. This was an antibiotic-specific phenomenon, with some resistant bacteria surviving less well than controls. Al-Mossawi *et al.* (1981) found increased survival in seawater of resistant coliforms, by a factor of 50%, another example of a resistance plasmid conferring an advantage.

Therefore, resistance to antibiotics or certain antibiotics can confer a selective advantage other than resistance itself. This seems logical as resistance is usually plasmid-borne, plasmids exist and are conserved in natural populations (Kobori *et al,* 1984) and therefore it is highly likely they carry a selective advantage. When resistance genes are also present in a plasmid, these will be one of various advantageous genes. This would result in the resistant gene being conserved in the environment. Furthermore, if some resistant bacteria from wastewater have an increased chance of survival, there is a higher chance of their transmitting their genes most probably by conjugation to the natural population once they reach the water environment.

Uptake of DNA encoding for antibiotic resistance directly by aquatic bacteria (transformation) is another possible route of resistance spread, not only from dead bacterial fragments, but also directly from antibiotic residues themselves. Webb and Davies (1993) found that antibiotic preparations are often contaminated with DNA fragments from the antibiotic-producing bacteria, these fragments have been found to encode resistance. Even if antibiotics resistance cannot be transferred into the environment, resistance arising from fish farms and other heavy antibiotic usage will persist in the environment at low levels and once selective pressure is applied, spread rapidly throughout the bacterial population.

There is no doubt that the high levels of antibiotic resistance found in many bacteria today, particularly pathogens and human commensals, is of an anthropogenic origin. However, some studies have shown that there always seems to be some level of resistance present in a bacterial population, no matter how remote it may be. Work carried out by Kobori *et al.* (1984) on bacteria from Antarctic samples (water column, ice, sediments and invertebrates) showed that 31% of isolates carried at least 1 plasmid. Seven resistant strains were found (five antibiotics tested for) among the 47 plasmid carrying strains and 2 among the 104 non-plasmid carrying strains. Not only was resistance present at a 6% level, but it seems it was mainly plasmid borne. Hughes and Datta (1983) looked at pathogens from the pre-antibiotic era and the beginning of the use of antibiotics, and they found pathogens contained plasmids, but the low level of resistance found in *Klebsiella* and *Proteus* was chromosomal. Experiments by Magee and Quinn (1991) found that 60% of non-faecal bacteria from a remote upland river were resistant to one of eight antibiotics present, over half of these where resistant to at least two compounds. There was no faecal pollution at the site, and no other human influence. Two plasmid curing methods where used unsuccessfully to try to demonstrate that resistance was plasmid borne.

Factors other than a gene encoded resistance, may explain the high resistance of aquatic bacteria in the environment found by Jones *et al.* (1986), Magee and Quinn (1991) and Boon (1992). High resistance may be phenotypic. Water is usually a very nutrient-poor medium for growth. This results in slow growth and long generation times, which would result in slower and reduced antibiotic uptake. Jones and Pickup (1989) isolated freshwater bacteria from lake water, and grew them on media with low and high doses of organic carbon. Bacteria grown on the low concentration were much more resistant to several antibiotics. Low concentrations resulted in physiological changes, particularly in the outer membrane.

By growing in a nutrient poor environment the cell envelope may have different characteristics. for example, loss of binding sites, as found for *Esherichia coli* grown in bay water by Chai (1983). Loss (or lack) of binding sites due to low nutrient levels would result in phenotypic resistance, resistance being due to physiological changes rather than genetic changes. This could be an explanation for the failure of the plasmid curing experiments carried out by Magee and Quinn (1991) on resistant bacteria isolated in remote water sources.

Research has also found that antibiotic resistance can be selected for when bacteria are exposed to heavy metals. The work of Wnorowski (1993) showed that selection of resistant bacteria to a range of heavy metals results in an increased antibiotic resistance. Again, this demonstrates that antibiotic resistance in the water environment is not a simple issue, there are many factors involved. One final possible factor influencing antibiotic resistance in the environment is the presence of naturally produced antibiotics. Their presence has never been shown outside of a laboratory environment. Should they be produced it is likely to be in very small amounts, and only in particular microenvironments, which would make them difficult to detect.

1.3.2.2. Bacterial biochemical well-being

Antibiotics at above Minimum Inhibitory Concentrations (MIC) are only likely to be found in very localised areas in the aquatic environment, such as immediately around fish farms or close to sewage outfalls. Away from these antibiotics are likely to be present, if at all, at sublethal concentrations. It is possible that at these levels the antibiotics will affect the biochemical well-being of bacteria which may result in inefficient or reduced metabolic activity or physiological changes. In a microbial ecosystem changes in the metabolic activity of any individual organism could have an effect on dependant bacteria. Chopra and Linton (1986) reviewed some of the effects of low concentrations of antibiotics from a medical point of view, such as changes in bacterial surfaces, which can affect adhesion to surfaces. Lebrun *et al.* (1992) found that both penicillins and tetracyclines had profound effects on cell surface characteristics at concentrations an eighth of the minimum inhibitory concentrations. Another effect of exposure to low concentrations of antibiotics, which has been seen in laboratory experiments, is an increase in background resistance (Brady and Katz, 1992; Brady *et al.*, 1993). This is related to exposure to multiple antibiotics mixtures in particular, with an increase in resistance of 33.95% over controls. Two conclusions can be drawn from this. Firstly, exposure to low levels of antibiotics (100μg/l or less) has some impact on bacteria which results in the increased resistance. It is likely there are other, less easily detectable, effects. Secondly, bacteria in the environment are most likely to be exposed to combinations of antibiotics which, in the above experiments, result in the greatest changes in resistance. The presence of antibiotics in the environment could result in, at the very least, increased background levels of resistance.

An example of the toxicity of antibiotics to sensitive bacteria can be seen in the work carried out by Hansen *et al.* (1992). They looked at the effect of antibiotics on bacteria in marine fish farm sediment. During the first 20 days around a third of the bacteria present in the sediments disappeared, and a further 25% disappeared over the next 165 days. The number of bacteria in the treated sediment decreased to 50-70% of the numbers in the control sediment 2 days after treatment. The effect of the medication on bacterial activity was investigated by measuring sulphate reduction, an important function in the mineralization of the organic-rich sediments from fish farms. Seven days after treatment sulphate reduction was reduced to 10% of the control, this effect was still present after 29 days but had disappeared after 70 days.

Johnsen (1994) looked at the effect of antibiotics on the degradation of fatty acids in fish farm sediments and found that the presence of antibiotics had a temporary delaying effect on the rate of degradation of fatty acids, and therefore the rehabilitation of the site. Fatty acids are the result of anaerobic microbiological activity occurring when fish farm waste falls to the sea bed. The presence of oxytetracycline had also been shown to affect nitrification, an aerobic bacterial activity which removes ammonia toxic to fish from the water (Klaver and Matthews, 1994). Unfortunately only short term effects were looked at, over several days as opposed to weeks, so it is not known if this is a permanent effect (though this is unlikely) or if nitrification does recover and how long it takes to do so.

The study of bacterial efficiency and well-being is fairly difficult. Work has already been highlighted above (Hansen *et al.*, 1992; Johansen, 1994) which looked at the efficiency of bacteria in cycling nutrients in the presence of antibiotics. Another possible approach is to look at measurable cell functions, such as luminescence (Naveh *et al.*, 1984), and see how these are affected by the presence of antibiotics. This is not a method that has been used to determine the impacts of antibiotics on bacteria. Luminescence and other bioassays have been used to detect and measure the toxicity of chemicals (Hill *et al.*, 1993) but never with the aim of looking at their long term impact on the bacteria themselves.

Looking at the impacts of antibiotics on individual species is not enough. In the environment individual species form part of a community which as a whole fulfils varied and important functions in the ecosystem. The effect of a pollutant seen in the laboratory on an individual species may not reflect the impact it has on the community as a whole. Another possible approach to investigating the impact of antibiotics on freshwater microbial communities is to look for changes in the community structure and diversity that might reflect the presence of antibiotics and indicate possible impacts. According to Watve and Gangal (1996) there are three important dimensions of bacterial diversity: Species richness, relative abundance of each species and differences or taxonomic distances between biotypes. Antibiotics are likely to affect all of these in the laboratory. What really needs to be known is if the low levels of antibiotics that could be present in rivers and streams are sufficient to have a measurable impact on bacterial communities. It is possible that aquatic ecosystems are diverse enough and robust enough to absorb any changes caused by the presence of antibiotics, without detectable effects. This may or may not be the case and needs to be further investigated.

1.4. Conclusions

From the above it can be concluded that despite the large quantities of antibiotics used worldwide there is very little information on how much of these actually reach the aquatic environment. There is also limited information on the possible impacts these antibiotics may have and whether they are detrimental to the environment in the long term. Though there is an interest in all these areas very little work has been carried out. No work at all has been published on the fate of antibiotics from human use, though this is an area that should be of interest and deserves further attention. The other area of interest is the investigation of the impacts of these antibiotics on bacteria and the aquatic environment as a whole. The eventual aim of work in this field should be to obtain a clear picture of the fate and impacts of antibiotics from human and agricultural use on the aquatic environment, focusing particularly on freshwater microbial communities as these would be expected to be the first and most highly impacted components of the ecosystem.

Chapter 2: Use of Antibiotics in the UK

2.1 Introduction

It would be impossible to obtain a comprehensive view of the impacts of antibiotics from human use without some indication of what levels of antibiotics are used in the UK. From the results of initial enquiries, it became apparent that this would not be a simple task. However, the possible sources and levels of antibiotics that could be polluting the aquatic environment had to be identified and attempts made to quantify them.

2.2 Antibiotic Production

All major producers of antibiotics in the UK were contacted (14 pharmaceutical manufacturers in all), requesting data on amounts of antibiotics produced and enquiring about the existence of ecotoxicology or environmental impact studies carried on these. Eleven companies replied and none of these addressed either issue in any way.

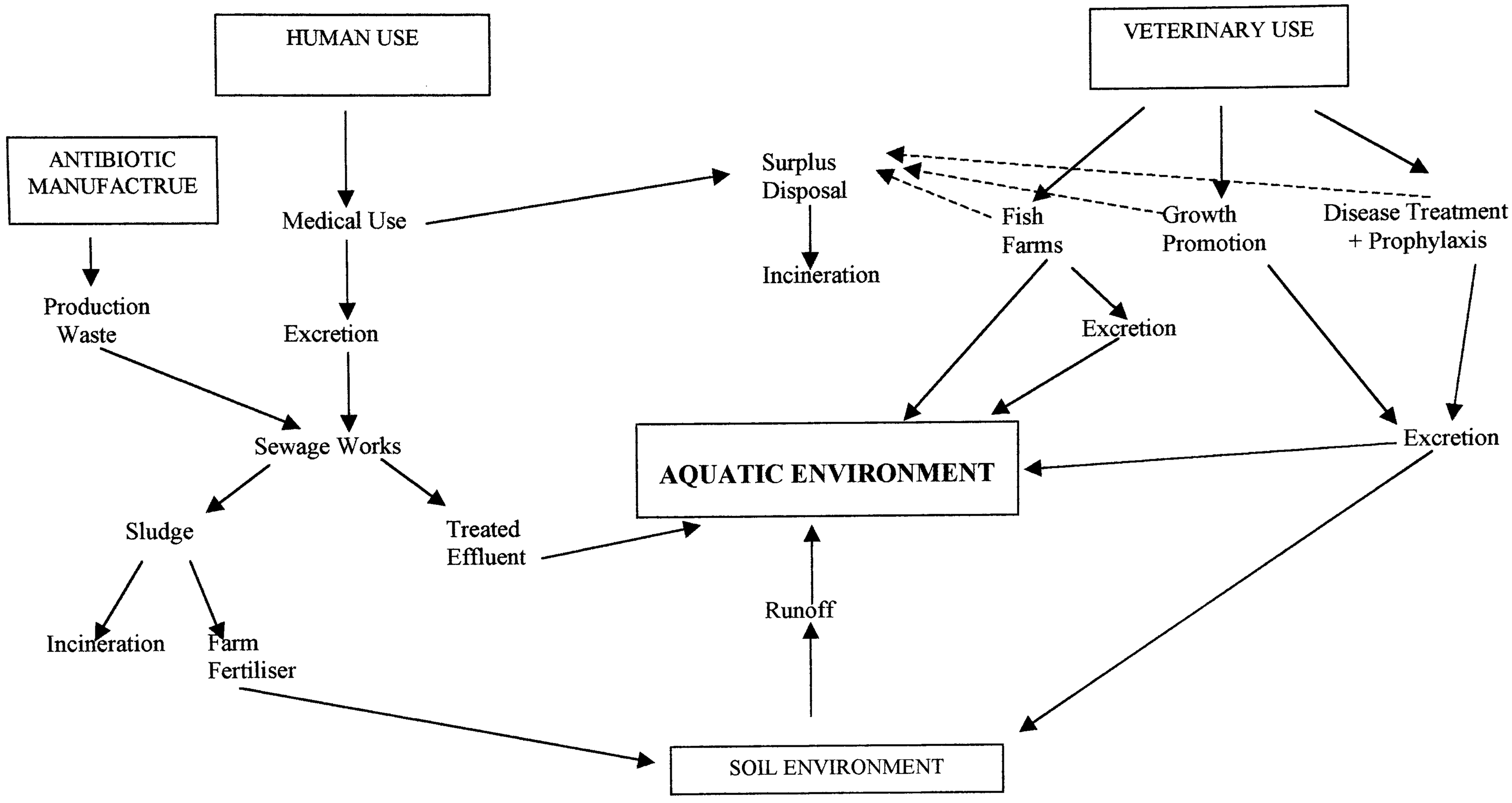
There is the possibility of large quantities of antibiotics entering the environment due to the manufacturing process. Waste from the fermentation vats used to produce antibiotics is usually treated on site to reduce the high Biological Oxygen Demand (BOD). Depending on the site treated waste then passes into the sewerage system to be treated again at a treatment works or it is poured directly into the receiving waters. Manufacturing companies again will not divulge any data on the levels of antibiotics contained in their waste.

2.3 Antibiotic Uses

The various antibiotic uses in the UK were outlined in Chapter 1. From all the information available it was possible to produce a diagram of all the routes for antibiotics that could be entering the aquatic environment. Sources could then be classified according to their likely importance in contributing to the antibiotic load reaching freshwater aquatic ecosystems.

In Figure 2.1, it can be seen that the only direct entry of antibiotics after use into the freshwater ecosystems is from the use of antibiotics in fish farms. The levels of use in this industry are impossible to calculate. No central records are kept of amounts used. The common method of adding antibiotic to feed is to add it in powder form and then mix it in a cement mixer, with no proper control of amounts used (R. Bawden, Aqua Systems Ltd, Pers. Com.).

Figure 2.1: Possible fate of antibiotics after use and likely routes of entry into the aquatic environment



A lack of understanding by veterinarians of fish farming practices may contribute to this lack of control. Method of disposal of surplus antibiotics is also unknown, theoretically they should be returned to a veterinarian or pharmacist for disposal but this is unlikely to be the case. Other veterinary uses of antibiotics are again difficult to estimate. Information is not available from manufacturers. No central record of amounts used exists. The only possible way of obtaining information would be to carry out an extensive survey of farmers to determine levels used. As can be seen in Figure 2.1, the antibiotics entering the environment from farms would probably do so from the excreta of animals receiving antibiotics as a growth promoter, prophylaxis or disease treatment. This slurry could produce runoff that reaches the aquatic environment directly, or if sprayed as fertiliser, again possibly reaching the aquatic environment as runoff.

Human medical use of antibiotics is strictly controlled. They are not obtainable in any form without prescription. Though every prescription is recorded, there is no central record of these. As can be seen in Figure 2.1 antibiotics from human use could be entering the aquatic ecosystem at unknown levels, via sewage treatment works. In addition, it is common practice for the sludge produced from treating sewage solids to be used on farmland as fertiliser. Any antibiotic not removed by treatment might then be released into the freshwater aquatic ecosystem as runoff.

2.3.1 Human Medical Antibiotic Use.

Human antibiotic consumption in the UK is strictly controlled. Antibiotics can only be obtained by prescription. Therefore, the only two sources of antibiotics for patients are GP or Hospital prescriptions. When considering the levels of antibiotics from these sources as environmental pollutants it is important to address the fate of antibiotics in the body and how they are excreted, if at all. Antibiotics from the same group have generally similar characteristics, though significant variations from one antibiotic to another do exist even within the same group.

The characteristics of each of the most significant antibiotics from the above groups are set out below in Table 2.1.

Table 2.1.Antibiotic fate: excretion route and percentage of antibiotic dose that leaves the body in active form.

Antibiotic group	Excretion route	% dose unchanged
Benzylpen & Phenoxymethylpen		
Penicillin V	Urine	15
Penicillinase Res Penicillins		
Flucloxacillin	Urine Bile	50 Some
Broad spectrum Penicillins		
Amoxycillin	Urine	50-70
Ampicillin	Urine	50
Cephalosporins		
Cephalexin	Urine	100
Cephardine	Urine	100
Cefuroxime	Urine	100
Ceftazidime	Urine	80-90
Cefotaxime	Urine	inactive
Ceftriaxone	Urine Bile	50 10-20
Cefaclor	Urine	70
Tetracyclines		
Tetracycline	Urine/Bile	50-70
Oxytetracycline	Urine	60
Doxycycline	Urine/intestine	100
Macrolides		
Erythromycin	not excreted	0
Sulphonamides and Trimethorpim		
Trimethoprim	Urine	90

It can be seen that significant proportions of antibiotics administered are excreted in their active form in urine and in faeces. This indicates that there is the potential for significant quantities of antibiotics to be reaching the aquatic environment.

2.3.1.1 GP Prescriptions

Data on antibiotic prescription from General Practitioners is collected through a system called Prescription Cost Analysis, introduced in 1991. This analysed all medication prescribed by general practitioners. The data that the UK Department of Health (DoH) collects was therefore extensive and if made available would have provided a great deal of information on levels and patterns of antibiotic use in the UK. However, in 1994, when this project began the data were considered very commercially sensitive and only a limited amount of information was made accessible, after signing various confidentiality and loss of revenue agreements. The laboratory work that was carried out in the present study was based on this limited information, which is detailed in Table 2.2. In addition, after further negotiations, list of the most prescribed individual antibiotics was also provided, with no mention of actual quantities.

Table 2.2 Number of GP prescriptions (in thousands) of the 7 most prescribed antibiotic groups in the UK from 1985 to 1995.

	Antibiotic group	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995
5.1.1.1	Benzylpen & Phenoxymeth.	4131	3988	3886	4125	4271	4256	4387	4138	4214	4156	4162
5.1.1.2	Penicillinase Res Penicillins	873	979	1030	1124	1191	1314	1502	1643	1729	1920	2091
5.1.1.3	Broad spectrum Penicillins	13936	14343	15489	15211	15437	17520	17520	17547	20206	19415	21737
5.1.2.0	Cephalosporins	3190	3178	3320	3451	3110	3297	3575	3741	4396	4229	4735
5.1.3.0	Tetracyclines	5125	4639	4424	4315	393	3767	4019	3946	4047	3783	3796
5.1.5.0	Macrolides	5117	5427	5635	5781	5537	5414	5923	5567	6018	5655	6538
5.1.8.0	Sulphonamides and Trimethoprim	4076	3781	3623	3633	4166	4151	4300	4026	4099	3605	3120

It can be seen that penicillins, particularly the broad-spectrum penicillins are by far the most prescribed antibiotics in the UK, which comprised 46% of prescriptions for 1995. Tetracyclines, which consist of 8% of total prescriptions for 1995, are also widely used in agriculture and aquaculture for animal husbandry, though it is impossible to say with any certainty in what quantities. Macrolides are also an important group, however they are not excreted in an active form from the body and therefore would not enter the environment.

From the data obtained from the DoH it was possible to ascertain that there was a seasonal variation in antibiotics prescriptions, as can be seen in Figure 2.2. Prescriptions for all groups were higher in the winter months. This seasonality would have an impact on the levels of antibiotics reaching the environment. This, however, may be counteracted by the fact that there is increased flow in rivers during the winter months, which would result in increased dilution of the antibiotics that may be reaching the aquatic environment.

In 1996 the DoH policy changed and retrospective information on the quantities, and cost, of all prescriptions made in England from 1991 were published in an annual Prescription Cost Analysis. When asked, the DoH stated that the reason for the previous restrictive policy was to protect the identity of patients receiving unusual prescriptions (Mrs A Custance, pers. com.). Apparently, this was now avoided by not including information on any items that carried fifty prescriptions or less. This change in policy meant that it was possible to calculate, in kilograms, from the data on GP prescriptions, the amounts of the most important antibiotics prescribed in England in 1996. The results are set out in Table 2.3.

Figure 2.2: Seasonal variations in antibiotic GP prescriptions for 1994/95 based on data provided by the UK Department of Health.

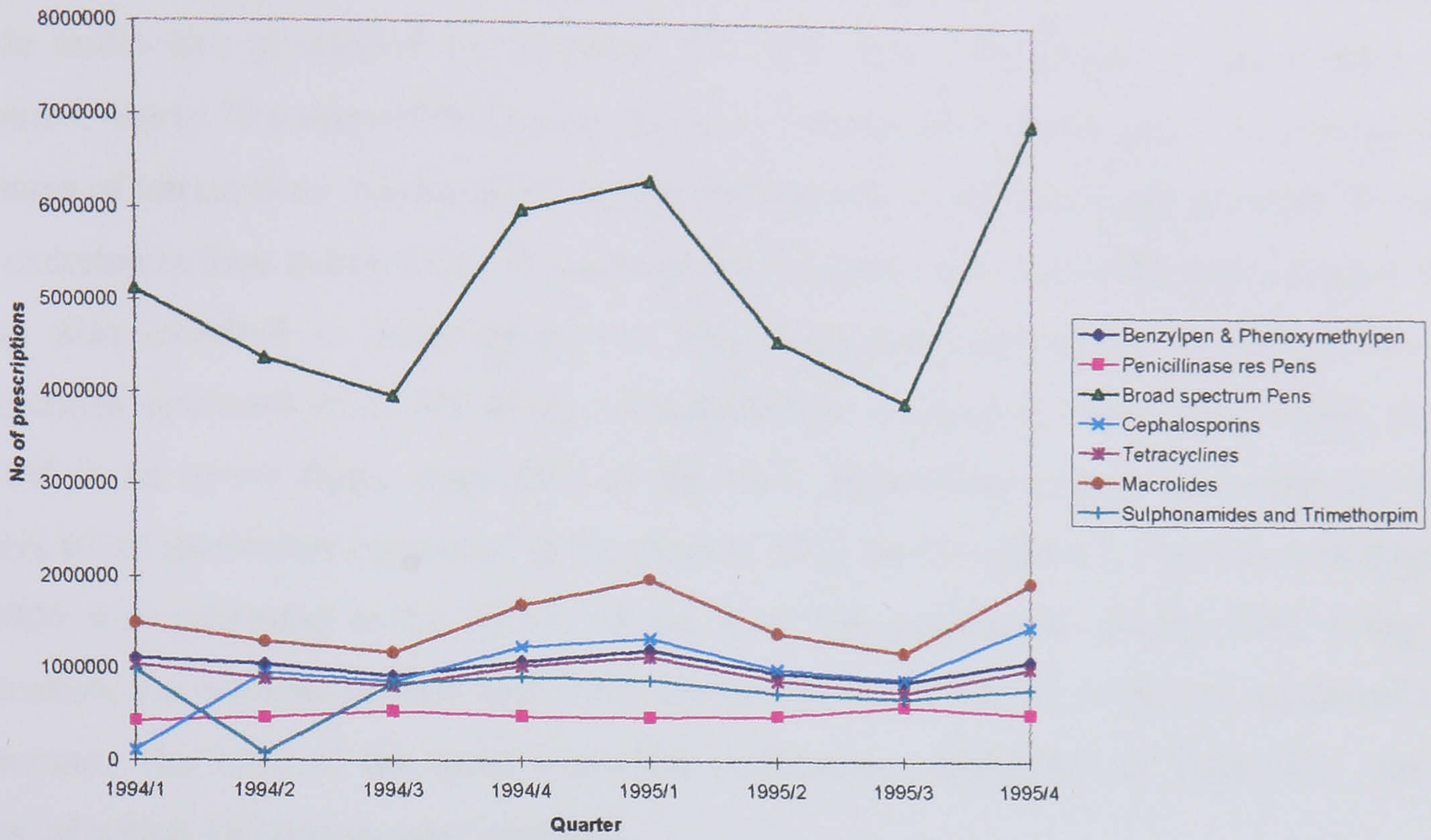


Table 2.3:
Summary of the quantities of the most commonly antibiotics prescribed by GP's in 1996 for England Calculated from data in the Prescription Cost Analysis, England 1996. (Government Statistical Services, 1997)

Antibiotic group	Total per Group (kg/yr)	Antibiotic	Total per Antibiotic (kg/yr)	Total excreted (kg/yr)
Benzylpen & Phenoxymethylpen	53097.9	Penicillin V	52653.1	7898.0
Penicillinase res. Penicillins	19036.5	Flucloxacillin	18585.5	9292.8
Broad spectrum Penicillins	123271.8	Amoxycillin	115281.5	69168.9
		Ampicillin	7115.3	3557.6
Cephalosporins	28375.7	Cephalexin	13666.5	13666.5
		Cephardine	4898.2	4898.2
		Cefuroxime	470.3	470.3
		Ceftazidime	28.0	23.8
		Cefotaxime	1.6	0
		Ceftriaxone	2.8	1.4
		Cefaclor	7617.6	5332.3
Tetracyclines	40575.8	Tetracycline	3143.1	1885.8
		Oxytetracycline	29105.5	17463.3
		Doxycycline	1185.9	1185.9
Macrolides	47738.8	Erythromycin	43313.4	0
Sulphonamides and Trimethorpim	8720.8	Trimethoprim	7424.3	6619.9
Total	**320816.7 Kg**		**304097.5Kg**	**141464.7Kg**

The data presented in Table 2.3 show the large amounts of antibiotics that are consumed in England. Over 320 tonnes of antibiotics were consumed in England in 1996, with at least 141 tonnes excreted by patients in their active form. This is only part of the picture as it does not include antibiotics prescribed by hospitals. In 1996 over 115 tonnes of amoxycillin were consumed, nearly 70 tonnes of this was excreted by patients in an active form. For tetracyclines 33 tonnes of tetracycline, oxytetracycline and doxycycline were consumed, of which 20 tonnes were excreted in their active form. Oxytetracycline was the most frequently prescribed of these. It was also excreted in large quantities. Broad-spectrum penicillins, cephalosporins and tetracyclines consisted of 57.3% of the total antibiotics consumed. Macrolides, which are not excreted in an active form, were 15% of the total. These four groups accounted for three-quarters of all antibiotics consumed in England in 1996 by GP patients. Population in England for 1996 was estimated to be 83.5% of the total UK population (58,801,100). Using this information, the estimate of total antibiotics prescribed in the UK for 1996 was calculated to be 384 tonnes. The total of the most important antibiotics, highlighted in Table 2.3, was 364 tonnes, of which 169 tonnes were excreted.

Population data for England and information for the Thames River catchment areas were combined with the information on antibiotic consumption to obtain a better understanding of the likely levels of antibiotics reaching the environment.

Table 2.4: Estimated annual and daily amounts of antibiotics prescribed and excreted in England as a total and per head of population

Antibiotic	Prescribed Antibiotic			Excreted Antibiotic		
	Per Year (kg)	Per day (kg)	Ab/head/day (mg)*	Per Year (kg)	Per day (kg)	Ab/head/day (mg)*
Amoxycillin	115281.5	315.84	6.43	69168.9	1898.50	3.86
Cephalexin	13666.5	37.44	0.76	13666.5	37.44	0.76
Cefaclor	7617.6	20.87	0.43	5332.3	14.61	0.30
Tetracycline	3143.1	8.61	0.18	1885.8	5.17	0.11
Oxytetracycline	29105	79.74	1.62	17463.3	47.84	0.98
Doxycycline	1185.9	3.25	0.07	1185.9	3.25	0.07

English Population Estimate1996: 49,089,100 (Office of National Statistics).

It can be seen in Table 2.4 that considerable amounts of antibiotics are being excreted by humans in England on a daily basis and are entering the sewerage system. On average 3.9 mg of amoxycillin and 0.98mg of tetracyclines were excreted per person per day in England in 1996. Similar amounts could be expected on a national level.

Information on levels of antibiotics that could enter particular river systems could also be estimated using population data for particular cities. Oxford and Reading are two towns on the River Thames, and sewage from them is discharged into the river after treatment.

The population of Oxford was 114,000 and that of Reading was 145,851 in 1996. Estimates of antibiotics excreted by the population can be seen in Table 2.5.

Table 2.5: Estimates of yearly and daily quantities of antibiotics excreted by the populations of Oxford and Reading.

Excreted Ab	**Oxford**		**Reading**	
	Yearly (kg)	**Daily (g)**	**Yearly (kg)**	**Daily (g)**
Amoxycillin	160.6	440.0	205.5	562.9
Cephalexin	31.6	86.6	40.5	110.8
Cefaclor	12.5	34.2	15.9	43.8
Tetracycline	4.6	12.5	5.9	16.1
Oxytetracycline	40.8	111.7	52.2	142.9
Doxycycline	2.9	7.9	3.7	10.2

This table shows the levels of antibiotics which could be entering the Thames via Sewage Treatment Works (STW) on a yearly and daily basis (assuming no degradation occurs in the STW). This relates to just two sources for antibiotics entering the Thames.

2.3.1.2 Hospital Prescriptions

Having contacted various health authorities and the Regional Drug and Therapeutics Centre in Newcastle Upon Tyne it became apparent that there is no central or regional record kept of drug usage by hospitals. As a result of this, it was impossible to obtain an overall picture of antibiotics use in England. However, it was possible that data from hospitals could be used to obtain information on antibiotic use that could then be extrapolated to give an indication of use in England.

Various hospitals were then contacted directly. Antibiotic prescriptions are kept on record, but are uncompiled, as the data on quantities used are not utilised in any form by the pharmaceutical departments. Two hospitals were happy to supply data. The resulting information, summarised in Tables 2.6 and 2.7, was compiled from lists of every prescription of the most commonly dispensed antibiotics at the hospitals. This was very time consuming work and therefore limited the amount of the data that could be compiled and utilised.

Table 2.6: Amounts of most commonly prescribed antibiotics at St Peter's Hospital, Chertsey from April 1995 to March 1996.

ST PETER'S HOSPITAL					
400 Beds	**Annual Antibiotic (Grams)**				
	Quarter 2 1995	**Quarter 3 1995**	**Quarter 4 1995**	**Quarter 1 1996**	**TOTAL**
Amoxycillin	**10889.3**	**9856.8**	**10940.0**	**10926.9**	**42612.9**
Cephalexin	1225.0	1240.8	1109.5	1094.8	4670.0
Cefotaxime	2741.3	1922.3	1754.5	1577.0	7995.1
Total Cephalosporins	3966.3	3163.1	2864	2671.8	**12665.1**
Minocycline	2.4	0.7	2.3	2	7.4
Doxycycline	56.8	61.3	64	58.2	240.3
Tetracycline	152	92	124.5	9	377.5
Total Tetracyclines	**211.2**	**154**	**190.8**	**69.2**	**625.2**
				Grand Total (g)	**55903.2**

Table 2.7: Amounts of most commonly prescribed antibiotics at Wycombe Hospital, High Wycombe, from July 1996 to June 1997.

WYCOMBE HOSPITAL					
379 Beds	**Annual Antibiotic (Grams)**				
	Quarter 3 1996	**Quarter 4 1996**	**Quarter 1 1997**	**Quarter 2 1997**	**TOTAL**
Amoxycillin	**5915**	**9414**	**8272.8**	**6653.5**	**30255.3**
Cefaclor	245.4	365.4	427.25	242	1280.1
Cefuroxime	4051.25	4518.5	4599	4675.5	17844.3
Cephardine	1764.5	1337.5	1984	1978.3	7064.3
Ceftriaxone	24.5	0	0	222	246.5
Cefotaxime	212.5	468	794	401.5	1876.0
Ceftazidime	787	732.5	329	181.75	2030.3
Total Cephalosporins	**7085.15**	**7421.9**	**8133.25**	**7701.05**	**30341.5**
Oxytetracycline	2062.5	1606	2044	2312.5	8020.0
Doxycycline	164.6	182.2	145.1	28.5	520.4
Total Tetracyclines	**2227.1**	**1788.2**	**2189.1**	**2341**	**8540.4**
				Grand Total (g)	**69137.2**

Table 2.8: Antibiotic consumed and excreted per hospital bed for St Peter's and Wycombe Hospital

	St Peter's Hospital Total/ Year/Bed		Wycombe Hospital Total/Year/Bed	
	Consumed (g)	**Excreted (g)**	**Consumed (g)**	**Excreted (g)**
Amoxycillin	106.53	63.92	75.64	45.38
Cefaclor			3.20	2.24
Cefuroxime			44.61	44.61
Cephardine			17.66	17.66
Ceftriaxone			0.62	0.31
Ceftazidime			5.08	4.31
Cefotaxime	19.99	0.00	4.69	0.00
Cephalexin	11.68	11.67		
Minocycline	0.02	0.02		
Tetracycline	0.94	0.57	1.30	0.78
Oxytetracycline			20.05	12.03
Doxycycline	0.60	0.60		
Total	139.76	76.78	172.84	127.33

The data for the hospitals do not correspond to the same year and this must be remembered when looking at the information contained in tables 2.6, 2.7 and 2.8. Despite this, it is possible to draw various conclusions from the analysis of prescriptions. It can be seen that there are important differences between the prescription patterns of the two hospitals. Both were general hospitals, but one, St Peter's does not have a Genito-Urinary department. This is reflected in the levels of tetracycline antibiotics prescribed, as this was the department that prescribed the majority of tetracyclines in Wycombe Hospital. Levels of amoxycillin prescribed per bed were higher in St Peter's than at Wycombe Hospital. However, prescription of cephalosporins, another group of broad-spectrum antibiotics, per bed, was higher at Wycombe than at St Peter's. Other antibiotics for which data has not been included again showed very different patterns of prescription. For example, Flucloxacillin, a penicillinase resistant penicillin, was prescribed twice as much at St Peter's than at Wycombe. Some seasonal variations can be seen in both hospitals, with higher amoxycillin prescriptions for the winter months. No other antibiotics show this kind of seasonality.

All of the above factors make the use of results from individual hospitals to obtain a picture of national antibiotic use in hospitals in the UK, by extrapolation, highly inaccurate. However, other than surveying every single National Health Service trust in the UK (there are 342 in England alone) there is no other more reliable way of obtaining some indication of the level of antibiotic use in hospitals on a national level.

Figure 2.9 shows the very rough estimates calculated using hospital bed data for England obtained from the Department of Health. It must be remembered that the data used for the calculations do not all correspond to the same year. Hospital data for St Peter's was for 1995/96, for Wycombe hospital was for 1996/97 and hospital bed data for 1997/98. However, the results still give indications of the levels of antibiotics that have been prescribed in England over the last few years.

Table 2.9: Comparison between estimated hospital prescriptions for England and calculated GP prescriptions for England.

	Total Antibiotic Prescribed (St Peter's + Chertsey) g.	**Average /Bed (St Peter's + Chertsey) g.**	**Estimated Total Hospital Prescriptions/Year 1995-97 [1] kg.**	**Total GP Prescriptions/ Year 1996 kg.**
Amoxycillin	72868.2	93.54	18414.0	115281.5
Cefaclor	1280.1	1.64	323.5	7617.6
Cefuroxime	17844.3	22.91	4509.3	470.3
Cephardine	7064.3	9.07	1785.2	4898.2
Ceftriaxone	246.5	0.32	62.3	2.8
Ceftazidime	2030.3	2.61	513.1	28
Cefotaxime	9871.1	12.67	2494.5	1.6
Cephalexin	4670	5.99	1180.1	13666.5
Tetracycline	377.5	0.48	95.4	3143.1
Oxytetracycline	8020	10.30	2026.7	29105.5
Doxycycline	760.7	0.98	192.2	1185.9
Total			31596.3	175401.0

1- Total available beds in England 1997/98: 196,856 (DoH Statistics)
English Population 1996: 49,089,100

It can be seen in Table 2.9 that the majority of antibiotics in England are prescribed to GP patients. Amoxycillin is the most important antibiotic in both hospital and GP prescriptions. In addition, it can be seen that some of the cephalosporins are not prescribed in significant quantities by GPs but are important in hospital prescriptions.

2.4 Sewage Treatment Works

If enough data on hospital prescriptions were pooled, they could be combined with GP prescription data to estimate, in the case of any particular sewage treatment works (STW), the level of antibiotics passing through on a yearly or daily basis. The only the information needed would be the size of the population in the catchment area for the particular STW, together with the number of beds of any hospital present in the area.

Information made available by Thames Water plc on two treatment works that were sampled during work on this project can be used to illustrate this. Data from the two sewage works was combined with data from St Peter's Hospital and estimated levels of antibiotics excreted by the population to predict the level of various antibiotics reaching the sewage treatment works.

Table 2.10: Example of calculation of estimated antibiotic present in sewage arriving at Chertsey and Bracknell STW

	AMOXYCILLIN (Estimated)	
	CHERTSEY STW	**BRACKNELL STW**
Population Served	74000	71500
Average/head/day	3.86mg	
Total GP Prescriptions	**285.6g/day**	**275.9g/day**
Hospital	400 beds	none
Total Hospital Prescriptions	**70.1g/day**	**0**
Total Overall	**355.7g/day**	**275.9g/day**
Dry Weather Flow	19000m^3 /day	16705m^3 /day
Estimated Sewage Concentration	**18.7µg/l**	**16.5µg/l**

This estimate of 16.5µg/l of amoxycillin per litre of untreated sewage entering the treatment plant at Bracknell gives an indication of the level of antibiotics that can be expected in sewage. It can be seen that the presence of a hospital in the Chertsey sewage works catchment are has an impact on the estimated levels of amoxycillin in the sewage. These figures do not take into account seasonal or regional variations that may occur in antibiotic consumption. Using this method, estimations for all the antibiotics passing through both treatment works were made. Results can be seen in Table 2.11.

Tale 2.11: Estimated concentrations of some antibiotics in sewage arriving at Chertsey and Bracknell STWs.

	CHERTSEY STW	BRACKNELL STW
Amoxycillin	18.7µg/l	16.5µg/l
Cephalexin	3.6µg/l	3.5ug/l
Cefaclor	1.17µg/l	1.28µg/l
Tetracycline	0.46µg/l	0.47µg/l
Oxytetracycline	3.74µg/l	4.19µg/l
Doxycycline	0.31µg/l	0.30µg/l

From results detailed in table 2.11 it can be seen that the presence of a hospital in the catchment area of a STW is counteracted by the increased level of sewage passing through the works. This will not always be the case. Calculated levels are in the range of 20µg/l to 0.5µg/l.

2.5 Antibiotic disposal

When antibiotics are prescribed there is a very strong emphasis on the need to finish a course completely. This is important on medical grounds, but it also means that patients will not be left with any unfinished prescriptions that might have been directly disposed of into the sewerage system. Any unused or out of date antibiotics that are returned to pharmacists are incinerated (Mr. Odd, Royal Pharmaceutical Society, Pers. Com.) and therefore are not a threat to the aquatic environment.

2.5 Discussion

From initial investigations into antibiotic use in the UK it became clear that the possible impacts of antibiotics used both in human and animal medicine had hardly been considered in the past. Very few records were kept of antibiotic use, particularly with regards to its use in agriculture. However, as was seen in Chapter 1, this was also the area in which the role of antibiotics as environmental pollutants had mostly been considered, particularly with relation to fish farms. The importance of antibiotics from human medical use as pollutants had not been considered in the past. Because of this, there was no information at all on the levels of antibiotics that could be reaching the environment from this source.

The data obtained and analysed in this chapter have provided invaluable information on the levels of antibiotics that are used in the England. Figures are likely to be similar on a national level. The data on GP prescriptions showed broad-spectrum penicillins to be the most prescribed antibiotics, mostly amoxycillin. This antibiotic was also very important in hospital prescriptions. In St Peter's Hospital it was by far the most prescribed antibiotic in 1995/6. In the case of Wycombe Hospital, it was also the single most prescribed antibiotic. However, if the cephalosporin group is taken as a whole, they were prescribed as much as amoxycillin, which was not the case at St. Peter's. These differences highlight differences in prescription patterns that will occur in all hospitals. They will be due not only to different departments in the hospitals, even when they are general hospitals, but also to different prescription patterns as a result of hospital policy and consultant preferences. In addition, Wycombe prescription data are for a year later, 1996/7. During this time changes in general prescription trends could have resulted in this change. A survey of consultant prescribing habits carried out in 1990/91 (Halls, 1993) showed that broad-spectrum penicillins were the most prescribed groups of antibiotics in English hospitals. However, the most commonly used one was ampicillin, which was not prescribed at all at either of the hospitals whose data were analysed for the project. This shows how trends have changed over the last 7 years. The study, which covered five European countries, found widely varying patterns of prescription. For example aminoglycosides constituted less than ten thousand GP prescriptions per year in the UK over the last ten years (DoH data). They accounted for only 4% of therapy days in UK hospitals for 1990/91 but in Spain, they were used in 25% of therapy days. This illustrates how variations occur not only within national boundaries but also internationally.

A further example of these differences and changes of prescribing practices with time is a study carried out in a 800 bed Spanish hospital (Valero and Mateos, 1995). This showed that in 1990 the most utilised antibiotics were the aminoglycides and broad-spectrum penicillins. In 1992 it was the amonoglycosides and quinolones and in 1994 changed again cephalosporins. This differed from results of national surveys carried out in Spain since 1991 that showed the most prescribed antibiotic in Spanish hospitals to be amonoglycosides, until 1993. This then changed, due to resistance problems, to broad-spectrum penicillins. The findings from this hospital also highlight how large variation between individual hospitals make it difficult to obtain a realistic estimate of consumption, without sampling a large number of hospitals.

When looking at GP prescriptions form 1985 to 1995 it can be seen that, overall, there were no major changes in prescription patterns. The only large increase was for broad-spectrum penicillins that consisted of 38% of prescriptions of the 7 most important groups, to 47%. The only major decrease were the tetracyclines that were reduced from 14% to 8% of total prescriptions.

Data used to estimate antibiotic consumption for England showed that the levels consumed in hospitals, for the majority of the most important antibiotics were only a fraction of that consumed by GP patients. Results from Table 2.9 are only a very rough estimate of likely consumption patterns over the last two or three years, as has already been pointed out. However, they do highlight some interesting facts. Amoxycillin was the most prescribed antibiotic overall. Cephalosporins were widely prescribed by GPs and hospitals, but whilst GPs prescribed mainly first generation cephalosporins, hospitals prescribed mainly second and third generation ones. In the estimate of all the most commonly prescribed antibiotics in England, hospital prescriptions accounted for around 18% of the total. Because of this, when considering the environmental impact of antibiotics it can be concluded that amounts prescribed by GPs are the most important factor. This is fortunate because it was possible to obtain much more accurate figures for these prescriptions, as detailed in Table 2.3. The data that were not available were a seasonal breakdown, and as can be seen in Figure 2.1 seasonal variations in prescriptions do occur, particularly in the case of broad-spectrum penicillins. Seasonal variations were also found in hospitals but only in the case of amoxycillin. As already mentioned, the difficulty with hospital data available was that they were not compiled and converting individual prescriptions to data on actual antibiotic quantities was very time consuming. There were 782 amoxycillin prescriptions alone for one quarter at Wycombe Hospital. The aim was to use the data from the two hospitals, together with data on the number of available hospital beds in England obtained from the Department of Health to estimate the amounts of antibiotics prescribed in hospitals in England.

This aim was still addressed but the large differences in types of antibiotics prescribed between the two hospitals meant that the resulting figures cannot be seen as a reliable estimate, just an indication of level of antibiotics used in hospitals in the UK. The actual total quantities of antibiotics prescribed per hospital were in a similar range. This suggests that the major differences between hospitals will be in types of antibiotics prescribed not total quantities

When considering what levels of antibiotics could be reaching the environment from human medical use it was necessary to take into account two major factors. Antibiotics once taken can be broken down in the body before excretion. Recovery in urine, bile and faeces can vary from 0% to 100%. Once this was taken into account, some commonly used antibiotics were found to be of reduced or no importance environmentally. Macrolides are an example of this, as they are degraded completely in the body and excreted in an inactive form. The second factor that had to be taken into account was the dilution that would occur when excreted antibiotics entered the sewerage system.

It was possible to combine population data, average antibiotic excreted for England and daily flow through particular sewage treatment works to estimate levels of individual antibiotics reaching the works. The resulting calculations resulted in estimated concentrations of µg/l. (these levels are likely to be similar throughout the UK, though as already pointed out some regional variations would be expected). When all antibiotics were considered together, concentrations were much closer to the mg/l. Combination of antibiotics could prove more toxic to bacteria, due to the possibility of additive effects. Levels of individual antibiotics estimated to be arriving at the sewage works are lower than the concentration range of mg/l that is the minimum inhibitory concentration for the majority of bacteria for most antibiotics. It has already been shown antibiotics can have an impact at sub-Minimum Inhibitory Concentration levels (Chopra and Linton, 1986; Lebrun *et al.*, 1992). Whether these antibiotics impact bacteria at these concentrations is not known and is one of the issues addressed in this project. A further finding was that though, overall, hospital prescriptions were only a relatively small proportion of total antibiotics prescribed in England, the presence of a hospital in the catchment area of a STW could result in considerable increases in the levels likely to be present in sewage.

It has to be pointed out that the calculations do not take into account various factors that could have a considerable impact on the figures obtained. Variations in population health would affect levels of antibiotics consumed. This would be the case, not only seasonally with more antibiotics consumed in winter, but regionally. Poorer areas of the country suffer from worse health, and poor living conditions are known to increase diseases, such as respiratory tract infections, which are treated with antibiotics.

Another possible source of variation are the differences due to weather conditions. Increased rainfall increases flow through sewage works, thus affecting dilution of any antibiotics present. In addition, increased rain results in increased flow in rivers and greater dilution of sewage effluent. Because of this seasonal and year to year variations would be expected all over the country.

One final consideration must be remembered when looking at the data in Table 2.11, on antibiotics reaching sewage works. It is that treatment and then dilution of effluent in receiving river waters would result in a further reduction in the concentrations of antibiotics present. Treatment could result in the loss of antibiotics by binding to solids which are removed before treatment (though eventually residues could reach the environment as runoff from treated solids used as fertilisers). In addition, treatment could destroy antibiotics before they reach the aquatic environment. Dilution of final effluent in receiving waters could be anything from 1:2 (more likely in the summer in the case of small streams) to much larger ratios when major rivers are involved. This would partly be counteracted by the high number of STW effluent outfalls into a large river along its course.

When considering antibiotics in the environment it must still be remembered that unknown quantities of antibiotics from agriculture could potentially be entering aquatic ecosystems. Worldwide expenditure on antibiotics for agriculture was 24% of the total market in 1990 (Bird and Snale, 1992). However, this does not really give an indication of the amounts used because some antibiotics used commonly in agriculture, such as tetracyclines, are very cheap in comparison to, for example, cephalosporins which are one of the most commonly used antibiotics in human medicine. Antibiotics from agriculture could therefore be contributing a considerable proportion of the antibiotics reaching the environment.

2.6 Conclusions

Work contained in this chapter has for the first time reviewed the amounts of antibiotics prescribed for medical use in England. Conclusions from results showed that GP prescriptions were an estimated 80% of antibiotics consumed in the England. Broad-spectrum penicillins, in the most part amoxycillin, were the most prescribed antibiotics in 1996 and 1997 in both hospital and doctors' practices. Cephalosporins and tetracyclines were also important, particularly oxytetracycline.

Levels of antibiotics likely to be reaching the aquatic environment were low, below the levels that clinically inhibit bacteria, and were likely to be further diluted before reaching the aquatic environment. Concentrations could be substantially increased by the presence of a hospital in the catchment area. The possible impacts of these low levels of antibiotics individually, and as a mixture, on freshwater aquatic ecosystems are investigated further in the following chapters.

Chapter 3: Detection of antibiotics in environmental samples

3.1 Introduction

Detection of a particular pollutant in environmental samples is a vital part of the process involved in determining the impact and fate of pollutants. For this project information on the levels of antibiotics used in the UK, together with their chemical characteristics and fate in the body gave an indication of what levels of antibiotics could be reaching the environment and what their fate might be. These data suggested that antibiotics might be expected not only in water samples themselves, but also in the surrounding sediments. Methods of analysis used to detect them would have to take this into account. The aim of this work was primarily to determine if there were significant amounts of antibiotics in the freshwater environment, and then to apply any information obtained, together with data from the other areas of work, in the determination of any possible impacts.

There were two very different available methods for detection of antibiotics. The first one was biological. Bioassays are carried out using sensitive bacteria to detect the presence of antibiotics in samples by the inhibition of growth. Using the right array of bacteria, it is possible to identify antibiotics from different groups. However, it is very difficult to identify the exact antibiotic as antibiotics from the same groups often have the same mode of action. Because of this, bacteria are often sensitive to more than one antibiotic from the same group. There is also the possibility of other pollutants being present with an antibacterial effect, which would make interpretation of inhibition experiments much harder. Additionally, in this project the levels of antibiotics expected would probably be below the levels of detection for the majority of bioassay methods.

The second method available was chemical detection and analysis. This had the advantage not only of allowing the identification of specific antibiotics, but it was also possible to concentrate the samples before analysis, thereby making detection more likely. Quantification of the amounts of antibiotics present was also a possibility using this method. For all the above reasons, chemical analysis was decided upon as the most appropriate method for detecting antibiotics in environmental samples.

Antibiotics are non-volatile polar compounds and as such the most appropriate method for their chemical analysis is High Performance Liquid Chromatography (HPLC) followed by detection using ultraviolet (UV) light absorbance. This method is widely used in the pharmaceutical industry and there are many different published methods for the analysis of all types of antibiotics.

Indications from the early results of enquiries into the amounts of antibiotics used in the UK were that the levels that might be found in the environmental water samples would be very low. Because of this it was necessary to utilise a method that would concentrate the samples, making it easier to detect any antibiotic present.

Results from investigations of the types and levels of antibiotics used in the UK indicated that the two groups of interest were the tetracyclines and the β-lactams (both penicillins and cehpalosporins). Method development was carried out for the concentration and analysis of both these antibiotics groups. Once suitable methods were developed, analysis of samples began, whilst simultaneously attempting to improve sample extraction and analysis throughout the duration of the project.

3.2 Sampling

Method development had to take into account the various types of samples to be analysed throughout the project. In addition, all samples taken from the environment would have to be concentrated to increase the chances of detecting antibiotics. Throughout the project, sediment samples, river water samples, sewage samples and final treated effluent samples were all analysed.

River sediment samples were used for experiments to determine the fate of antibiotics in a water and sediment environment. All these samples had been previously spiked with antibiotics. Extraction procedures were carried out without concentration steps. River water was also sampled, concentrated and then analysed to determine if antibiotics were present. Both sediment and water were obtained from the River Thames at Medmenham.

Sewage samples were collected from Chertsey and Bracknell sewage treatment works from the settling tanks, as settled sewage (SS) This is untreated sewage as it arrives at the works, which has been held in tanks to allow the gross solids to settle out. This fraction contained the part of the sewage that is then treated. This treated final effluent (FE) would then be discharged into the receiving stream or river waters. Both treatment works were of a similar size and used the same treatment methods (Table 3.1). They were chosen because of this, the main difference between them being that Chertsey sewage treatment works (STW) received the waste water from a general hospital, whilst Bracknell STW had no hospital in its catchment area. It was also possible to obtain samples of the river water from Bracknell, upstream from where the effluent was discharged into the river

Table 3.1: Characteristics of Chertsey and Bracknell Sewage Treatment works.

	CHERTSEY STW	BRACKNELL STW
Population Served	74000	71500
Dry Weather Flow	19000m^3 per day	16705m^3 per day
Primary settlement tanks	6	7
Percolating filters	20	17
Digested sludge	18000m^3 per year	Not known

The reason for analysing sewage before and after treatment was twofold. Firstly, it was important to try to find out if treatment reduced the levels of antibiotics reaching the freshwater environment. In addition, it was possible that whilst levels of antibiotics reaching sewage works might be detectable, treatment might reduce antibiotic levels to below the limit of detection. This was a distinct possibility in the case of penicillins as they are readily biodegradable.

3.3 Method Development

Detection of antibiotics in such complex matrices as sewage was not something that had been attempted in the past. There were no published methods in the literature. However, methodology did exist for the recovery and analysis of antibiotics from other difficult media. An example of this is the extraction and analysis of antibiotics in meat products (Boison *et al.*, 1991) and foodstuffs (Oka *et al*, 1995). It was therefore decided to use these kinds of methods as the basis for developing methods appropriate for this project.

3.3.1 Materials

All methods were tested on a Hewlett Packard 1050 HPLC Package using a Zorbax C8 Bonded column with 3μm particles. This column was selected because it had suitable characteristics for antibiotic extraction. It was stable at low pH and did not strongly absorb basic compounds. The reduced particle size (usually 5μm) resulted in improved peak shape and separation, though it did require increased running times.

Solid phase extraction (SPE) was carried out using two different types of matrices. Firstly, C18 Bond-Elut 2.5g extraction columns were used. Method development was carried out using these columns. These were able to concentrate a maximum of 500ml of samples (with difficulty). It was decided that larger samples needed to be concentrated and analysed so as a result, 3M Empore extraction discs (also C18) were used. These were able to concentrate up to 10 litres of water.

Particulate removal was attempted by both centrifugation and filtration, using various kinds of filters. After evaluation of the results, filtration before solid phase extraction using 0.2μm membranes to remove any particulates was found to be the most efficient method. Vacuum pressure was used to increase filtration rates. All solvents and chemicals used were of HPLC grade and supplied by Sigma. Antibiotics used were also obtained from Sigma in powder form and made up in solutions using HPLC grade solvents.

Various kinds of standards were used during these experiments:

Standard solutions:

Antibiotic solutions of known concentrations made up in the same injection solute as samples. Results from their analysis were used to plot calibration graphs and produce equations to calculate concentrations.

Standards in eluted samples:

Standard solutions made up in eluted control samples. No antibiotic added before SPE. These controls were used to determine, by comparing to chromatograms of standard solutions, if the presence of co-eluents from SPE of sewage and river water samples interfered with the levels of antibiotics detected.

Spiked samples:

Spiked water and sewage samples that had antibiotic added before SPE extraction was carried out. These samples were used to determine the effectiveness of the methods for extraction and analysis of antibiotics. These samples were also used to determine limits of detection.

3.3.2 Tetracyclines

A literature review of available methods resulted in an extraction method by Oka *et al.* (1995) and an analysis method by White *et al.* (1993) being chosen for future work. Both these methods were tested and proved satisfactory after some modifications.

For extraction, a C-18 bond elute column was conditioned by washing with 5ml of methanol, followed by 2ml of water. The sample was then passed through the column using a vacuum manifold to pull it through as fast as possible. 100 ml took about 30 minutes to pass through the column. It was important not to let the flow of the sample stop at any stage. If the column dried out at any stage then the concentration step had to stop. This was the main difficulty associated with this method, as the samples involved were large. Once the entire sample had passed through the column, it was washed using 20 ml of water. The column was then dried and the sample eluted, using 0.01M oxalic acid in methanol.

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The original method used 10ml to do this. In order to determine if less eluent could be used to obtain a more concentrated sample, consecutive fractions of 2ml of eluent were used to extract the antibiotics from spiked samples and then analysed. Results can be seen in Table 3.2. It can be seen that all except the smallest amount of both tetracyclines was present in the first wash. Negative values represent peaks that, though visible, are not large enough to give a quantitative value to. This was because peak area was so small that concentration calculated using the standard equation gave negative values. This approach significantly reduced the amount of eluent used and therefore increased fivefold the concentration of antibiotic in the final sample that was used in the HPLC analysis.

Table 3.2: Fractioned extraction of tetracyclines. Final expected concentration 100mg/l

	Tetracycline		Oxytetracycline	
Fraction (2ml)	**Peak area**	**Conc. (mg/l)**	**Peak Area**	**Conc. (mg/l)**
1	5149.1	102.0	3192.6	81.2
2	44.8	0.9	23.0	0.2
3	16.1	0.4	11.3	nd
4	13.3	0.3	12.3	nd
5	9.7	0.2	7.0	nd

nd: not detectable

In addition, wash samples were collected and analysed to determine if antibiotics were passing through the columns unbound. The original sample containing the antibiotics at 2mg/l concentration was analysed and no antibiotic was detected. The water that was used to wash the column before elution was carried out was also analysed. Again, no antibiotic was found. This showed that no antibiotic was lost during the washing step of the extraction process. Because of all of these results this method was appropriate for the aims of this project.

Extraction efficiency of this method was also tested. Results in Table 3.3 show that there is no problem in recovering the entire sample. In the case of the tetracycline samples there appears to be an overestimation of the concentration present. Separate samples gave very similar results. It is likely that the reason for this is either that the samples contained too much antibiotic initially (error in adding antibiotic to sample) or that an error in the standards used has resulted in a miscalculation of the level of antibiotic present.

Table 3.3: Extraction efficiency of tetracycline SPE method. Initial duplicate samples: 100 ml of water with 1mg/l of each antibiotic. 2.5 ml of eluent solution used.

Sample	Peak Area	Conc.(mg/l)	Exp. conc.	% Efficiency
Oxy A	2699.8	41.1	40	102.8
Tet A	3070.4	60.2	40	150.6
Oxy B	2563.6	39.1	40	97.7
Tet B	3114.3	61.1	40	152.8

A further experiment was carried out to determine the efficiency of the extraction method when working with more complex samples, such as river water and sediment. Results, in Table 3.4. show that extraction efficiency is high, though, not surprisingly, it is lower than when extracting from cleaner samples. It can also be seen that at higher concentrations extraction efficiency improves. A tenfold increase in antibiotic present results in a 10% improvement in detection.

Table 3.4: Extraction efficiency of tetracyclines in sewage and river water samples during SPE. 50ml samples containing 0.1mg/l or 10mg/l of each antibiotics extracted and analysed.

	Oxytetracycline (mg/l)		**Tetracycline (mg/l)**	
Expected final conc.	2.5mg/l	25mg/l	2.5mg/l	25mg/l
Actual conc.				
Bracknell RW	1.75	25.5	1.48	21.5
Chertsey SS	2.09	20.5	1.79	19.3
Chertsey FE	2.12	23.0	1.76	18.8
Bracknell SS	1.76	20.8	1.53	19.1
Bracknell FE	2.25		1.63	
Efficiency of extraction	79.9%	89.3%	65.4%	78.7%

Due to the difficulties associated with filtering more than 500ml of sample using the solid phase extraction columns, 3M Empore C18 membranes were substituted. With these, it was possible to filter up to around 10 litres of sample. There was difficulty in this due to the very slow, constant flow of sample required, around 1 – 2 ml per minute. As with the columns, if the filter dried out at any point, filtering had to stop and analysis had to be carried out. The extraction efficiency of tetracyclines using this method was maintained at over 60%, even though the samples involved were much larger and the concentrations of antibiotics as low as 0.02mg/l.

The recommended method of extraction utilised by the manufacturers was 10ml of eluent. This was changed to two or three 5ml fractions, as a greater proportion of the antibiotics were eluted in the first fraction (though they were present in all three fractions), thus increasing the chances of detecting any tetracyclines in the samples.

Acidification of samples before extraction was attempted for tetracyclines to determine if it improved the method. However, it resulted in a greatly reduced extraction of all three tetracyclines. Peak area for oxytetracycline when the sample was acidified was 1.3% of the area when sample was not acidified. Similar results were obtained for tetracycline (0.49%) and doxycycline (1.1%).

The analysis method of White *et al.* (1993) was adapted and used for all analyses of tetracyclines during this project. Various running times and gradient compositions were tested. The most efficient at delivering compact peaks were used for analysis.

Doxycycline was not initially one of the antibiotics being studied. However, after taking into consideration data relating to use in the UK, it was incorporated into the analysis. All that was required for this was to increase the running time by 6 minutes. A summary of the method as it was used in this project can be seen in Table 3.5.

Table 3.5: Method for the analysis of tetracyclines (adapted from White *et al.* (1993).

	Analysis of tetracyclines
Column	Zorbax SB C8 (3.5µm; 4.6mm internal diameter x 15 cm long)
Guard Column	Zorbax (5µm) – prevents particles blocking column
Mobile phase	ACN* 0.05M Oxalate Buffer + 0.01M TMA*
Solvent Gradient	85:15 (Buffer:ACN) at 3 min. 64:36 (Buffer:ACN) at 16 min.
Run time (per sample)	No Doxycycline : 16 min. + 4 min. post time Doxycycline: 22 min + 4 min. post time
Injection Volume	20 to 200µl
Flow Rate	0.5 ml/min.
UV Wavelength Detection	355nm
Solid Phase Extraction Eluent	0.01M Oxalic acid in Methanol (1 in 2 dilution with 0.05M Oxalate Buffer before analysis)

ACN: Acetonitrile TMA: Tetramethylammonium chloride

When combining the SPE and HPLC analysis as detailed, it was necessary to dilute the sample obtained from SPE before injection into the HPLC column. The eluent for SPE was methanol. This was a stronger eluent than the mobile phase. Injection of the samples in this medium would have resulted in a large proportion of the antibiotics being eluted in the solvent front (large initial peak) and being undetected. Dilution with buffer removed this problem. A one in two dilution was carried out using the buffer used in the mobile phase.

Injection volumes were varied throughout the project, depending on the particular experiment. However, it was found that injection volumes of more than 50µl generally resulted in reduced detection of antibiotics. This was likely to be because of the large volume of sample being washed through the system with the solvent front resulted in smaller peaks.

Comparisons between analysis of standards made up in mobile phase solvents and those made up in the eluent after SPE had been carried showed that there was no difference in the levels of antibiotics detected. An example of this can be seen in Table 3.6.

Table 3.6: Comparison of chromatogram peak areas of various concentrations of tetracyclines into two different solutes. Std. Sol.: antibiotics in standard solution. Wash 1: antibiotic solutions in the first elution fraction from SPE of 10 litres of concentrated river water 26.9.97.

Antibiotic	Oxytetracycline		Tetracycline		Doxycycline	
Conc.	Stnd. Sol.	Wash 1	Stnd. Sol.	Wash 1	Stnd. Sol.	Wash 1
10mg/l	1754	1661	2004	2060	1299	1335
5mg/l	865	866	931	1070	641	699
1mg/l	181	184	183	221	134	134

Therefore, any antibiotic present in the water and sewage samples would produce a peak that could directly be compared to the standard peaks to determine the concentration of antibiotic present.

3.3.3 Penicillins

After an extensive search through the literature the most appropriate methods available for the reverse phase HPLC analysis of penicillins were tested (Fletouris *et al.*, 1991; Boison *et al.*, 1991 and Kirkland *et al.*, 1994). Of these, the Kirkland method, once adapted by changing the proportions of the mobile phase components to improve separation, proved the quickest and most sensitive for analysis of penicillins. This method could separate and detect amoxycillin, ampicillin and penicillin. These were the three most important antibiotics in the penicillin group. It was also possible to use this method to detect three cephalosporins at the same time. Cephalexin, cefaclor and cefotaxime could all be separated out using the same method. Further work resulted in the determination of the best UV wavelength to be used to measure the amount of antibiotic present. A typical chromatogram can be seen in Figure 3.1. Figure 3.2 shows the effect of changing the detector wavelength on peak area. Amoxycillin and cefotaxime absorbed produced larger peaks at 240nm and cefaclor; cephalexin and penicillin at 265nm.

Figure 3.1 Chromatogram of a 10mg/l standard solution of amoxycillin, cefotaxime, cefaclor, cephalexin and penicillin. UV detection 240nm

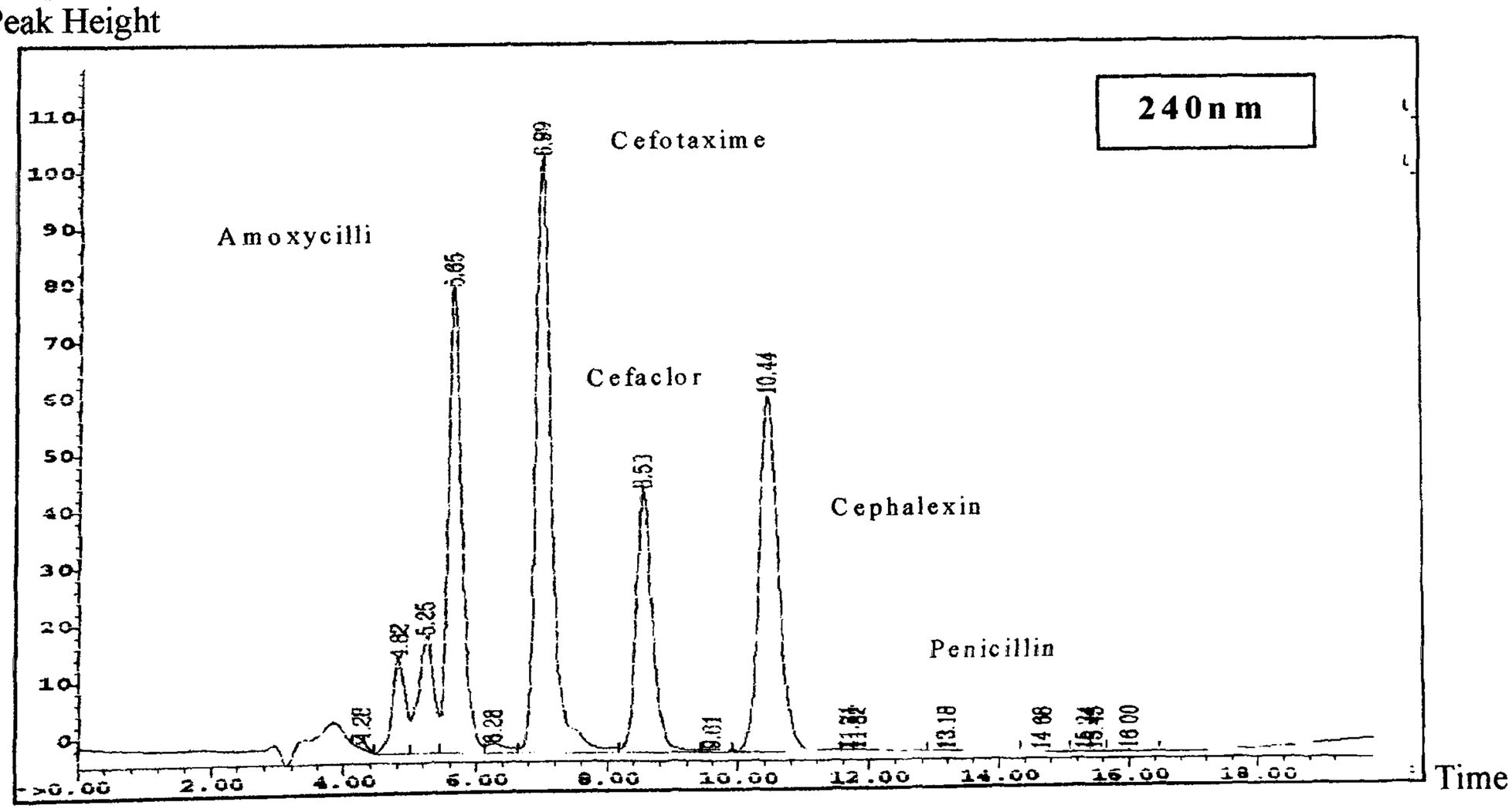
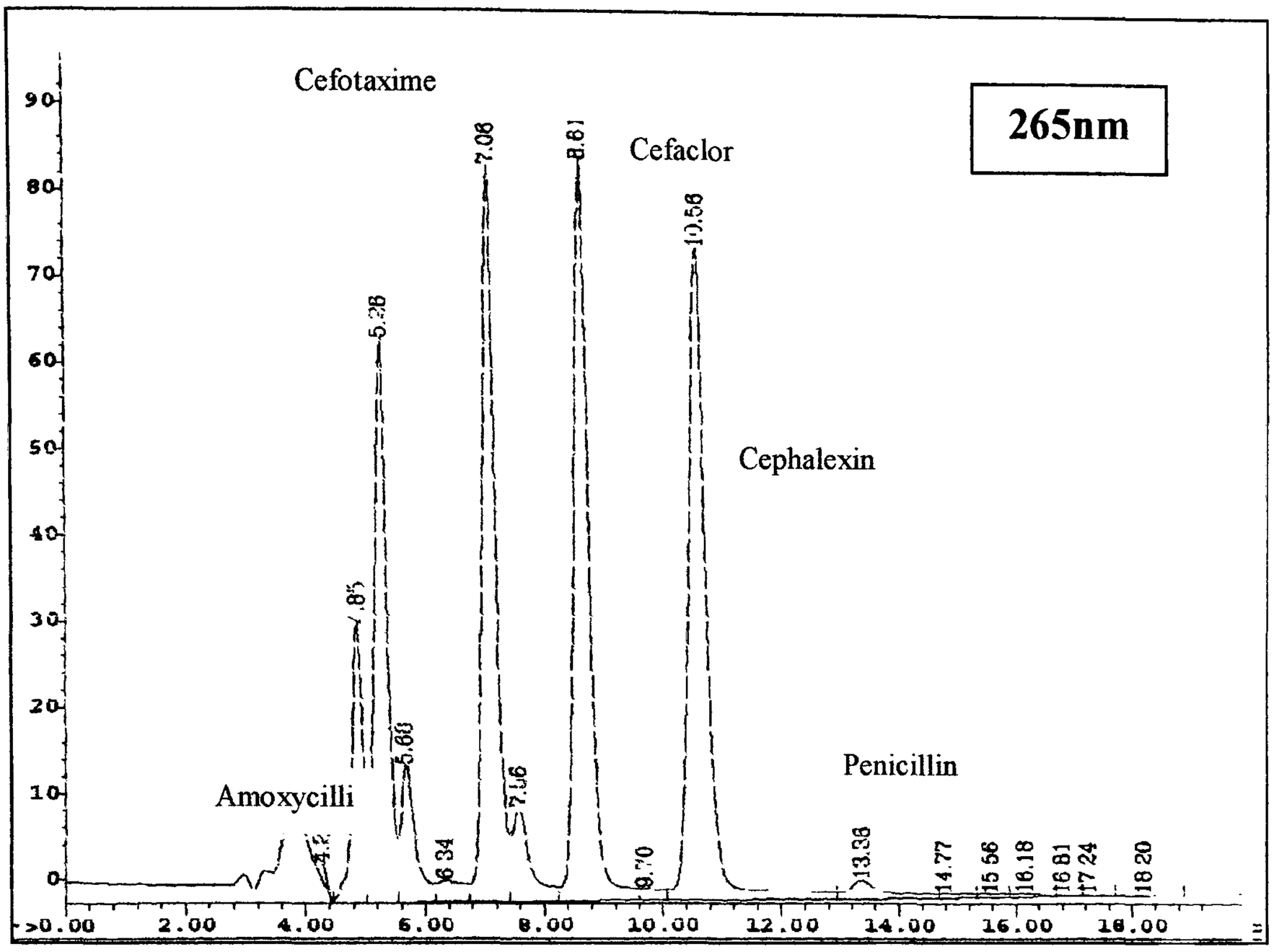


Figure 3.2 Chromatogram of a 10mg/l standard solution of amoxycillin, cefotaxime, cefaclor, cephalexin and penicillin. UV detection 265nm
Peak Height



Unfortunately using this method ampicillin and cephalexin were co-eluted, resulting in one single peak when both were present. The result was that the presence of ampicillin was masked by the cephalexin as this showed much higher absorbance of UV than the ampicillin. Despite this, the method was used because there was clear separation of all the other antibiotics and in addition, it was most likely that any antibiotic detected would be cephalexin. There were two reasons for this, firstly cephalexin is used in much larger quantities medically and secondly, it is more resistant to degradation than ampicillin. It would therefore be the more likely of the two to be present. Other than this problem, this was the best and least time consuming method for the analysis of these particular antibiotics.

Cefotaxime was not included in any analysis until later stages of the project because its importance was not known until the data from hospital prescriptions were analysed.

Once an analysis method was decided upon, it was necessary then to develop an extraction and concentration method that was compatible with this. Because HPLC is based on the differences in polarity that exist between the solution that carries the antibiotics and the solid phase, it was necessary to take this into account when developing a solid phase extraction method, which also worked due to differences in polarity. The aim was to produce a concentrate of the original samples, which contained as much as possible of the antibiotic and as little of any other interfering matter, in a solvent that was then acceptable for HPLC analysis.

The first attempt at SPE carried out used the mobile phase of the HPLC method as an eluent for the columns. This proved to be a very inefficient eluent with only 0.3% of amoxycillin detected, 1.9% of cefaclor and 6.8% of cephalexin detected at expected concentrations of 500mg/l of antibiotics.

It was decided to adapt the Boison *et al.* (1991) method by adding a phosphate buffer to the extraction eluent solution and higher concentration of ACN, thus making the solution less polar. Buffer was also then added to the original mobile phase to make it as similar as possible to the injection solvent containing the sample, with the aim of improving sample detection

In addition samples were spiked with antibiotic after particle removal and refrigerated for six days before analysis. Results can be seen in Table 3.7.

Table 3.7: Results from SPE experiment to determine the extraction efficiency of β-lactams using Boison *et al.* (1991) method. River water and sewage spiked with various antibiotics. All samples extracted on 12/12.

	Extraction Efficiency							
Final max. conc. 20mg/l	**Spiked 6/12**				**Spiked 12/12**			
	Amx	**Cef**	**Cph+Amp**	**Pen**	**Amx**	**Cef**	**Cph+Amp**	**Pen**
FE 240 mg/l	8.7%	2.7%	14.6%	nd	31.7%	32.0%	31.9%	nd
SS 240 mg/l	nd	0.4%	6.2%	nd	25.6%	32.9%	31.5%	nd
RW 240 mg/l	24.0%	11.6%	30.3%	nd	21.3%	31.8%	31.9%	nd

nd: not detectable

It can be seen that there are large differences in the levels of antibiotics detected among the different samples. The differences could have been related to the original bacterial content of the samples before particle removal, as β-lactamas are highly biodegradable. To counteract this filtration, through a 0.2μm filter, was used instead of centrifugation after this experiment to ensure sterilisation of samples.

Due to the relatively low extraction efficiency for the antibiotics, together with the evidence of degradation occurring before analysis, further work was carried out to try to improve extraction efficiency. Acidification of samples would prevent any bacterial degradation of any antibiotic present after their collection. In addition, a change in pH could improve the extraction of the antibiotics from the column.

Chromatography at an acid pH converts penicillins from salt to acid forms, which are much more strongly retained on reversed-phase packing (Moats, 1992)

Table 3.8: Efficiency of extraction of Amoxycillin and Cephalexin during solid phase extraction. Samples are compared to peak areas for spiked samples. Maximum possible concentration in extracts samples 100ug/ml. Acidification was carried out using 2ml 5NHCl per litre, end pH of sample was 2.

	AMOXYCILLIN – 240nm		CEPHALEXIN – 265 nm	
	Acid	Non Acid	Acid	Non Acid
Pre Centrifugation	383 (4.8%)	244 (1.8%)	5923 (46.3%)	2052 (16.1%)
Pre SPE	400 (5.0%)	411 (3.7%)	7373 (57.9%)	3369 (26.5%)

Unfortunately, it was possible to see that the level of amoxycillin that was present in the final eluent and analysed was only 5% of the original antibiotic. This was lower than in the previous experiment, even though the method was the same. Extraction was improved by over half by the addition of acid, so that was not the cause of the reduction. The improvement was all due to reduction of antibiotic loss before solid phase extraction as there was no difference in antibiotic levels detected when antibiotic was added between particle removal and extraction steps.

In the case of cephalexin, extraction of antibiotics was greatly improved, by over 200%, when acid was added to the solution. As can be seen in Table 3.8 the improvements were seen in both stages. There was improvement if acid was added before particle removal, either due to an antibacterial effect which reduced degradation or to a chemical effect which reduced binding of antibiotic to the removed particles. In addition, acidification increased the amount of antibiotic that was recovered from solid phase extraction by over 100%.

The results of all the above work were combined to produce the method detailed in Table 3.9, which was used for the extraction and analysis of β-lactam antibiotics from river water and sewage samples.

Table 3.9: Method for the analysis of B-lactams (adapted from Kirkland *et al.*, 1994)

	Analysis of β-lactam antibiotics
Column	Zorbax SB C8 (3.5μm; 4.6mm internal diameter x 15 cm long)
Guard Column	Zorbax (5μm)
Mobile Phase	20% ACN* 77.5 % Triflouroacetic acid (0.1%aqueous soln.) 2.5% 0.2M phosphate buffer
Run Time (per sample)	20 minutes
Flow rate	0.5ml/min.
Injection volume	20 – 40 μl
UV detection	Penicillins 240nm Cephalosporins 264nm
Solid Phase extraction eluent (sample diluted 1 in 2 with 0.1% TFA before analysis)	40% ACN 55% TFA (0.1%aqueous soln.) 5% 0.2M phosphate buffer

* Acetonitrile

In the final experiment of this project, the eluent solution for the SPE column was modified in an attempt to increase the recovery of antibiotics. This involved using three different combinations of the individual solvents, in succession, to elute the column, in order of least to most polar. The different combinations were as follows, in increasing polarity order:

A. - 60% ACN, 35% TFA and 5% Phosphate Buffer

B. - 40% ACN, 55% TFA and 5% Phosphate Buffer

C. - 30% ACN, 65% TFA and 5% Phosphate Buffer

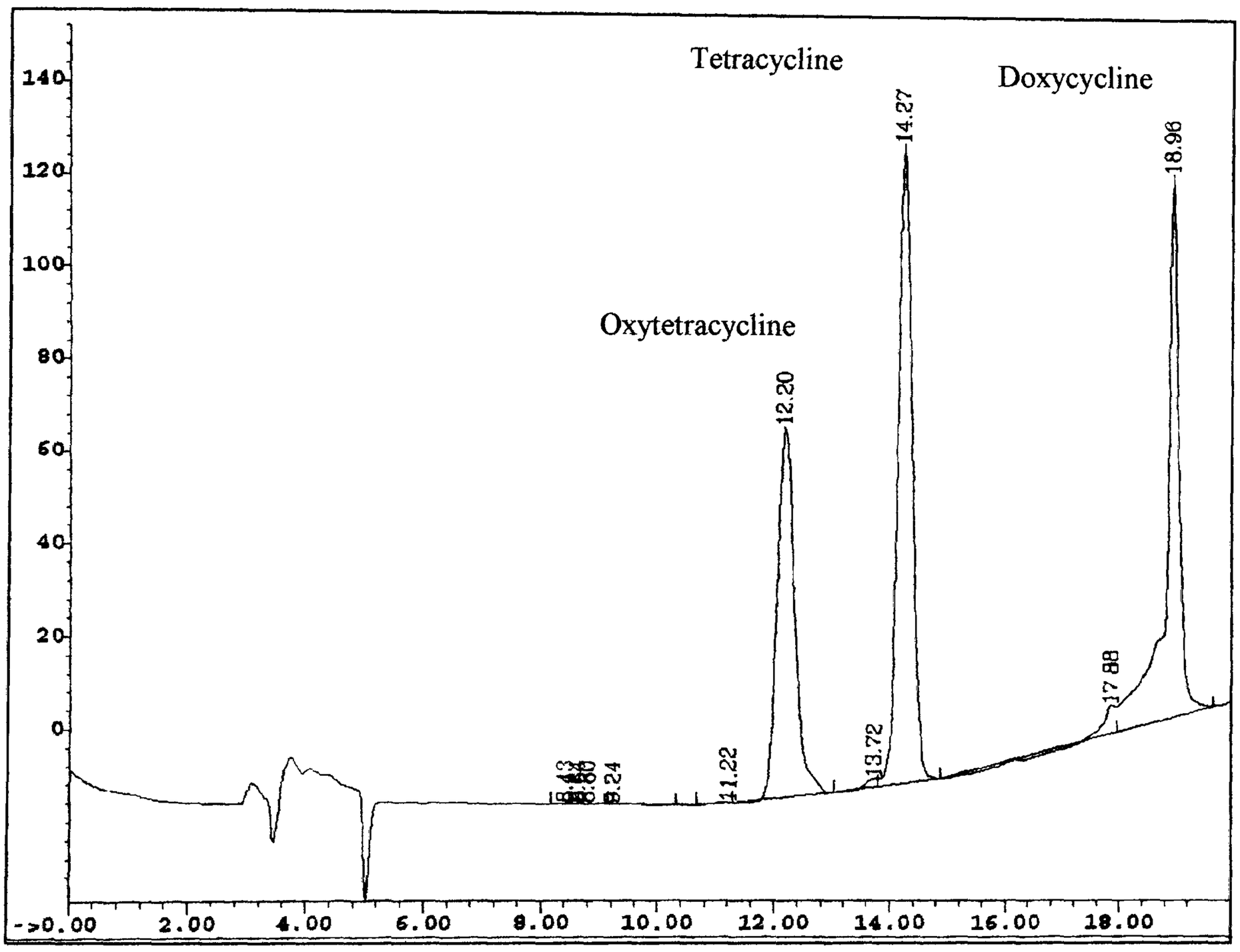
The sample consisted of 10 litres of river water spiked with 0.05mg/l of each of five penicillins. Combination A was by far the most effective at eluting the antibiotics.

3.4 Results

3.4.1 Tetracyclines

Results from the development method of antibiotics showed that the method used, as it stood at the end of the project could easily detect 0.02mg/l concentrations of all three tetracyclines. It is likely that half this concentration could be easily detected if the sizes of the peaks from the samples, as can be seen in Figure 3.3, are considered. This would be an estimated concentration of 10 micrograms of antibiotic per litre of river water.

Figure 3.3: Chromatogram of river water sample (10 litres spiked with 0.02mg/l of oxytetracycline, tetracycline and doxycycline). Injected sample 50µl; (26.9.97).



The above is a typical chromatogram of the three antibiotics. The relative positions of all these always remained the same throughout all the experiments carried out. However, there was some variation in elution times during different experiments. These changes could also sometimes be seen throughout a single experiment (the run time for a whole set samples for an experiment, usually around 20 samples, was around 10 hours).

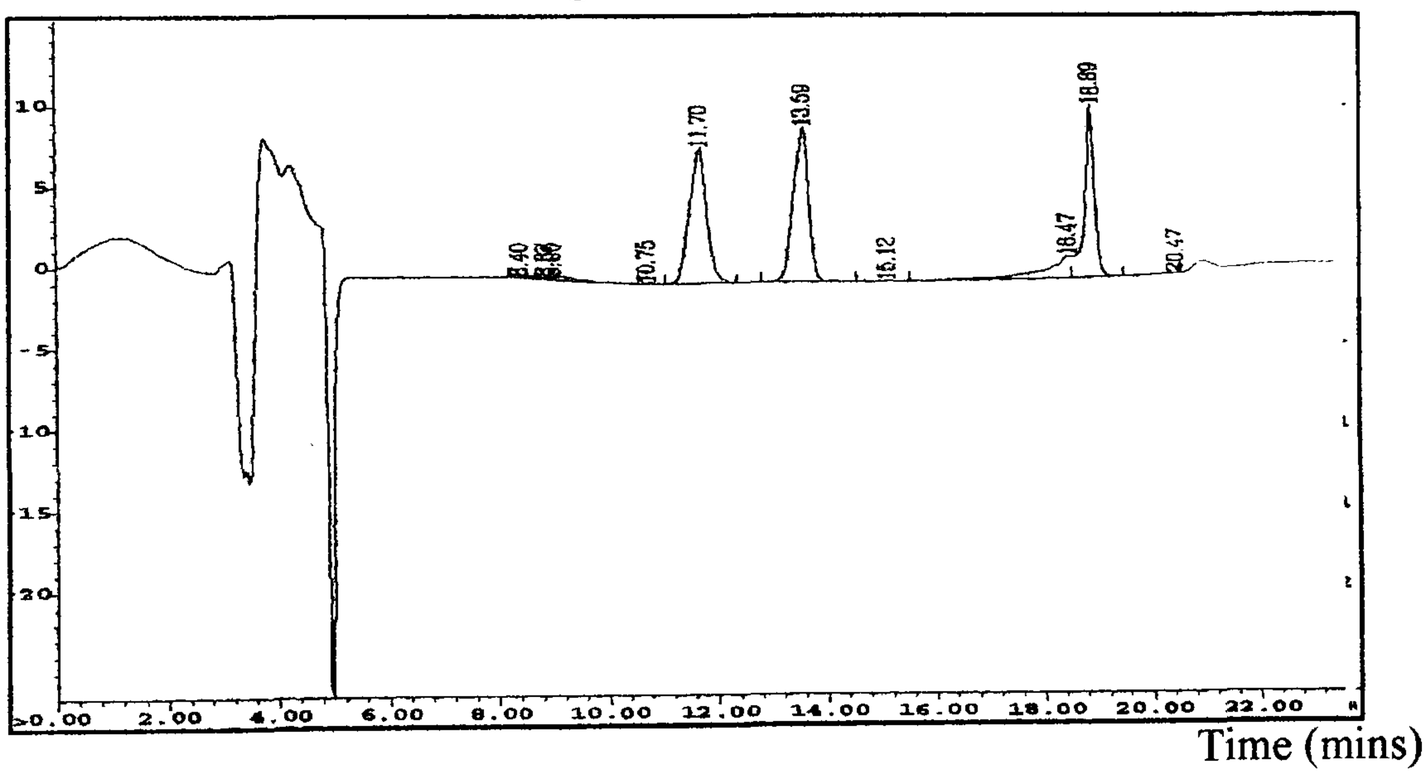
Examples of these changes can be seen in Table 3.10, and figures 3.4a and 3.4b. There are various possible explanations for these variations in elution times. Changes in environmental temperature would affect elution times. Small changes in the run of the solvent gradient, due to a mechanical fault, would also affect these. Attempts were made to control both of these, though they were not entirely successful. This did not pose a problem when analysing chromatograms of standards or spiked samples. It was, however, a problem when interpreting the results from environmental samples.

Table 3.10: Examples of some of the variations in elution times for the three tetracycline antibiotics

Experiment Date	**Elution Times in Minutes**		
	Oxytetracycline	**Tetracycline**	**Doxycycline**
1.11.96	11.82 - 12.60	13.66 – 14.82	
30.8.97	11.74 - 12.09	13.67 – 13.90	18.82 - 18.97
26.9.97	11.70 – 12.20	13.80 – 14.27	18.98 – 19.18
12.11.97	12.24 – 13.20	14.27 – 15.17	19.34 – 19.54
13.11.97	12.44 – 12.73	14.32 – 14.71	19.22 – 19.38

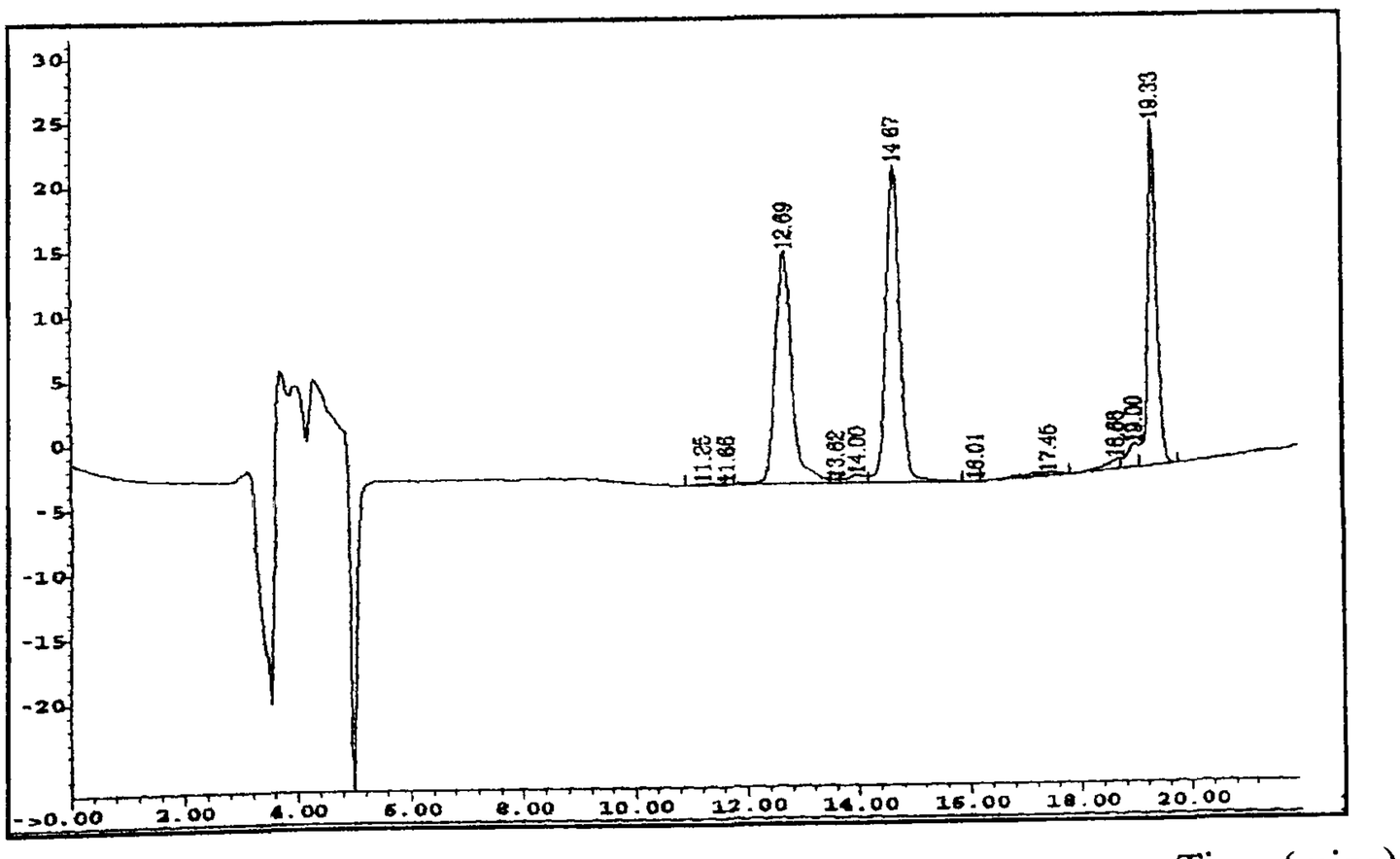
Figure 3.4: Chromatogram from two different experiments. Samples were standard solutions of 1mg/l of tetracyclines.

Figure 3.4a



Peak Height

Figure 3.4b



Various experiments were carried out during the project, which involved increasing amounts of samples. Initially both sewage works were sampled, with low volume samples analysed (100ml). Samples were filtered and SPE carried out results. A typical chromatogram of a spiked sample (0.1mg/l of antibiotics) and unspiked sample of Chertsey STW settled sewage sample can be seen in Figure 3.5 and 3.6.

Figure 3.5: Example of a spiked chromatogram. 100ml of Chertsey sewage sample spiked with 0.1mg/l of antibiotic

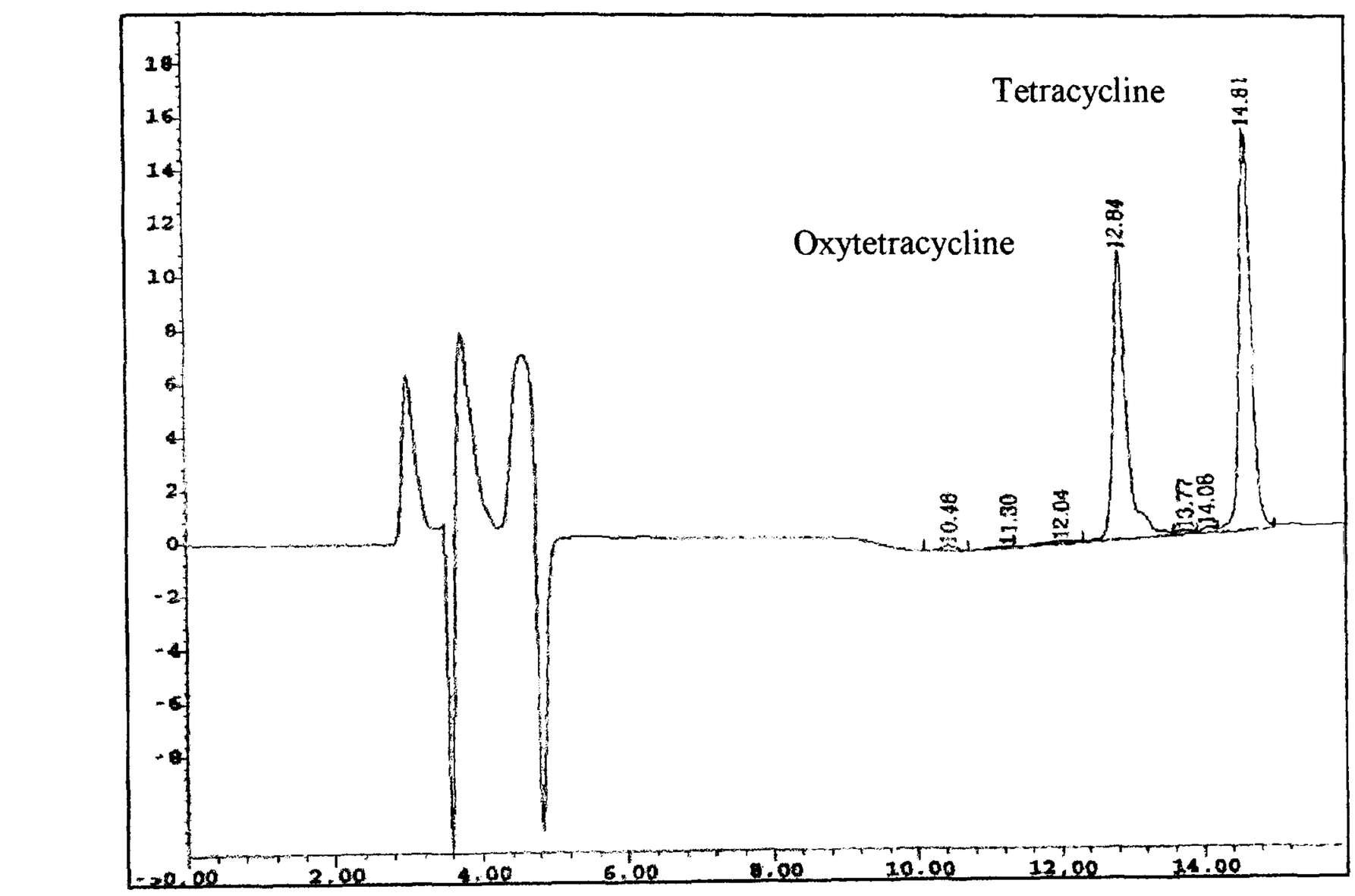
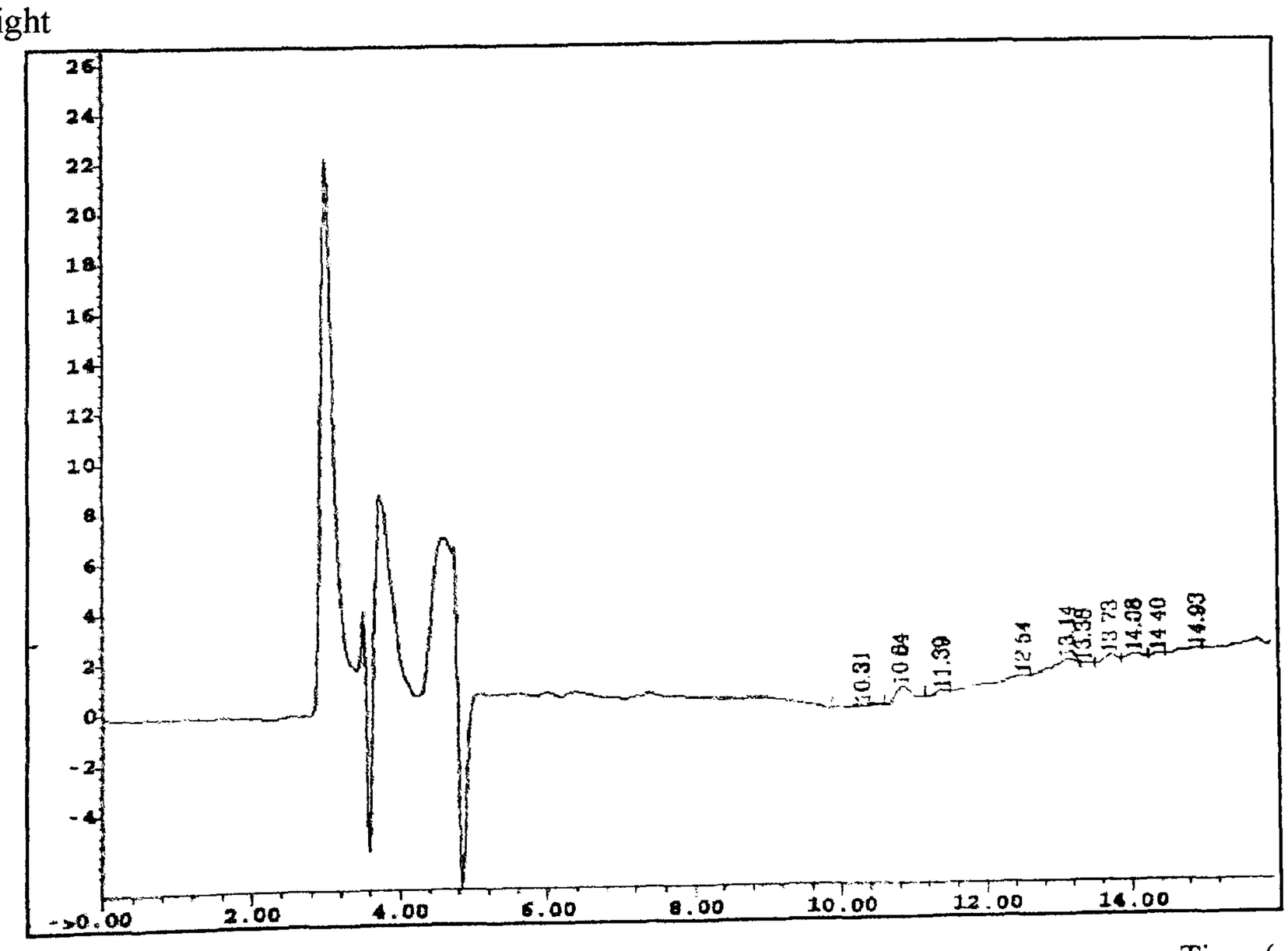


Figure 3.6: Chromatogram of 100ml sample of Chertsey settled sewage sample.



The first chromatogram is of a 0.1mg/l spiked settled sewage sample, the second is unspiked. As can be seen from comparing both chromatograms they are very similar, with similar unknown peaks eluting at the same time in both samples. The two antibiotic peaks in the spiked sample are not present in any form in the unspiked one. Results from the other samples were similar: no antibiotic was detected.

It was decided that the most important factor in determining whether antibiotics, if present, would be detected was the size of sample analysed. Increasing the water sample resulted in a large increase in the time required for sample concentration and extraction. Because of this, it was necessary to reduce the number of samples taken. For this reason, it was decided to concentrate on the samples whose content would most directly affect the environment. This was the final sewage effluent, as this was what was released into river waters. In the first experiment final effluent from Chertsey STW was analysed. Chertsey was the STW that received sewage from St. Peters General Hospital, which meant that it was the site that antibiotic was most likely to be detected at as Bracknell STW did not receive sewage from any hospitals. Results from this experiment can be seen in Figures 3.7, 3.8 and 3.9. One 3 litre unspiked sample was analysed and extracted, together with two spiked samples to which 0.5mg/l of each antibiotic was added. One sample was 2.25 litres and the other one was 1.6 litres.

Figure 3.7 shows how clearly all antibiotics were separated and identified in the spiked 2.25 litre sample. Figure 3.8 shows how concentrations as low as 0.005mg/l of antibiotic were clearly detectable when added to the 3 litre extractant before injection for HPLC analysis. Figure 3.9 shows how the 3 litre sample of sewage effluent showed no peaks at all and therefore no antibiotic was present at more than 0.005mg/l concentration in the final eluted sample.

Samples, once diluted after extraction, were 20ml in volume. This 20ml sample, with 0.005mg/l of antibiotic, would contain 0.1μg of antibiotics in total. In a 3 litre sample that would be (taking into account an 80% extraction efficiency) the equivalent of an antibiotic content of 0.04μg/l in the final effluent. This gives an indication of the limit of detection for tetracyclines that might apply in the case of sewage samples.

Figure 3.7: Chromatogram of a 2.25 l extracted sample (Wash 1) of Chertsey final effluent spiked with 0.5mg/l of each antibiotic. 30.8.97.

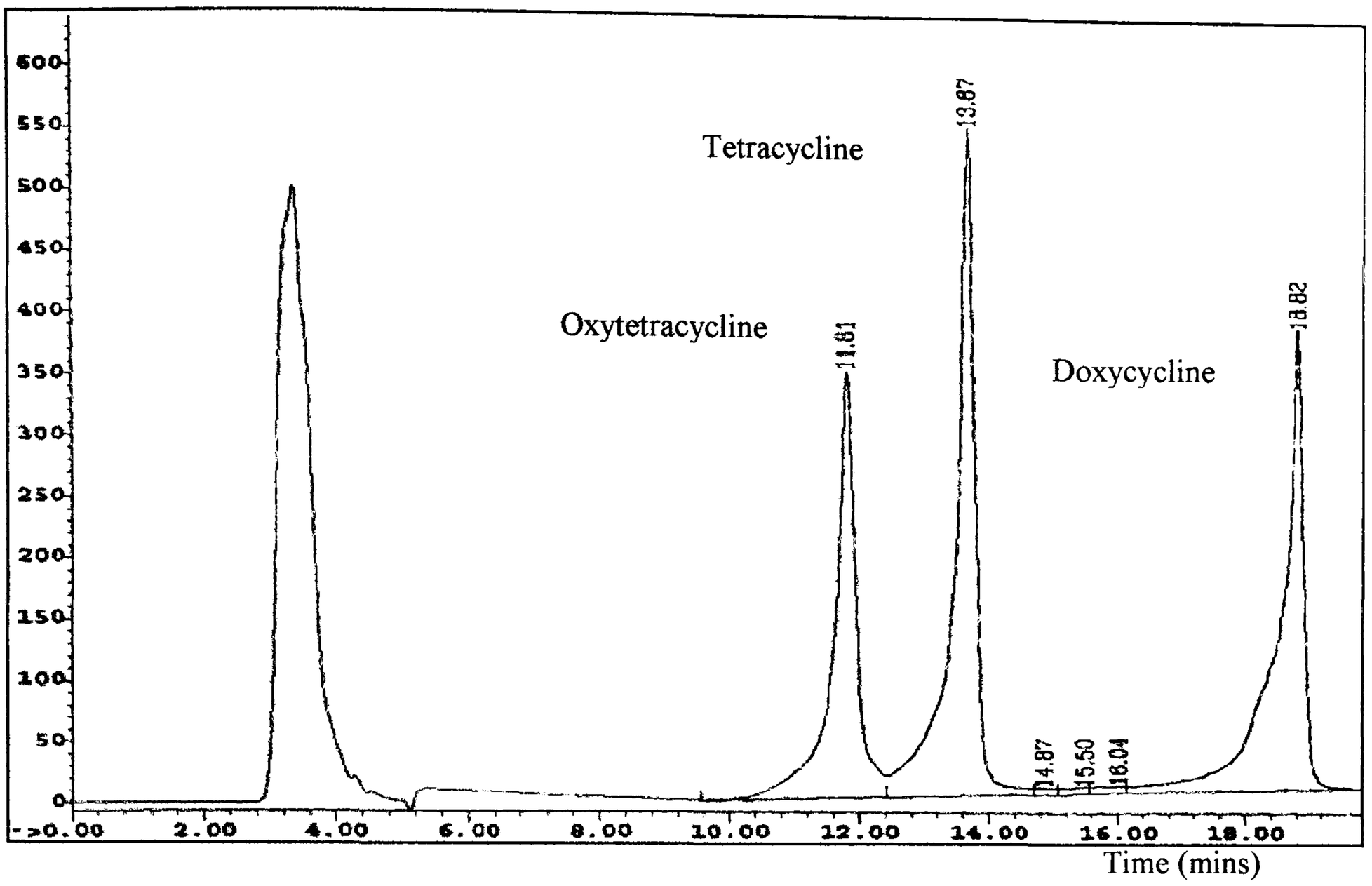


Figure 3.8 Chromatogram of extracted sample (Wash 1) to which 0.005mg/l of each antibiotic was added before analysis. 30.8.97

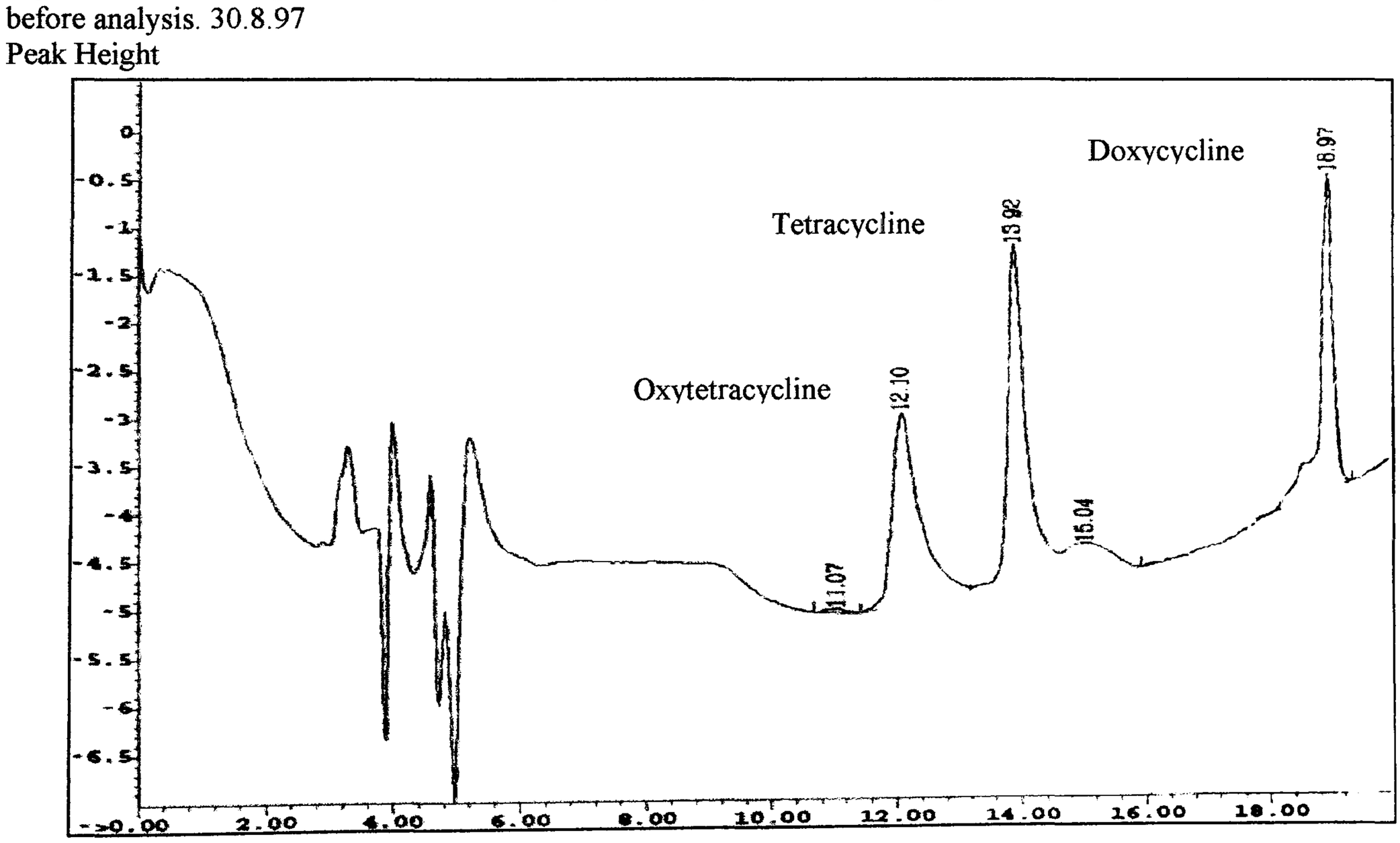
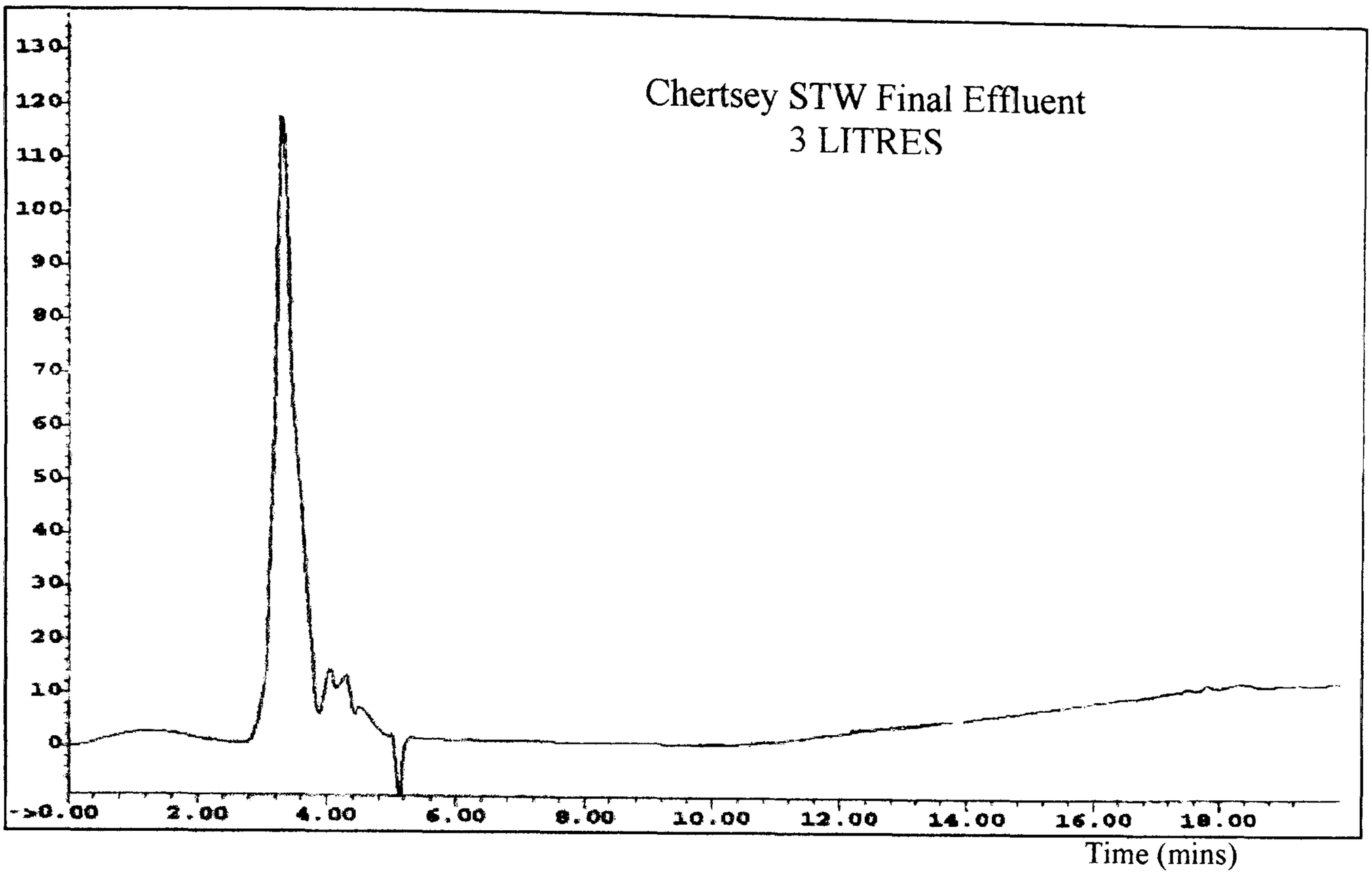


Figure 3.9: Chromatogram of a 3 litre sewage sample (Wash 1) from Chertsey STW. 30.8.97
Peak Height



Results from the analysis of all the samples gave an indication of the suitability of this method for detecting the low levels of antibiotics that were used as spikes in this experiment.

Table 3.11. Extraction efficiency of tetracyclines

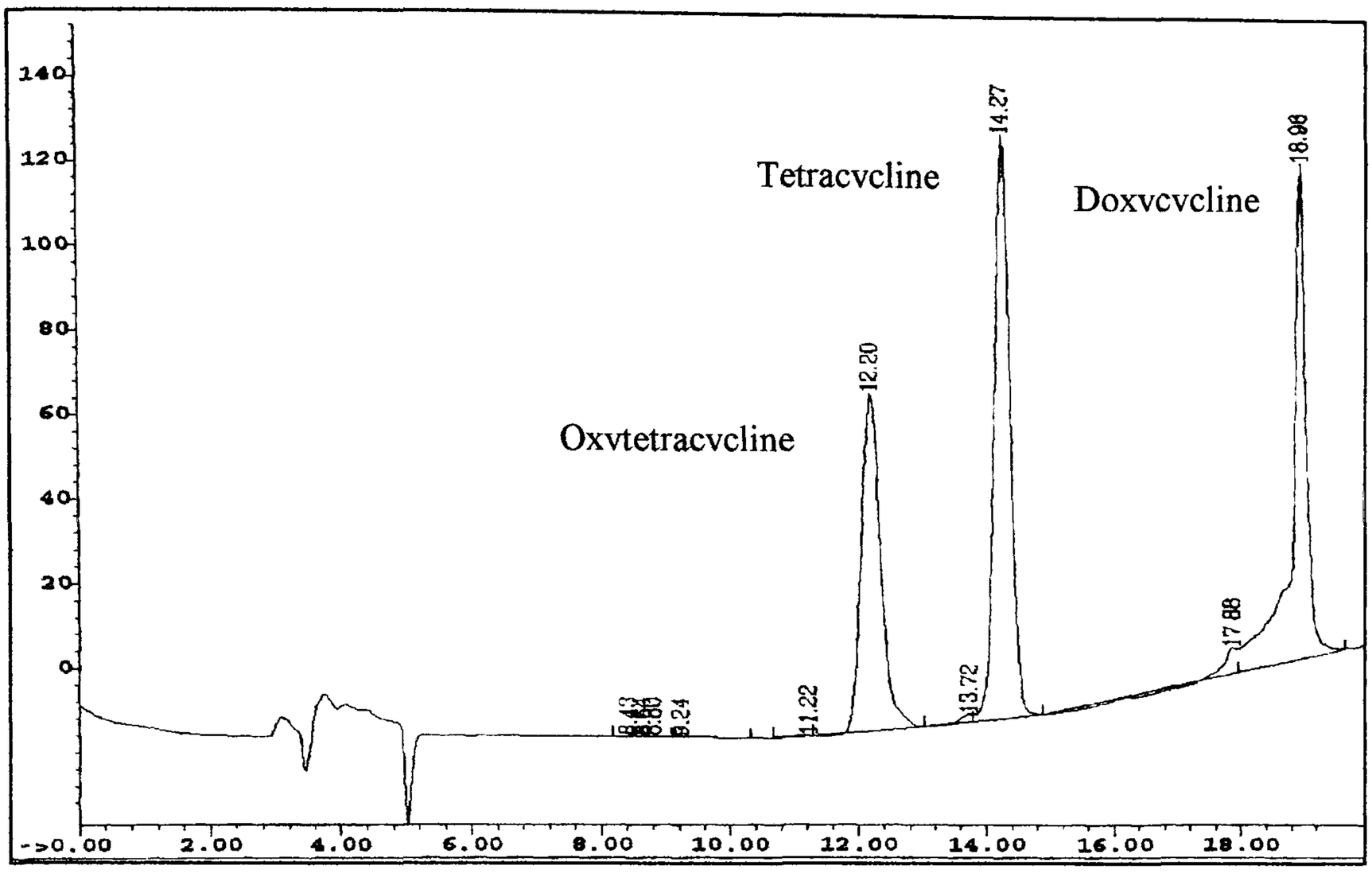
Antibiotic	Extraction efficiency (%)	
	Sample 1 (2.25 litres)	Sample 2 (1.60 litres)
Oxytetracycline	73.1	64.5
Tetracycline	81.8	68.3
Doxycycline	69.0	63.1
Sample antibiotic concentration - 0.5mg/l of each antibiotic		

It can be seen in Table 3.11 that the extraction efficiency of this method remained high and was in the same range as when extracting much smaller samples of 50ml (see Table 3.4).

Following these results the sample size was increased again, this time to 10 litres and a 3M Empore membrane used for extraction. There would be great difficulty in pre-filtering and then passing such a large volume of sewage sample through the membrane. Time was limited, so it was decided to concentrate on river water samples, as this was the part of the ecosystem in which it was important to know if antibiotics were present. Two 10 litre samples of river water were collected from the Thames at Medmenham, and one was spiked with 0.02mg/l of each of the antibiotics. Extraction and analysis was then carried out as usual, with the addition of a third 5ml wash being passed through the membrane during extraction.

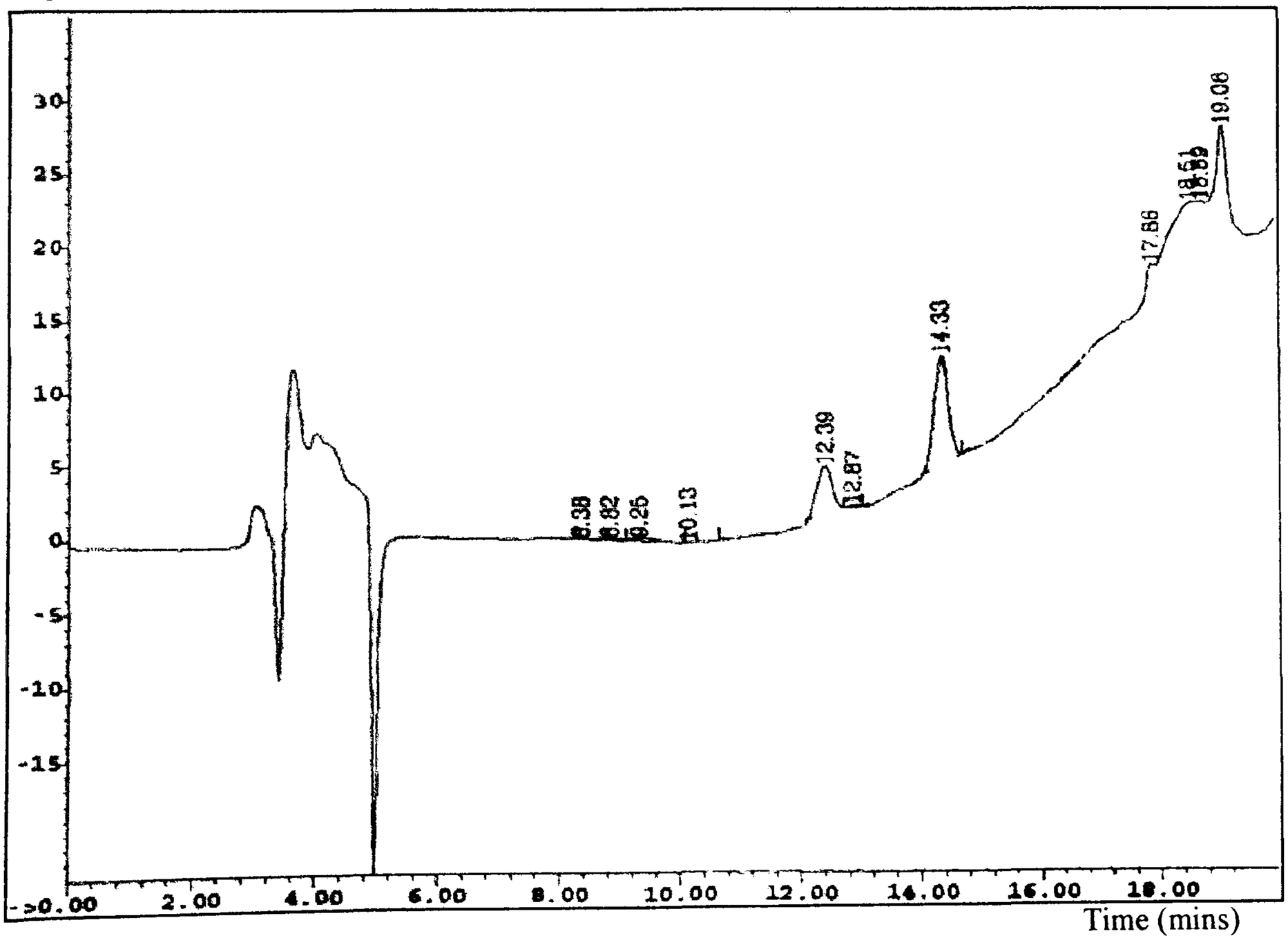
The chromatogram for the first washes of both samples can be seen in Figures 3.10 and 3.11. It can be seen that the antibiotic is clearly detectable in the spiked sample.

Figure 3.10: Chromatogram of a 10 litre water sample spiked with 0.02mg/l of each antibiotic



In addition, it can be seen that there are corresponding peaks in the unspiked sample that appear to indicate the presence of Oxytetracycline (12.39 minutes), Tetracycline (14.33 minute) and Doxycycline (19.06 minutes).

Figure 3.11: Chromatogram of a 9 litre unspiked river water sample showing three possible antibiotic peaks.



All three antibiotics were detected in all three washes of the spiked sample. Between 50 and 60% of the total amount of each antibiotic added (200µg in 10 litres) was present in first wash. Between 20 and 25% of each antibiotic was present in the second wash and between 2 to 3 % of each antibiotic was present in the third wash. Between 20% and 30 % of the antibiotics were not recovered. Figures for each antibiotic can be seen Table 3.12.

Table 3.12. Extraction of tetracyclines from a 10 litre sample of river water

	% Antibiotic Recovered			Total Antibiotic Recovered
	Wash 1	Wash 2	Wash 3	Spike - 0.2mg
Oxytetracycline	49.39	19.74	2.52	0.143 mg
Tetracycline	59.16	21.20	2.86	0.166 mg
Doxycycline	55.66	26.86	2.67	0.170 mg

The three peaks in the unspiked water sample appear to indicate the presence of all three antibiotics. The sample was run again after washing the columns and passing through various blank samples. This was to ensure that the peaks seen were due to the actual presence of the antibiotic in the sample and not due to bound antibiotic from previous samples being eluted. This had not been a problem in previous experiments. The same peaks, with the same areas were eluted the second time. This was proof that the antibiotic seen was actually present in the samples. Antibiotic was detected in all three washes for the river water sample in diminishing quantities. Because the amounts were so small in the last two washes it was not possible to use these to calculate levels present. Results from the first wash (Figure 3.7) were used to calculate the levels present in the original water sample. Results can be seen in Table 3.13

Table 3.13. Levels of tetracyclines detected in a 9 litre river water sample.

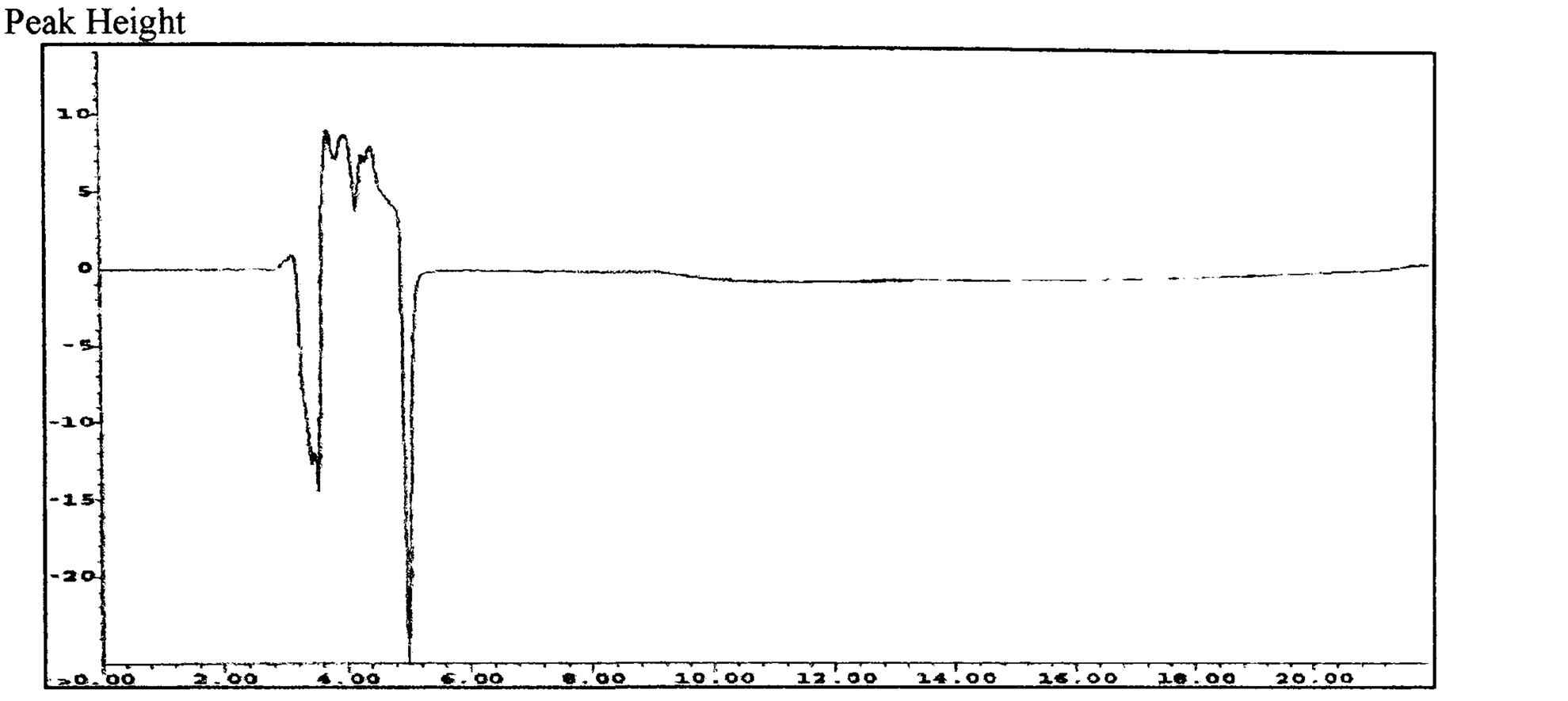
	Total Antibiotic in Sample - Wash 1 (µg)	Estimated Total Antibiotic in River Water Sample (µg)	Estimated Antibiotic Concentration (µg/l) in River Water Sample
Oxytetracycline	2.24	4.53	0.50
Tetracycline	6.25	10.57	1.17
Doxycycline	2.92	5.25	0.58

The resulting concentrations of micrograms per litre that were calculated were so low that it was necessary to consider the possibility that the antibiotics detected were due to cross contamination having occurred in the laboratory. Another factor was the presence of all three antibiotics in the spiked sample also being present in the unspiked sample, though the proportions were different.

The next experiment was carried out using all new tubing to the connect pumps to the sample, with all containers and filtering equipment acid-washed to remove any trace of antibiotics. In this experiment 8 litres of river water were concentrated and analysed for tetracyclines. Due to time constraints, no spiked sample was analysed at the same time.

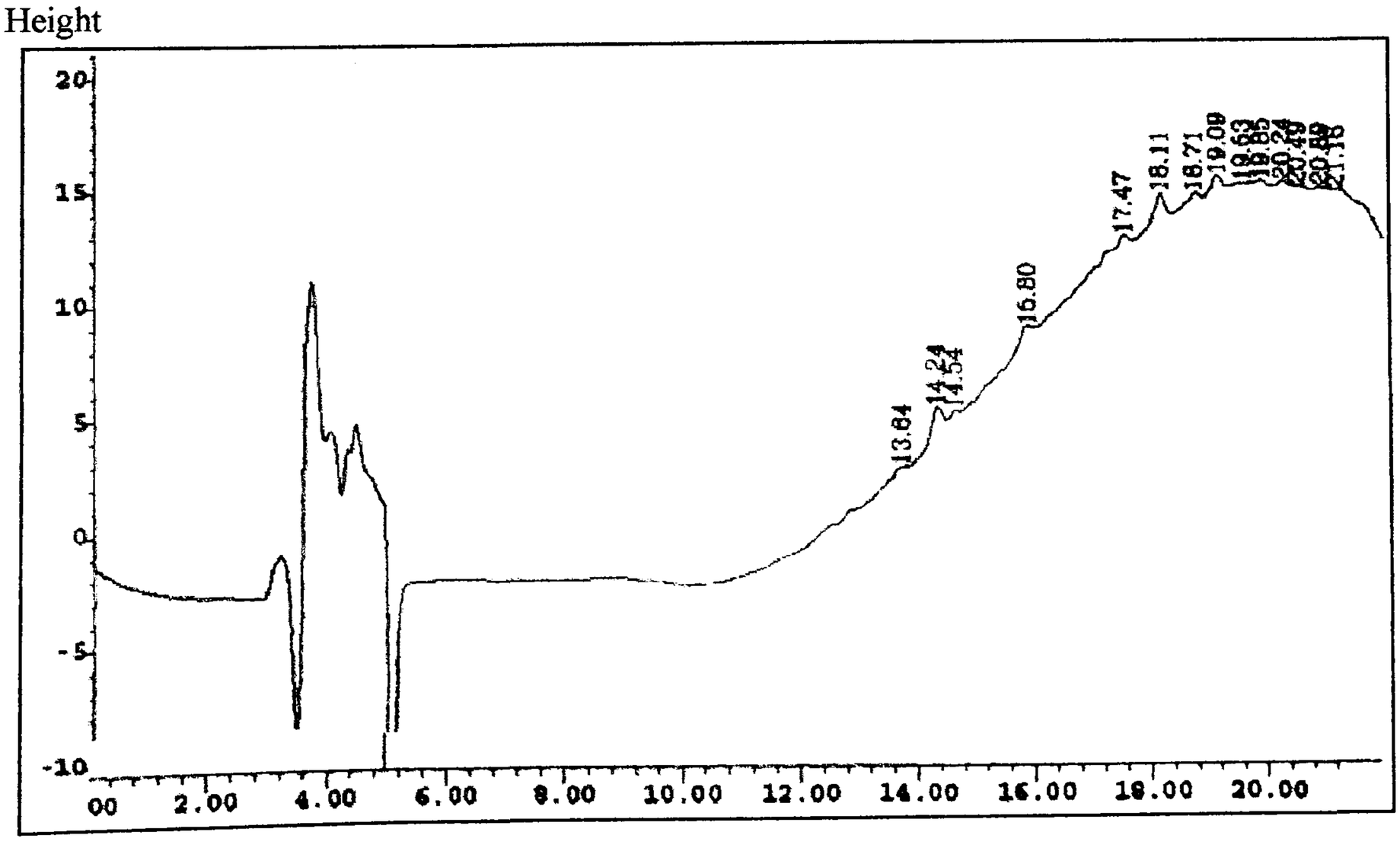
The chromatogram for a blank sample can be seen in Figure 3.12. This showed that there was no antibiotic present on the column from previous experiments that might be eluted when further analyses were carried out and produce false peaks.

Figure 3.12: Chromatogram of a blank run before river water samples on HPLC column



The chromatogram in Figure 3.13 shows the results from the analysis of an 8 litre sample of river water. This again showed, as in the previous experiment, that there are peaks present in the sample. If this chromatogram is compared with that of the 1mg/l standard (Figure 3.14), it can be seen that many of the same peaks are present in both. Calculated concentrations of antibiotics can be seen in Table 3.14

Figure 3.13: Chromatogram of river water samples on HPLC column

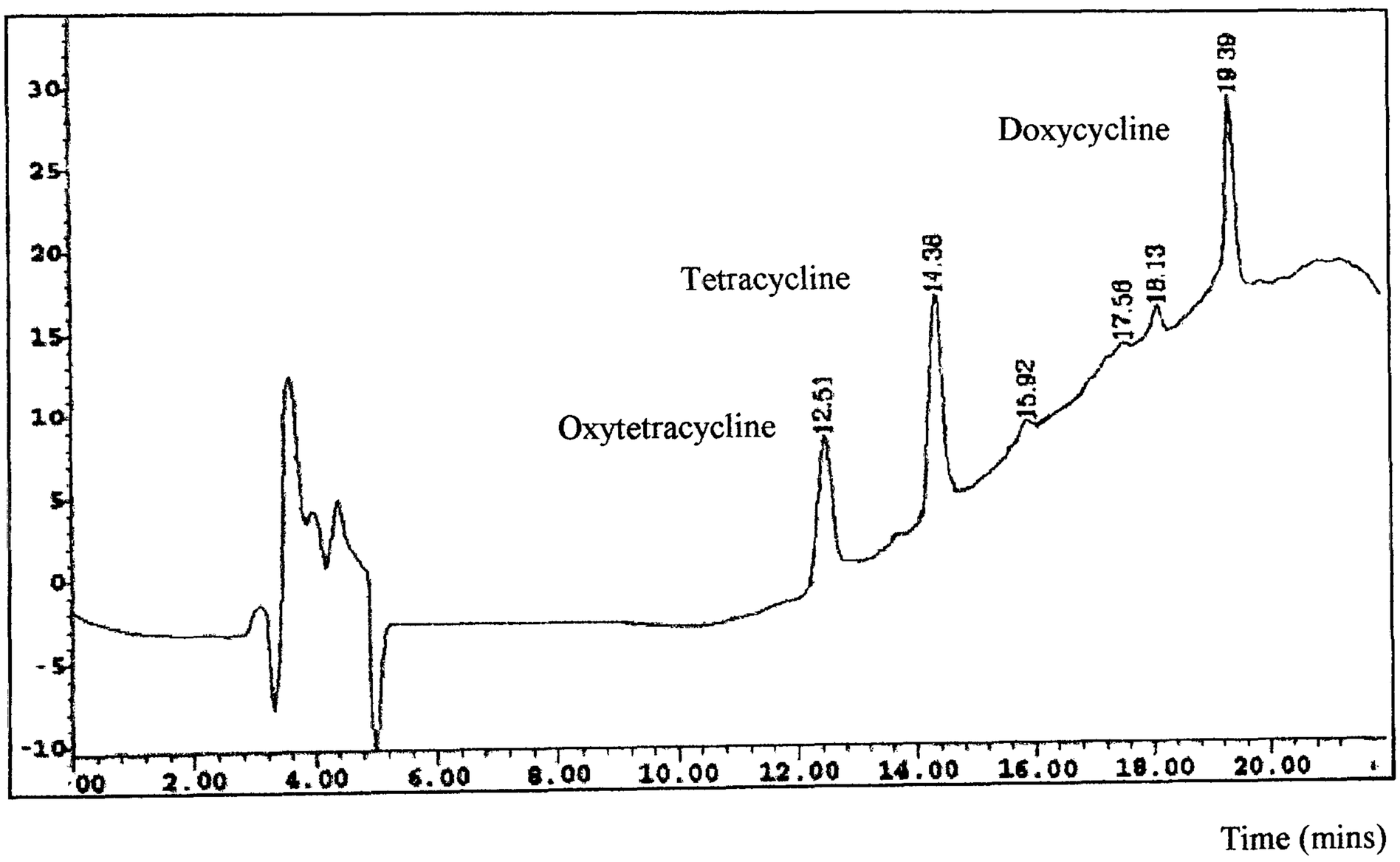


The antibiotic peaks (Oxytetracycline, 12.51; Tetracycline, 14.36 and Doxycycline 19.39) are present, though eluting slightly earlier in the river water sample (Oxytetracycline 12.5; Tetracycline 14.24 and Doxycycline19.09).

Table 3.14. Levels of tetracyclines detected in an 8 litre sample of river water. 14.11.97

	Total Antibiotic in Sample - Wash 1 (µg/l)	% Extraction Efficiency	Estimated Total Antibiotic in River Water Sample (µg)	Estimated Antibiotic Concentration (µg/l) in River Water Sample
Oxytetracycline	0.53	49	1.09	0.14
Tetracycline	0.23	59	0.40	0.05
Doxycycline	0.34	56	0.60	0.08

Figure 3.14: Chromatogram of an 8 litre river water extracted sample spiked with 1mg/l of each tetracycline.



There were also further peaks present in the samples that could represent other, unknown, compounds present in the river water. Some, like those at 17.55 and 18.13 minutes, appeared in many of the large volume samples analysed.

3.4.2 Penicillins

3.4.2.1. River Sediment Experiments

One of the first experiments carried out after the method for penicillins was decided upon was the extraction (with no concentration step) of penicillin antibiotic added to sterilised sediment samples (using formaldehyde). An extraction method by Boison *et al.* (1991) was used to extract the antibiotics. Results from this experiment were unexpected because there was very little antibiotic to be found in the samples. Large peaks were visible in the samples but not at the expected points. Because of this, the experiment was repeated overnight to determine whether these peaks were due to the presence of antibiotics or not. In addition, extraction was carried out using two methods, the above one and a second one using only of water.

Table 3.15. Peak areas from chromatograms of extraction experiment. (5g of sediment extracted, supernatant injected directly)

	Amoxycillin				Cephalexin			
	Amox		Unknown		Ceph.		Unknown	
	Time	Area	Time	Area	Time	Area	Time	Area
Experiment 1 (7 Days)								
Amox Spnt.	5.3	207	6.5	15201				
Ceph Spnt.					9.09	260	10.12	45046
Boison Extraction Method								
Amox Sed.	5.2	4.6	6.6	1003				
Ceph Sed.					9.1	20.4	10.6	2053
Experiment 2 (1 Day)								
Supernatant	5.2	156.4	6.39	21810	9.05	151	10.23	41406
Spnt. (spiked)	5.2	1622			9.07	2189		
Boison Extraction Method								
Sediment	5.2	15.5	6.59	2752	9.14	451	10.53	8316
Sed. (spiked)	5.2	1479			9.07	2018		
Water Extraction Method								
Sediment	5.2	13	6.62	1478	9.17	40	10.59	3648
Sed.(spiked)	5.2	770			9.1	1300		

The results in Table 3.15 show that the peaks are indeed likely to be some form of the two antibiotics added as the same peaks occurred in both sets of experiments. It can also be seen that the majority of the antibiotics remained in solution rather than binding to the soils. In addition the extraction method by Boison *et al.*(1991) and the use of only water produced the same result, with the new peaks present in both samples. This meant that the extraction procedure was not the cause. It can be seen that washing with water alone results in a reduced extraction of the antibiotics.

3.4.2.2. Sewage Analysis

The first experiment carried out using sewage samples was not successful, with only a small percentage of the spiked antibiotic being eluted.

Two 500ml samples of water from each site were spiked with 100mg/l and 1mg/l each of amoxycillin, cefaclor, cephalexin and penicillin. The following sites were sampled: Bracknell STW – Upstream; Bracknell STW – Settled sewage (SS); Bracknell STW – Final Effluent (FE); Chertsey STW – Settled Sewage (SS); Chertsey STW - Final Effluent (FE).

No peaks were detected in the unspiked samples that corresponded with the peaks in the spiked samples. Extraction efficiency was very low, and the method was subsequently changed to improve this, however both amoxycillin and cephalexin were detectable at a concentration of 1mg/l. They were therefore not present, at this concentration, in the samples analysed

Some of the samples showed one distinguishable peak, largest in the Bracknell and Chertsey settled sewage, which was eluted at 6.79 minutes. An example of this can be seen in Figure 3.15. This peak was only just detectable in the smaller spiked samples, but it was there.

Figure 3.15: Chromatogram of a 500ml sample of Chertsey SS extracted and analysed for β - lactams.

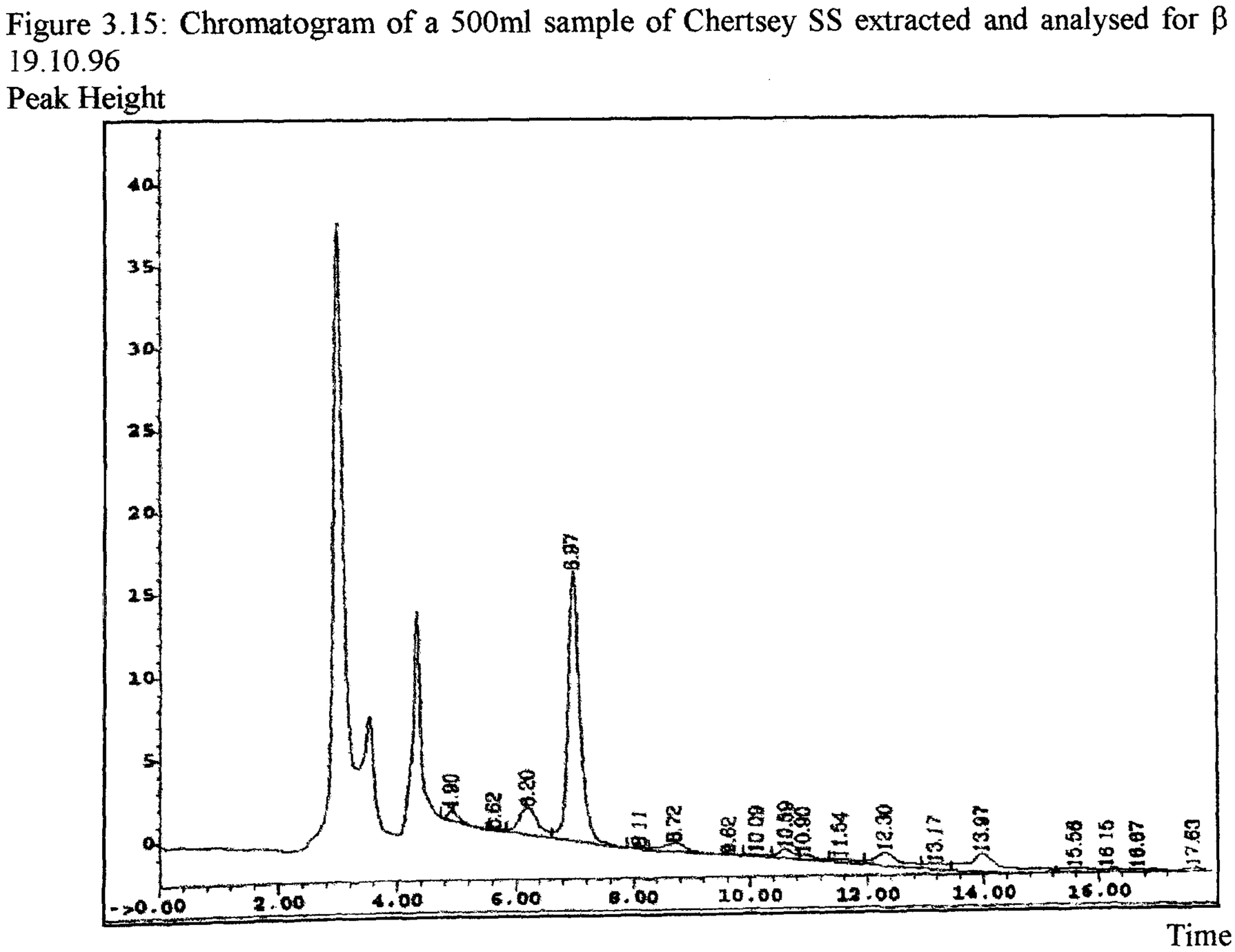


Table 3.16: Elution time and area of unknown peak found in 500ml samples from both Bracknell and Chertsey sites.

Sampling Point	Elution Time	Peak Area
Bracknell STW – Upstream	6.97	11.8
Bracknell STW – SS	6.97	233
Bracknell STW – FE	6.97	67
Chertsey STW - SS	6.97	1004
Chertsey STW - FE	6.96	12.4

This peak, did not correspond to any of the antibiotics with which the samples were spiked (amoxycillin, cefaclor, cephalexin and penicillin). Peak area and elution times are detailed in Table 3.16. However, it did have a very similar elution time to the peak that was seen during both soil experiments and that seemed to be associated with the presence of amoxycillin in the sample.

The next experiment was similar but used modified eluent for SPE. In this experiment sewage from Bracknell STW only was analysed. There had been a pollution incident at Chertsey STW and sampling was not possible. Bracknell river water, settled sewage and final effluent were collected. 50 ml samples of each were spiked with 1mg/l of β-lactams, filtered and refrigerated for six days. Unspiked samples were also refrigerated, at day 6 a further set of 50ml samples were spiked. Analysis of all these samples plus the unspiked sewage samples was then carried out. These consisted of 400ml each of final effluent and upstream river water, and 300ml of settled sewage.

3.17: Detection of antibiotics in spiked sewage samples. Spiked on day 0 and day 6. Analysed on day 6

Final max. conc. 20mg/l	**Spiked 6/12**				**Spiked 12/12**			
	Amx	**Cef**	**Cph+Amp**	**Pen**	**Amx**	**Cef**	**Cph+Amp**	**Pen**
FE mg/l	1.7	0.5	2.9	nd	6.3	6.4	6.4	nd
SS mg/l	nd	0.1	1.2	nd	5.1	6.6	6.3	nd
RW mg/l	4.8	2.3	6.1	nd	4.3	6.4	6.4	nd

The first difference is between the samples that were analysed a week after antibiotics were added and the ones where antibiotic was added and extracted the same day. In all three samples, there was a reduction in antibiotics. This reduction was not the same for the three samples. The greatest reduction was seen in the settled sewage sample, with most of the antibiotic undetectable, followed by the final effluent. The river water showed only a slight reduction in antibiotics. This pattern could be related to the levels of bacteria present in the original samples.

Samples were centrifuged to remove particulates and bacteria before antibiotics were added. However, this would not be sufficient to ensure that the samples were sterile and bacterial activity could still be expected to be present. It can therefore be concluded that the antibiotics could have been degraded. This could also be the case of any antibiotics that might have been in the samples when they were collected.

Another interesting factor was the appearance of two peaks in the samples that had been refrigerated for 6 days that were not present in the later samples. These peaks were only present in the river water and sewage samples, not in the sterile standards that were also refrigerated for six days. Chromatograms showing these differences can be seen in Figures 3.16 and 3.17. It can be seen that in addition to the levels of antibiotics being reduced over six days, there are two additional peaks at 6.14 and 6.84 minutes. This pattern is repeated in the three samples from different sources. These could be breakdown products of the antibiotics added.

Figure 3.16: Chromatogram of 50ml extracted Bracknell FE sample to which 1mg/l of amoxycillin, cefaclor and cephalexin were added before extraction.

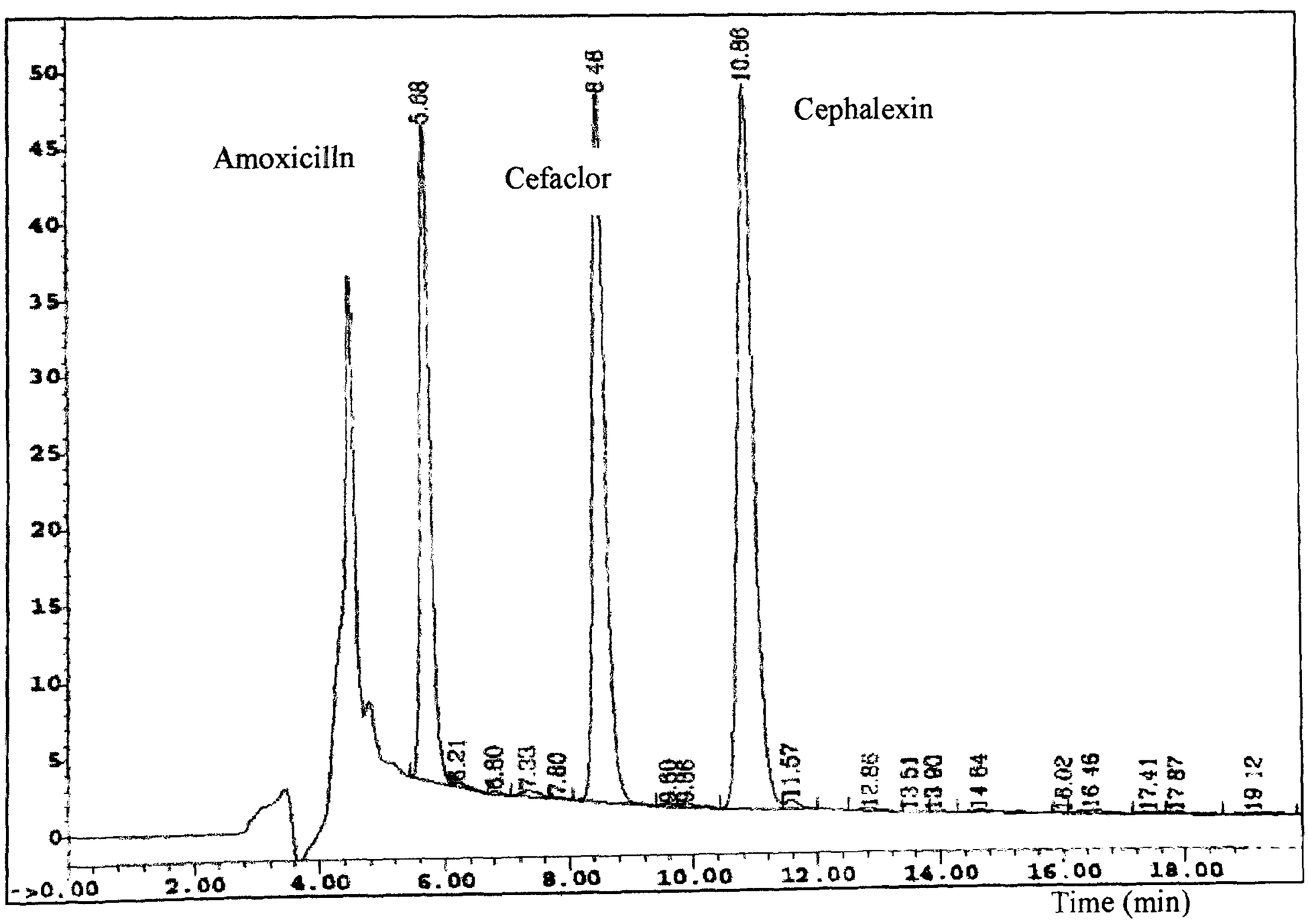
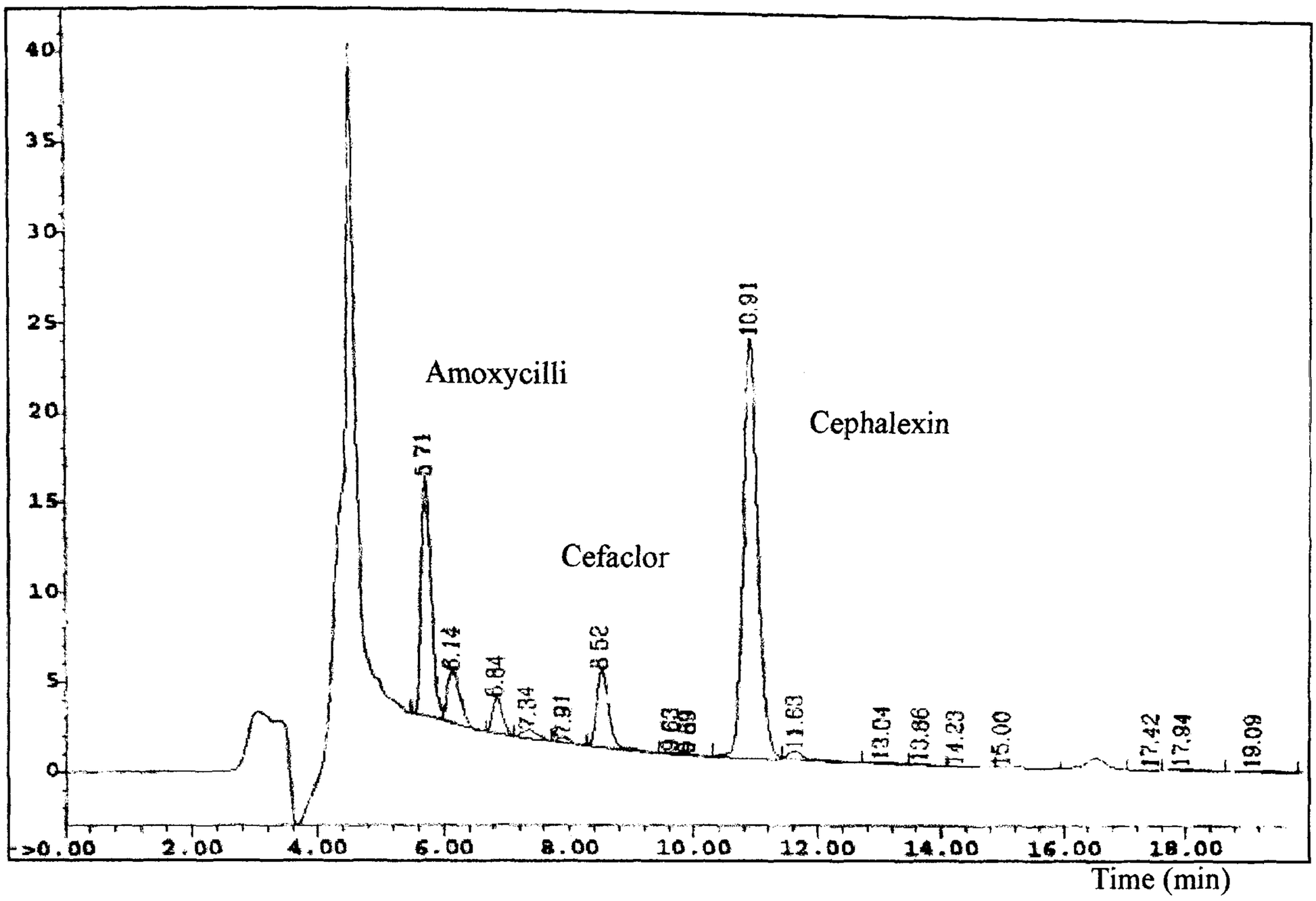


Figure 3.17:Chromatogram of 50ml extracted Bracknell FE sample to which 1mg/l of amoxycillin, cefaclor and cephalexin were added, refrigerated for 6 days and then extracted.



Results from the three unspiked collected taken showed different results. The river water and final effluent samples (400 ml of each), Figures 3.18 and 3.19, showed small peaks that could possibly have been traces of antibiotics. However, as can be seen in Figure 3.16, none of them corresponds to the elution times observed for the antibiotics in the spiked samples. There was however, in the settled sewage sample, Figure 3.20, a peak at 6.10 minutes, which could be the same peak seen in the previous experiments. This may be related to the presence of one of the antibiotics, possibly a breakdown product.

Figure 3.18: Chromatogram of a 400ml sample of Bracknell River water. 6.12.96

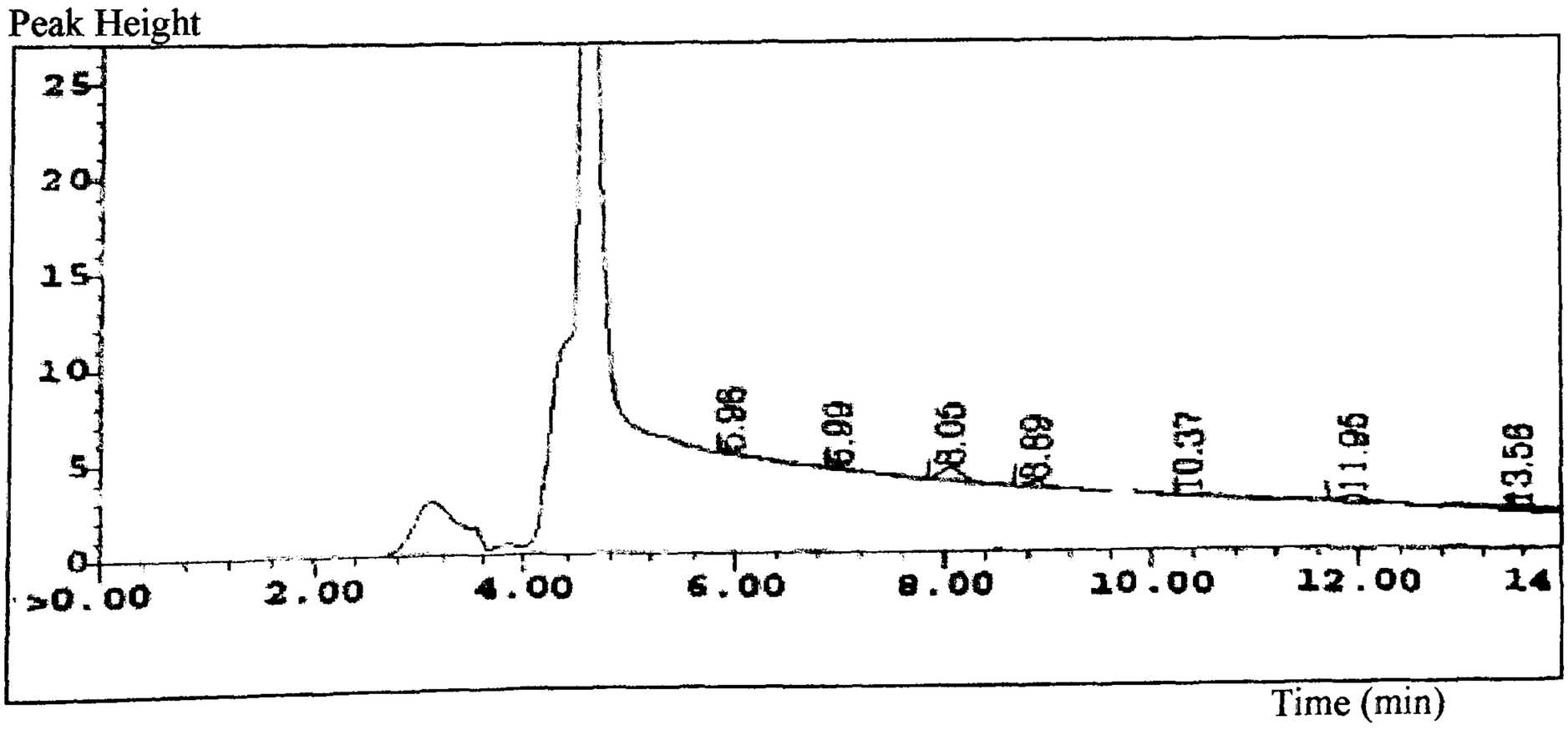


Figure 3.19: Chromatogram of 400ml sample of Bracknell Final Effluent. 6.12.96
Peak Height

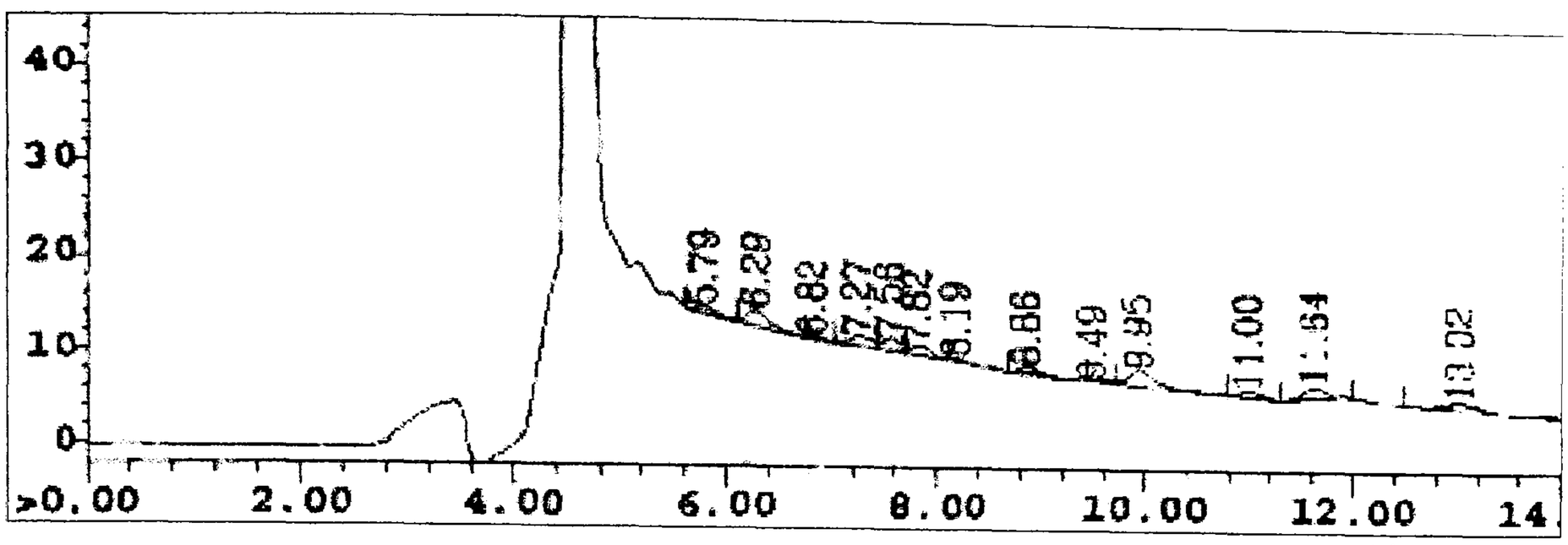
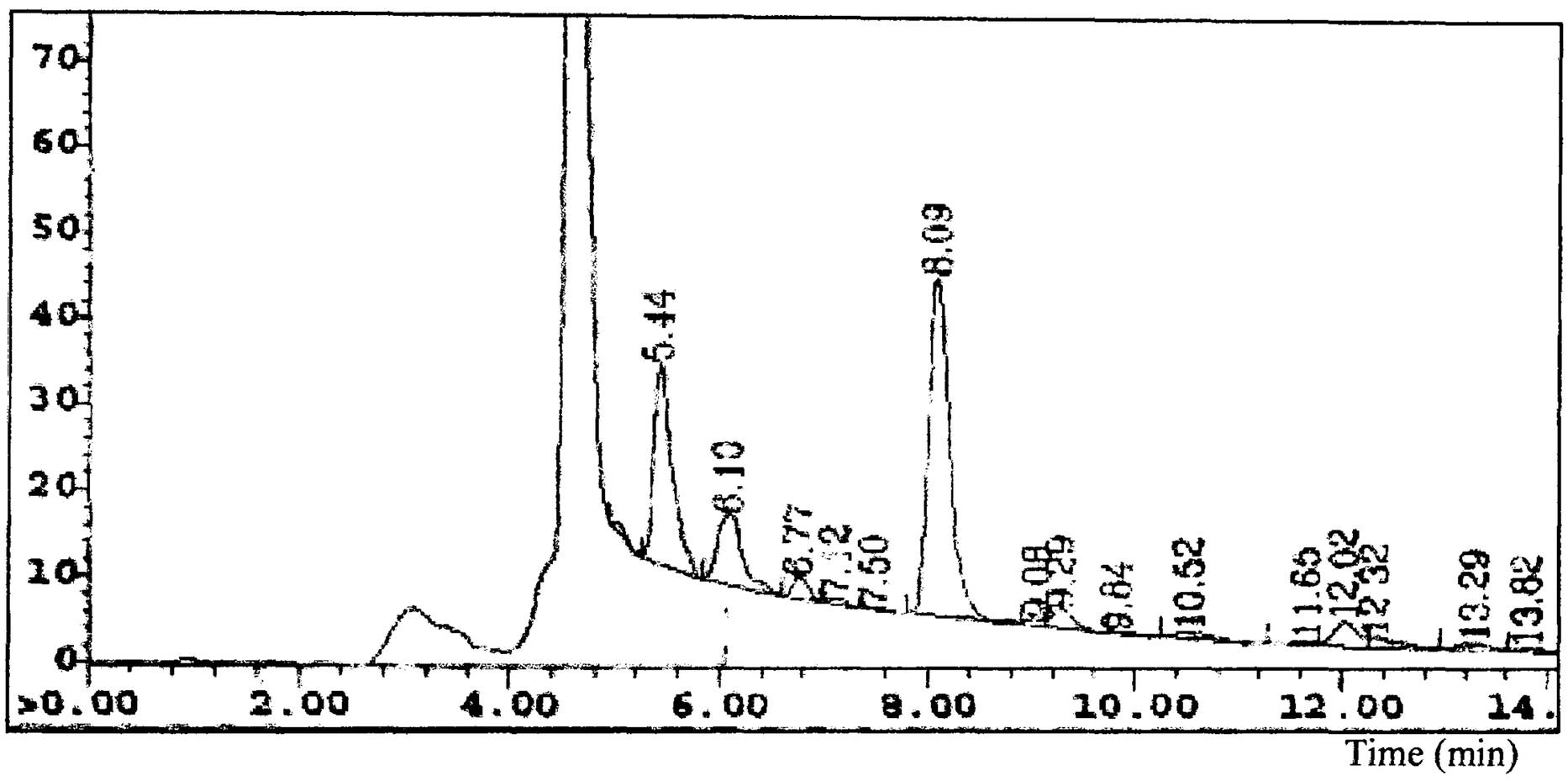


Figure 3.20: Chromatogram of a 300ml sample of Bracknell Settled Sewage 6.12.96
Peak Height



The final sewage experiment carried out used acidification of samples in an attempt to improve antibiotic recoveries. 400ml samples of settled sewage from Chertsey STW were analysed. Samples were spiked with amoxycillin and cefaclor before analysis. Results from this experiment showed that neither of the antibiotics was present in the unspiked samples.

An example of a spiked and unspiked sample can be seen in Figures 3.21 and 3.22.

Figure 3.21 Chromatogram of a 400ml sample of Chertsey SS, spiked with 1mg/l each of amoxycillin and cefaclor. Acidified sample.

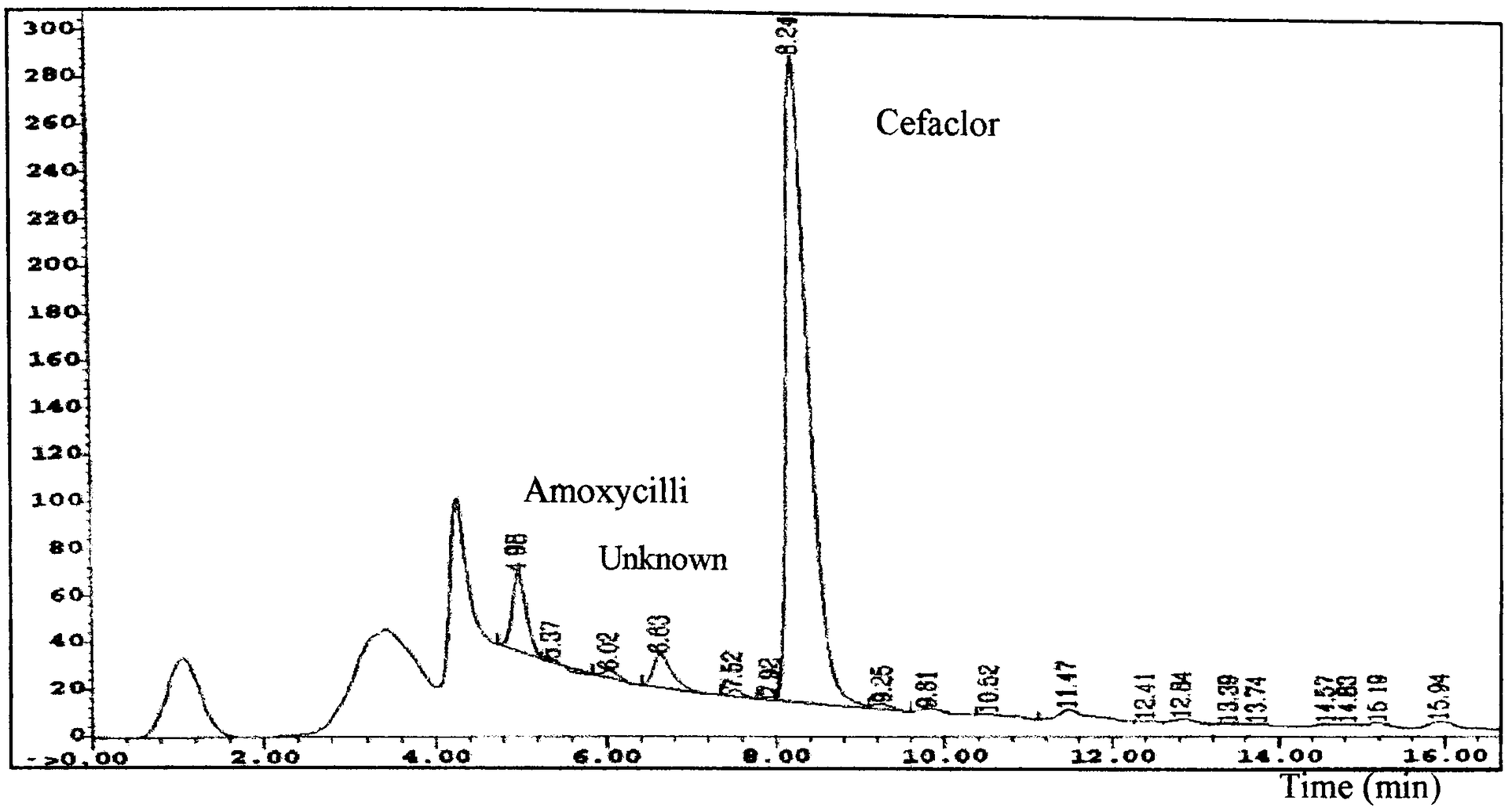
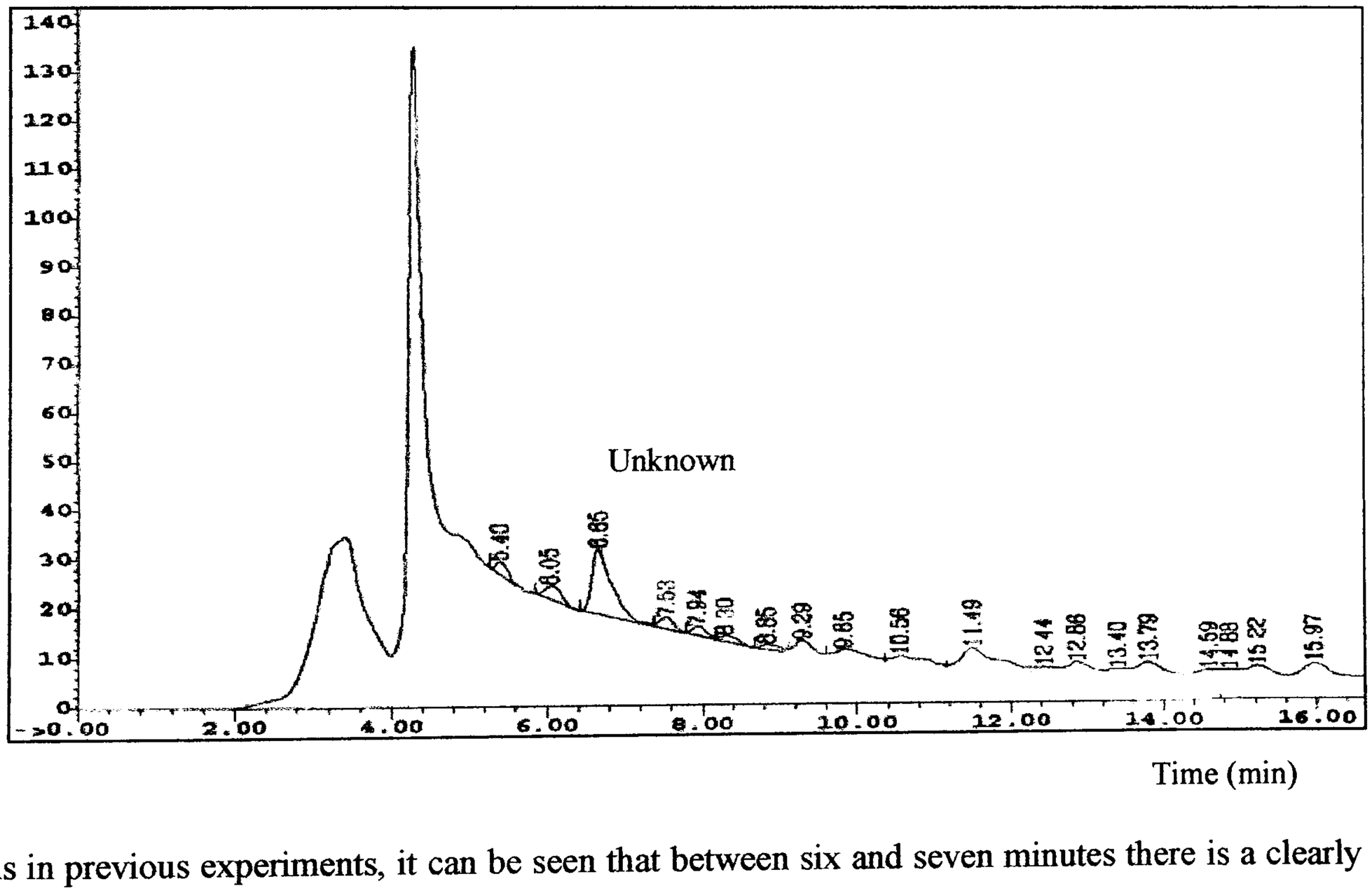


Figure 3.22: Chromatogram of a 400ml sample of Chertsey SS, unspiked. Acidified sample



As in previous experiments, it can be seen that between six and seven minutes there is a clearly detected peak eluted. This peak appeared in all the samples. Elution times and peak areas are shown in Table 3.18. Data include absorbance at the two different wavelengths used to detect the different antibiotics. Using two different wavelengths gave extra information on the eluting compound.

Table 3.18: Elution time and area of unknown peak detected in 400ml SS samples from Chertsey STW.

Antibiotic Added:	**240nm**		**265nm**	
	PA	**Time**	**PA**	**Time**
Non Acidified Samples				
Pre Centrifugation	169	6.66	395	6.67
Pre SPE	269	6.66	613	6.64
Blank – no antibiotic	186	6.64	431	6.64
Acidified Samples				
Pre Centrifugation	215	6.65	543	6.64
Pre SPE	279	6.65	639	6.65
Blank – no antibiotic	233	6.65	485	6.65
PA = Peak Area				

It can be seen that elution time was the same for all samples and all showed the same absorption patterns. The peak showed greater absorbance at 265nm than at 240nm. Acidification of the sample resulted in increased detection. The peak area was greatest when antibiotics were added to the sample after centrifugation and before solid phase extraction.

3.4.2.3 River Water Analysis

Following the results from the above experiments, it was decided to attempt the extraction of a large volume of river water with the aim of determining if there were detectable levels of antibiotics in river water.

A ten litre sample of spiked river water was extracted and analysed. This had 0.05mg/l of each of amoxycillin, penicillin V, cephalexin, cefaclor and ceftriaxone added. 6.4 litres of unspiked river water were also extracted and analysed. The three eluents of different polarities described in the method development section were used in succession in an attempt increase the levels of antibiotics extracted from the SPE column. The least to most polar were used in succession to elute the antibiotics.

Results from this experiment showed a very poor detection of antibiotics. Of the cephalosporins, ceftriaxone was almost completely recovered, whilst recovery of the remaining two antibiotics was below 10%. Penicillin V was just detectable and amoxycillin was not detectable at all. Results can be seen in Table 3.19.

Table 3.19: Extraction efficiency, using three different eluents in succession, of cephalosporins added to a 10 litre river water sample spiked with 0.05mg/l of each antibiotic. 14.11.97

	Eluent A		**Eluent B**		**Eluent C**	**Total**	
	Total μg	**% of spike**	**Total μg**	**% of spike**	**Detected**	**Total μg**	**% of spike**
Cex	410.9	82.2	50.9	10.2	+	461.8	92.4
Cef	14.0	2.8	1.3	0.3	+	15.3	3.1
Ceph	33.1	6.6	5.1	1.0	+	38.3	7.7

Two peaks present in the spiked sample were not present in the unspiked sample and had a similar elution time to amoxycillin. This may have been related to the presence of that antibiotic or possibly one of the other compounds. Chromatograms can be seen in Figures 3.23 and 3.24.

Figure 3.23: Chromatogram of a 400ml sample of Chertsey SS, unspiked. Acidified sample
Peak Height

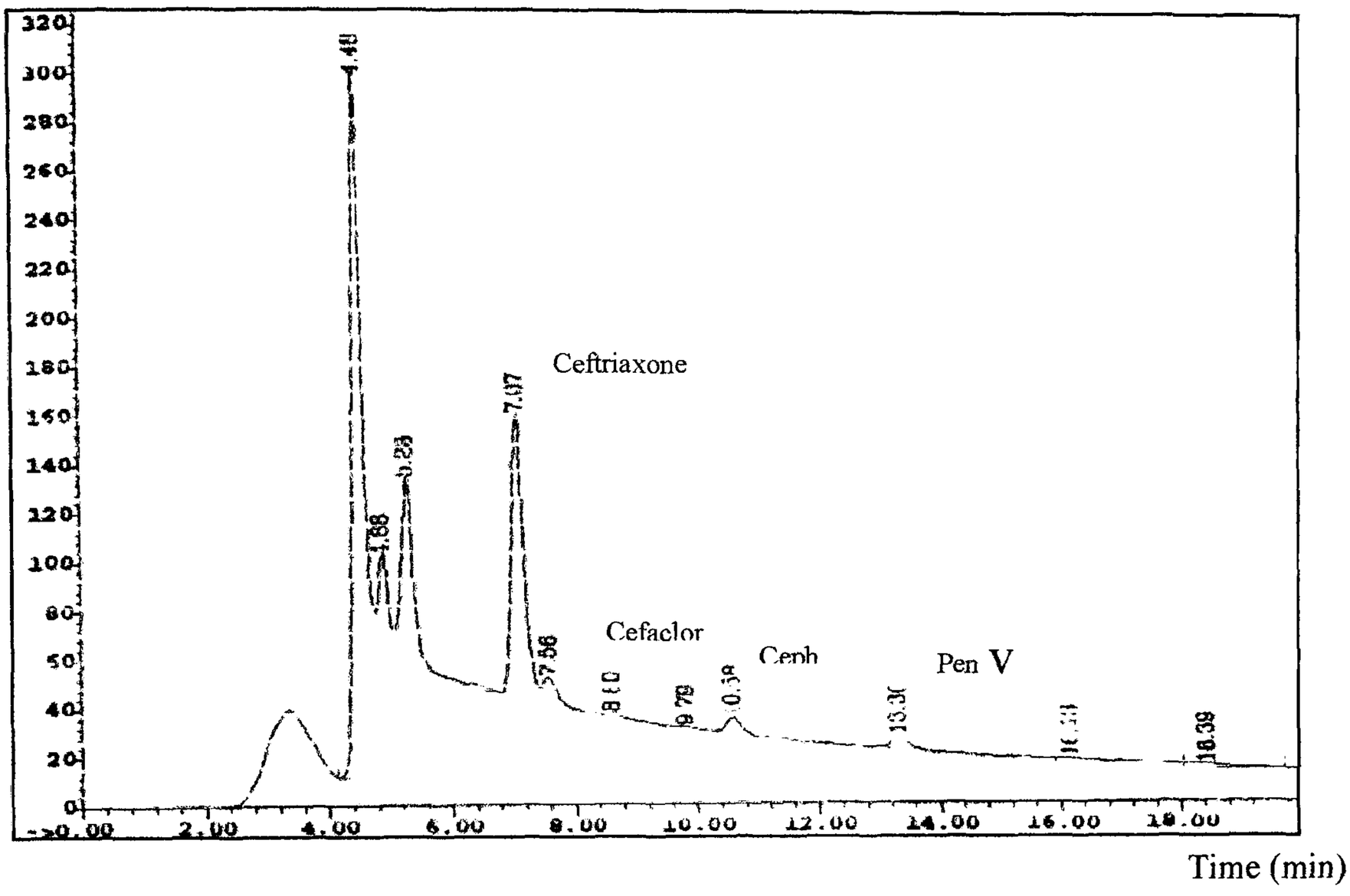
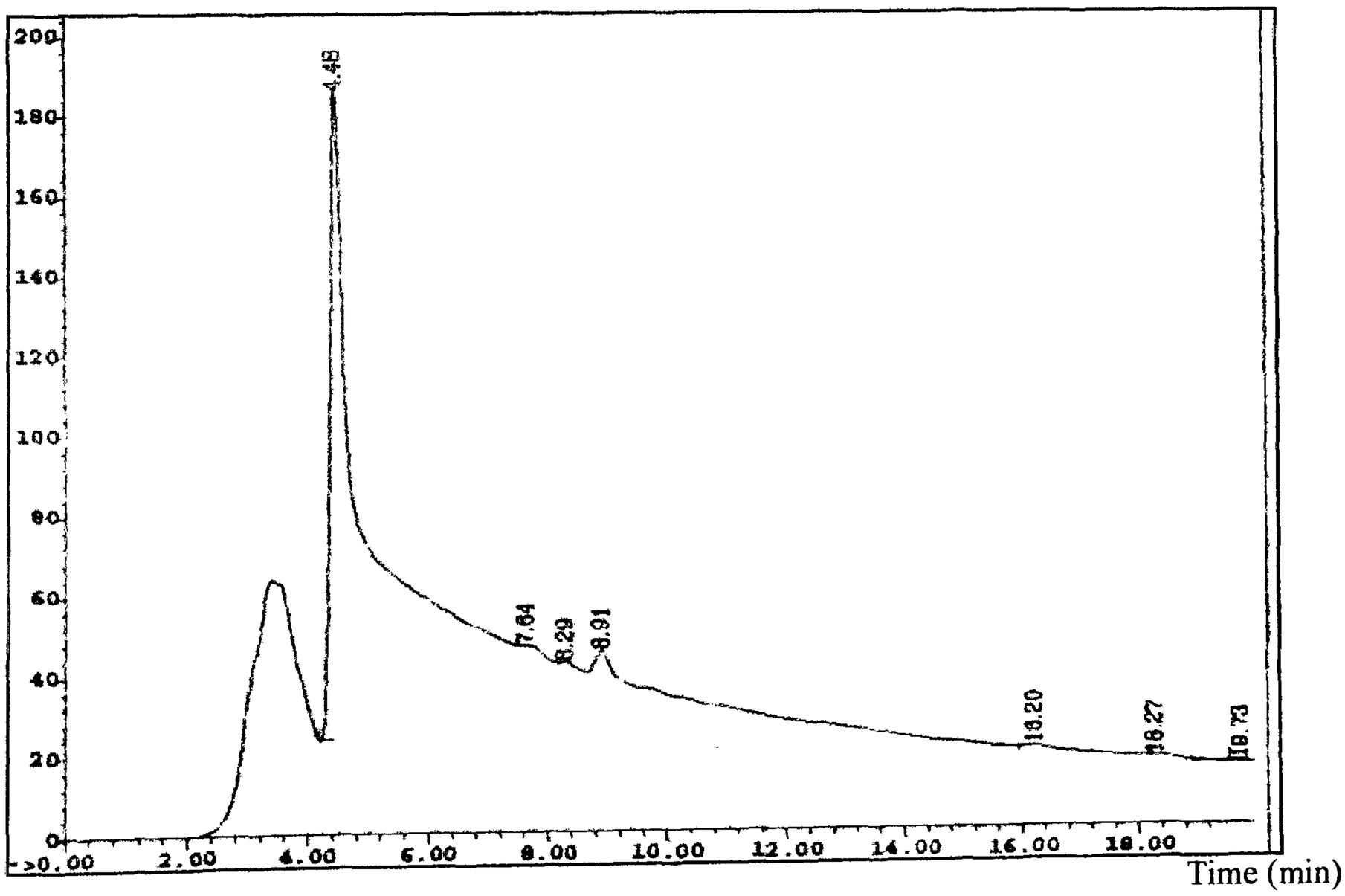


Figure 3.24: Chromatogram of a 6.4 litre river water sample. Eluted with Eluent A (60% ACN, 35% TFA and 5% Phosphate Buffer).
Peak Height



3.5 Discussion

3.5.1 Tetracyclines

There were various reasons already outlined for deciding to include tetracyclines in this project, even though they are not the most used antibiotics in the UK. The most important reason was the fact that they are non-biodegradable compounds that are only slightly degraded in any other way. Because of this, and their long history of use in human and veterinary medicine, it was important to try to determine if they were present in the environment in concentrations that could be harmful.

The initial experiments carried out used low volume samples. No previous work had been carried out in the past to determine the presence or absence of antibiotics in sewage or river water. It was therefore necessary to ascertain whether the methods used in the laboratory in clean solutions would be effective in the analysis of tetracyclines in environmental samples.

Extraction efficiency for oxytetracycline and tetracycline in clean water samples was almost 100% (Table 3.3). In sewage samples efficiency was reduced, though for most of the antibiotics, at least 65% was recovered when analysis was carried out. This was so even when the levels were as low as 0.02mg/l (20ppb). In addition, at these low concentrations the antibiotic peaks were clearly detectable. This indicated that there was potential for extracting and detecting even lower concentrations of tetracyclines.

Results from the analysis of sewage samples showed that there was a lot less interference from other compounds present than expected. The chromatograms from the sewage samples did present some small peaks corresponding to other compounds, but they were not large enough or frequent enough to have interfered with the detection of antibiotics present in any significant quantities. The one problem was found to be the drifting of extraction times during the long running times, though the distance between antibiotic peaks was maintained. This made it harder to decide confidently whether a particular unknown peak was related to the presence of a particular antibiotic.

The first large volume analysis of sewage showed that it was definitely possible to detect antibiotic present in samples at 0.5mg/l concentrations. In addition, indications from samples spiked after elution were that concentrations as low as 0.005mg/l in the final eluted sample would be detectable. This was equivalent to a concentration of 0.04µg/l in the original sample. No antibiotics were detected in the sewage sample, indicating that if present, they were at concentrations likely to be at concentrations lower than 0.04µg/l.

This was not surprising, their use has been greatly reduced in the UK over the last decade (5.1 million GP prescriptions in 1985 fell to 3.7 million in 1995) and expected concentrations were very low. In chapter 2, estimates of concentrations of tetracyclines reaching sewage works were around 0.05ug/l. This did not take into account any antibiotic that might be lost by binding to particulates. However, tetracyclines are not biodegraded in any way. Because of this, it must be assumed that most of the tetracyclines used over the years must be reaching, and remaining in, the environment one way or another. In England, in 1996, 31.7 tons of tetracycline were prescribed by GPs and at least 19.3 tons of this was excreted by patients in its active form. There are also the tetracyclines prescribed in hospitals to take into account, which are harder to quantify. In Chapter 2 estimates showed hospital contributions to be less significant, around 3.5 tons a year for England. In addition, any tetracyclines used in animal husbandry and fish farming could also be reaching the environment in unknown quantities.

Analysis of river water was carried out to determine if any of these antibiotics were detectable in the freshwater environment. The River Thames was sampled at Medmenham. Before it reaches this point, the river will have received sewage effluent from towns such as Oxford, Reading and many other smaller towns. In addition, there will be the runoff from the farmland that the river travels through before reaching this point. These factors made it important to attempt to detect if any were present. Tetracyclines do bind reversibly to soils (Sithole and Guy, 1987b) and are highly soluble.

The analysis of river waters meant that it was necessary to analyse much larger sample volumes. The difficulties involved in concentrating these large volumes made it impossible to carry out large sample runs. It was not possible to analyse multiple samples simultaneously and it was not feasible to analyse more than one spiked sample at a time.

Results from the two experiments where tetracyclines were found in river water samples show that the levels present are in the μg/l range of concentrations (see Table 3. 20).

Table 3.20. Levels of a tetracycline antibiotics detected in river water sample.

	Estimated concentration in River water (μg/l)	
	29.9.97	**14.11.97**
Oxytetracycline	0.50	0.14
Tetracycline	1.17	0.05
Doxycycline	0.58	0.08

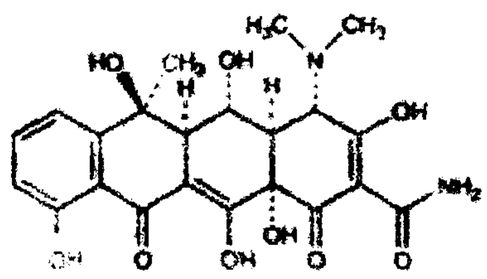
These concentrations are very low indeed, a thousand times lower than the mg/l concentrations that is the minimum inhibitory concentration range of most antibiotics, including tetracyclines. They are in the range of the levels expected in sewage. Sewage would be diluted by receiving water, which would reduce the concentrations. In addition, some proportions of the tetracyclines present in sewage are likely to be bound to solids and be removed with them during treatment. This suggests that the levels found are higher than would be expected if the only contribution were from antibiotics used. However, medical use of tetracyclines has diminished due to problems of resistance. GP prescriptions in 1995 were 73% of those for 1985 (Table 2.2, chapter 2). Therefore, there exists the potential for a large reservoir of tetracyclines in the aquatic environment as a result of past use of tetracyclines. This could be contributing to the levels detected during this project in river water. It must be remembered that Samuelsen *et al.* (1994) found tetracycline in sediments to have no antibacterial effect. This suggests that tetracycline is only active when not bound to sediment. In addition, Lunestad and Goksyr (1990) found that only 5% of oxytetracycline in sea water was in its active form, to what extent this also applies to freshwater is unknown.

Any impacts these concentrations may have on bacteria in the freshwater aquatic ecosystems would only be likely to be seen in the long term. The fact that it was possible to detect antibiotics on both occasions suggests that they might be present consistently in the river water. Tetracyclines being present at very low concentrations in drinking water supplies also becomes a possibility.

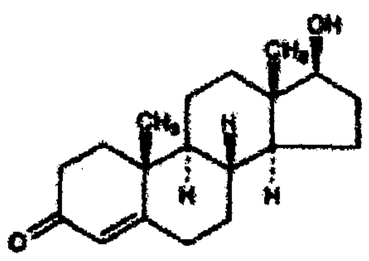
The concentrations found are similar to those in the only previous report of tetracycline in river water (Watts et al, 1983), where concentrations of around 1µg/l where found in Thames water. A recent review (Stan and Heberer, 1997) brings together a large number of old reports of levels of pharmaceuticals in sewage and water samples. These reports were largely ignored in the past. They show that well over 20 pharmaceutical compounds have been found in water samples, including groundwater and the North Sea, at concentrations that range from µg/l to ng/l. In view of this, the finding of tetracyclines in the River Thames becomes much more important and less unexpected. These low concentrations of antibiotics could have other impacts. It has been suggested that tetracyclines may have a hormonal effect on the human body (Golub and Ramamurthy, 1984; David Tucher, pers com). They have also been reported to lower testosterone levels by 20% when administered (Pulkkinen and Maenpaa, 1984). This suggests that tetracyclines could have hormone-mimicking characteristics. If so effects might be expected at much lower concentrations than those at which they show antibacterial effects. Hormones are active at ng/l concentrations. These are the concentrations at which tetracyclines have been found in the environment during this project.

The structure of tetracycline and testosterone can be seen in Figure 3.25. It can be seen that both have a 4-ring structure. Whether this could be an issue of interest or not would largely depend on what other, possibly more active, compounds are present in water at the same time.

Figure 3.25: Chemical structure of testosterone and tetracyclines



Oxytetracycline



Testosterone

There are various possible sources of these antibiotics, as mentioned above. It was not possible to detect the presence of antibiotics in the sewage. If more time had been available, further repeat analysis would have been desirable. It is possible that if larger volumes of sewage had been analysed that tetracyclines would have been detected. They may have been present in the same or higher concentrations in sewage, but not detectable because of interference from other compounds present. The chromatograms from the Bracknell sewage works showed very few peaks at all which might suggest this. However, these chromatograms only showed compounds that absorb UV light at, or around, 355nm. There may be a large number of compounds in the solution, interfering with the detection of the tetracyclines, not seen in the chromatogram because they absorbed light at a different wavelength.

These antibiotics bind reversibly to soils and sediments. It is therefore possible that the levels of antibiotics detected are ubiquitous in river waters and are there as a result of the long term usage of antibiotics. Sediments may act as a reservoir, with equilibrium being reached between levels in water and levels in sediments. Depending on conditions, more or less tetracyclines may be released into river water.

Concentrations were around ten times higher in the first analysis than in the second. The differences may be due to difficulties in converting the information contained in the chromatograms into actual antibiotic concentrations. In both cases, the antibiotic peaks were clearly visible.

However, the peaks were small and close to the limit of detection, which make variations that may occur during area calculations more significant. In addition, calculations of actual concentrations, by comparison with standards, become less reliable as concentrations get smaller.

It is possible that the difference seen could be a real effect. Various explanations for the variations seen are possible. Increased rainfall in the winter months would result in dilution of antibiotics present. There are seasonal variations in prescriptions (see Chapter 2) which might counteract this, though, as seen, levels being used in the UK are decreasing. Another, possible explanation is that changes in the river water environment triggered a release of antibiotics from the sediments. However Sithole and Guy(1987b) showed how binding to soil increased with decreasing pH. Therefore, acidification of waters due to pollution would result in decreased levels of tetracycline in solution, though this is unlikely to occur in the Thames.

3.5.2 Penicillins

Broad-spectrum penicillins and cephalosporins are the two most prescribed groups of antibiotics in the England accounting for over 40% of all antibiotics prescribed. Because of this, even though they are easily broken down by resistant bacteria, and therefore not expected to persist long term in the environment, it was important to determine if there were detectable levels present.

The first experiments carried out to determine the fate of amoxycillin and cephalexin when added to sediemnts gave very interesting results. Very little of the antibiotic was recovered in its original form. In the 7 day experiment antibiotic was only just detectable in the sediment sample, with only slightly higher levels detected in supernatant. In the 24 hour experiment similar results were obtained. Again, large peaks, which did not directly correspond to the antibiotic the sample was spiked with, were present. In all these results, including when the samples were extracted using only water, these new peaks appeared. These peaks were not present when antibiotics were mixed in with sediment and extracted immediately, nor were they present in blank soil samples.

The most obvious conclusion was that these peaks were related to the presence of amoxycillin or cephalexin and could be a modified form of both. In both cases, elution was delayed by about one minute, which suggested that there was a similar change occurring in both antibiotics, which changed their polarity and therefore their retention in the column.

Degradation of β-lactams involves the breaking of one of the C—N bonds in the second ring and the addition of a double bond oxygen molecule and a hydrogen molecule. This would make the whole compound more polar and affect both polar and non-polar interactions in the column. As result a change in retention time would be expected.

It was possible that these changes in extraction times were a result of changes due to the extraction method used and were not occurring during the experimental period. However, not only were the same patterns seen in the supernatants, which were analysed directly without an extraction step, but also extraction using water on duplicate samples in the second experiment produced the same results. The presence of the formaldehyde used to sterilise the soils samples could well have been affecting the antibiotic samples. Formaldehyde is a strong reducing agent and could have been breaking down the antibiotics. Alternatively, there might have been a chemical reaction with the soil that affected the antibiotics. There was also the possibility that, though the soil had been sterilised using formaldehyde, sufficient bacteria survived and flourished to be able to start degrading the antibiotic. However, the change was also seen in the second experiment when samples were only incubated overnight, at a low temperature (15°C). It is unlikely that there would be sufficient time for a significant bacterial population to establish itself in this time. Regardless of the reason for these changes, the results indicated that there was a strong possibility that the breakdown products of amoxycillin and cephalexin could be present in environmental samples, rather than the active antibiotics themselves.

The half-life of β-lactams in the environment was expected to be short (Feinman and Mathesson, 1979), a few weeks at most. In these experiments only traces of these antibiotics were recovered, even though the original quantities added were high, 50mg/kg of sample. This was the case even in samples that were analysed just 24 hours after adding the antibiotics. The results from these experiments indicated that their half-life was very low indeed, definitely less than a week, probably just a few days.

In the case of both, antibiotics most of the compound, modified or not, was present in the supernatant, and not in the sediment. This indicates that most of the penicillin antibiotics reaching the freshwater environment would remain in solution and not bind to particulates or sediments. It also suggested that, unlike with the case of tetracyclines, there would be no reservoir of antibiotics present in the sediments. Therefore, when attempting to determine levels in the environment, detection in water samples would be the best approach.

Recovery rates in the tetracycline and sewage experiments were over 60% even when analysing large volume samples. In the case of penicillins, the best recovery rates obtained were 30%, often less than this. Ceftriaxone was the only β-lactam., of those used that was easily recoverd. However, it was still possible to determine what concentrations were detectable by extracting spiked samples. 1mg/l concentrations were easily detectable in 400ml samples. The last experiment carried out was analysis of 10 litres of river water spiked with 0.05mg/l of five antibiotics. Ceftriaxone was easily detected, whilst cefaclor, cephalexin and penicillin were only just visible as peaks. It is possible to say that these antibiotics are therefore not present at concentrations of 0.05mg/l or more. Amoxycillin was not detectable.

There was no antibiotic detected in any of the unspiked river water or sewage samples analysed. It is possible that samples analysed were not large enough for antibiotic to be detected. Another possibility is that most of the antibiotics excreted are degraded or removed in the sewage system, before they reach the environment, or shortly after they enter the environment.

Results from all experiments showed that there were certain peaks appearing often in the samples. These peaks eluted between 6 and 7 minutes. They were larger in the sewage samples and smaller in the Bracknell river water samples, but it was not visible in the Thames river water sample. Data summarised in Table 3.13 showed that its detection was improved by acidification of the samples before extraction. Levels detected were higher in samples that had been spiked with antibiotics, in both acidified and non-acidified samples. This again appears to indicate that the peak is somehow a breakdown product of one of the antibiotics and that it is present in the samples when they were collected. In the sediment experiments of 7 days it was clearly visible in all samples including the blanks (sediment to which no antibiotic had been added). In the sample that had been spiked with amoxycillin, there was much larger peak and no other peak for amoxycillin. The presence of this peak could have been caused in some way by the extraction procedure, but it was not present in the last experiment carried out, which shows that this was not the case.

The peak eluted at 6.7 to 6.9 minutes. This suggests that there is a compound consistently present in environmental samples, which is similar in characteristics to the β-lactam antibiotics. It is most similar to amoxycillin that has been in soil or water for some time. In addition, there were two peaks present in spiked samples, eluting at between 5 and 6 minutes, that were not seen in the unspiked samples. These could be some sort of breakdown product or modified form of the antibiotic. All the results obtained, particularly in relation to amoxycillin, confirm that these compounds would be inactivated quickly, and that their breakdown products remain in solution.

3.6 Conclusions

From the results obtained in the work detailed in this chapter it can be concluded that there is a possibility of tetracyclines being present in very low concentrations in most freshwater aquatic environments, though further work would have to be carried out to confirm this. Concentrations found were too low to have a direct bactericidal or bacteriostatic effect on bacteria, however they might have a chronic long-term effect. There is also the possibility of a hormone-mimicking effect of tetracyclines. Should this be the case then the levels found become much more important, from both an ecosystem health and a human health point of view. The effects of the presence of tetracyclines in drinking water supplies should also be considered.

No penicillin or cephalosporin compounds were identified in the sewage or river water samples analysed. The method developed did not produce good recovery rates. However, these antibiotics are used in larger quantities than any other in the UK and are excreted in large quantities by patients. Indications are that these compounds are very quickly degraded and not present in active forms in freshwater ecosystems. Instead, results from this work highlighted the possibility that the inactivated forms and breakdown products of these antibiotics could be present in large quantities. The effect of the presence of these compounds in the environment is completely unknown.

Chapter 4:

The use of bacterial assays to investigate the impact of sublethal antibiotic concentrations on freshwater aquatic ecosystems

4.1 Introduction

In addition to determining the amount of antibiotics being utilised and the presence or absence of antibiotics in the water environment, it was important for this project to determine at what concentrations antibiotics could have an impact on bacteria. It was particularly important to try to determine this for environmental samples. Various approaches were used in trying to determine the biochemical well-being of bacteria in the laboratory.

The first method used, looking at antibiotic inhibition of growth, was an attempt to determine what is likely to be the final indication that antibiotics are having an impact on bacteria. Lorian (1993) highlighted the effect of sub-inhibitory concentrations of antibiotics on ultrastructure of bacteria and the excretion of enzymes. Lebrun *et al.* (1992) also showed effects of sub-inhibitory concentrations of bacteria on expression of surface antigens. The second method, developed by Bains (1993), measuring changes in the mid ultra violet spectrum of bacteria (MUVS), was an approach that was used with the aim of determining if it was possible to detect changes in bacterial characteristics before changes in growth were detected. Work with bacterial luminescence, which has been shown to be sensitive to antibiotics, was also attempted but was completely unsuccessful. This was mainly due to the difficulties in carrying out a growth assay instead of an exposure assay (where bacteria are gown up, exposed to antibiotic and luminescence measured within minutes). Details of the luminescence work are not included in this chapter.

4.2 Materials and Methods

4.2.1 Antibiotic Inhibition of Growth

A method developed by Pearson and Inglis (1993) for determining the concentration of antibiotics in water samples was used to determine the effects of antibiotics on bacterial growth. It used microtitre plates filled with 100µl of nutrient agar containing a bacterial inoculum (National Type Culture Collection (NTCC) cultures, *Bacillus subtilis* 10320, *Bacillus cereus* 10400 and *Ecsherichia coli* 10650; river water bacteria; or river sediment or water samples). Brilliant black, a dye that changed from blue to yellow in the presence of a bacterial growth was also added. 100µl samples of serial dilutions of various antibiotics in sterile distilled water were then placed on top of the agar.

Plates were covered and incubated at 37°C (NTCC cultures) or 22°C (environmental samples) for up to 96 hours. To obtain more accurate results a microtitre plate reader was used to obtain values for each well. Highest absorbance was found to be at 450nm. The wells containing the unseeded agar (blue and clear) were used to zero the reader and readings were then obtained for all the wells. Consequently it was possible to give each well a value that could then be used to draw an inhibition curve for each antibiotic, as opposed to plus or minus readings as were obtained with Pearson and Inglis' method.

4.2.2 Mid Ultraviolet Spectrum (MUVS) of bacteria

This work used an adaptation of the method of Bains (1993). Bacteria were grown up in Oxoid Iso-sensitest broth, containing various concentrations of antibiotics, until mid log phase. This was turbidity of between 0.1 and 0.3 at 600nm. Duplicate samples of each antibiotic concentration were grown up, except controls, where up to three samples were grown up. Samples were then centrifuged at 6000rpm and resuspended in water. The culture was diluted in water until a reading between 1 and 2 was obtained at 200nm in a 1 cm path length quartz cuvette. Absorbance of the bacterial sample was measured compared to a control cell containing only water. To normalise for slight variations in the number of cells between experiments, optical density (OD) values were divided by the OD at 280 nm, which was due to absorbance of protein in cells and reflected cell density. All the results in this chapter are normalised OD values. Analysis of variance was carried out on results using Excel 7.0 data analysis. Results are detailed in Appendix 1.

4.3 Results

4.3.1 Antibiotic Inhibition of Growth

The effectiveness of the microtitre method was tested by using the three NTCC species and three antibiotics, oxytetracycline, tetracycline and amoxycillin. The Minimum Inhibition Concentration (MIC) was taken to be the lowest antibiotic concentration at which there was no growth and no colour change. In addition, a no effect concentration (NEC) was determined, which was the concentration at which no visible differences from controls were seen. Results can be seen in Figures 4.1, 4.2 and 4.3. The minimum inhibitory concentrations together with the no effect concentrations for each antibiotic are set out in table 3.1.

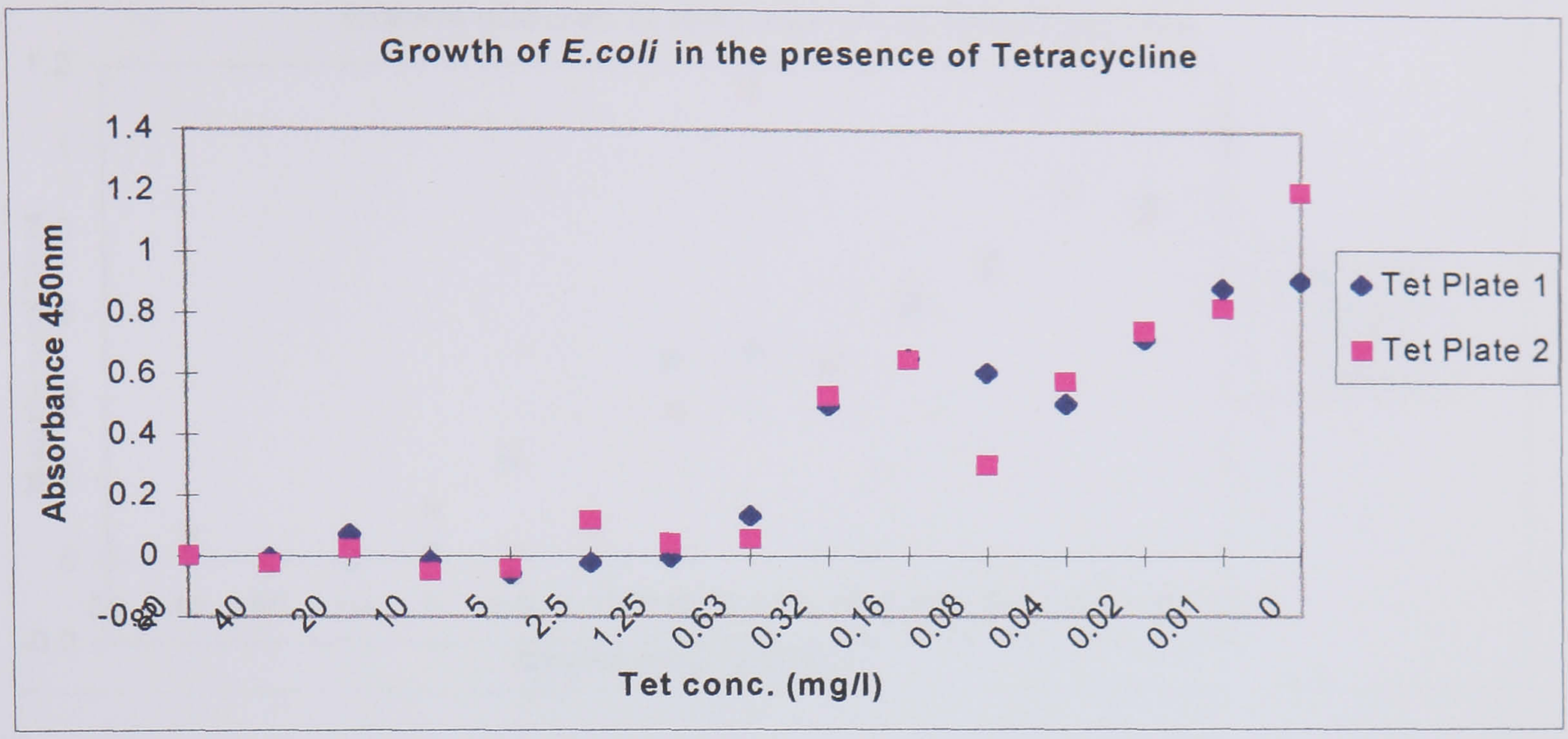
Table 4.1: Minimum Inhibitory Concentration (MIC) and No Effect Concentrations (NEC) obtained for three antibiotics using a bioassay method by Pearson and Inglis(1993). Plates incubate at 37°C for 24 hours.

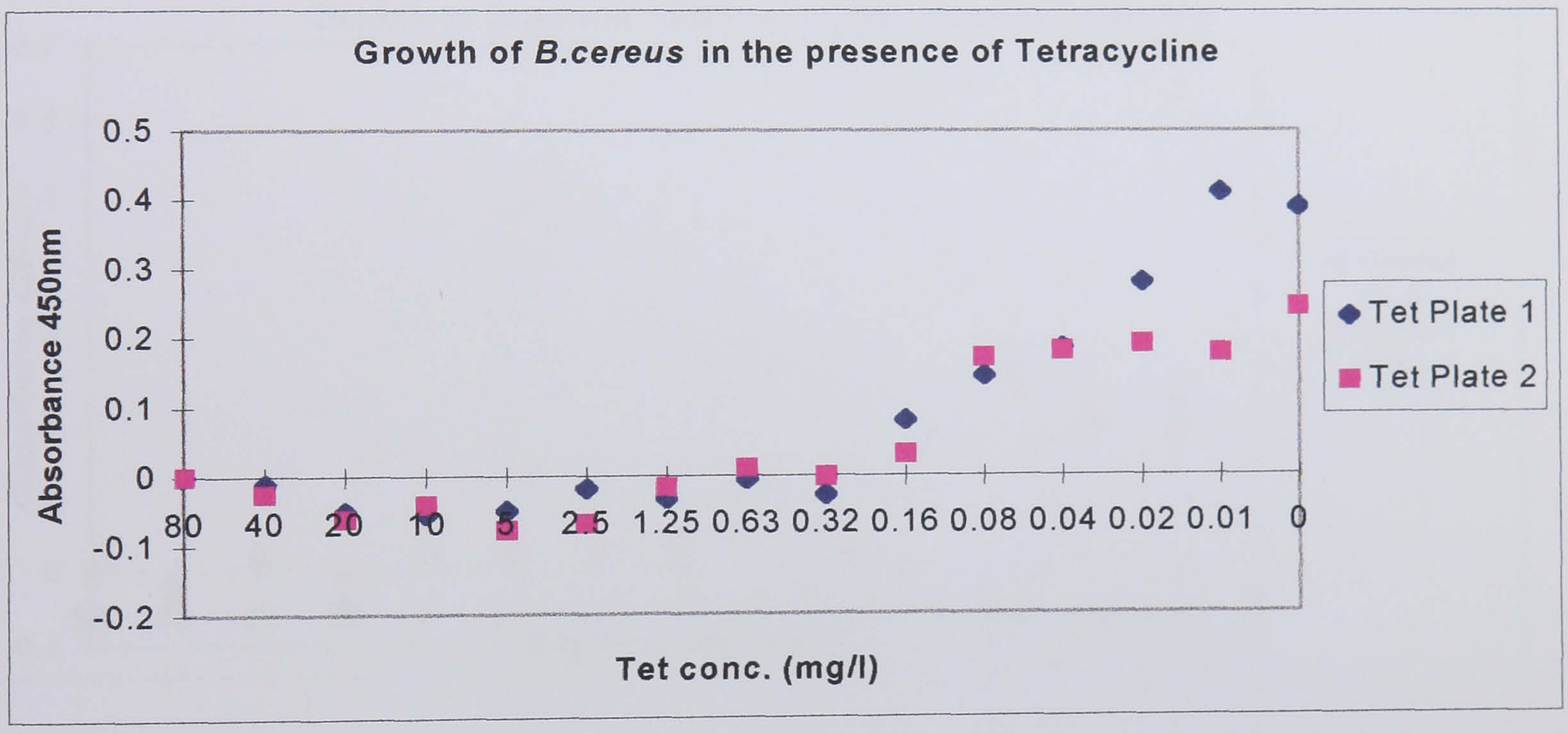
Antibiotic(mg/l)		*E. coli*	*B. cereus*	*B. subtilis*
Oxytetracycline	MIC	1.25	0.63	2.50
	NEC	0.04	0.16	0.32
Tetracycline	MIC	0.63	0.63	0.63
	NEC	0.04	0.04	0.04
Amoxycillin	MIC	0.32	Resistant	0.16
	NEC	0.16	2.5	0.08

The use of a microtitre plate reader allowed the gradual effect that decreasing concentrations of antibiotic have on bacterial growth to be seen. There were considerable differences between the MIC, the lowest concentration at which all growth was inhibited, and the NEC, the highest concentration of antibiotic at which there was a difference from controls. In addition, by measuring the presence or absence of growth using the plate reader, MICs obtained were lower than those that would have been estimated by eye.

Comparing the results from the different bacterial species showed that the response to the tetracyclines was much more consistent than that to amoxycillin. In addition, the response of *B.cereus* to amoxycillin showed, in this case, that even when judged to be resistant at higher concentrations there was still reduced growth compared to controls.

Figure 4.1: Growth of three bacteria in the presence of decreasing concentrations of tetracycline





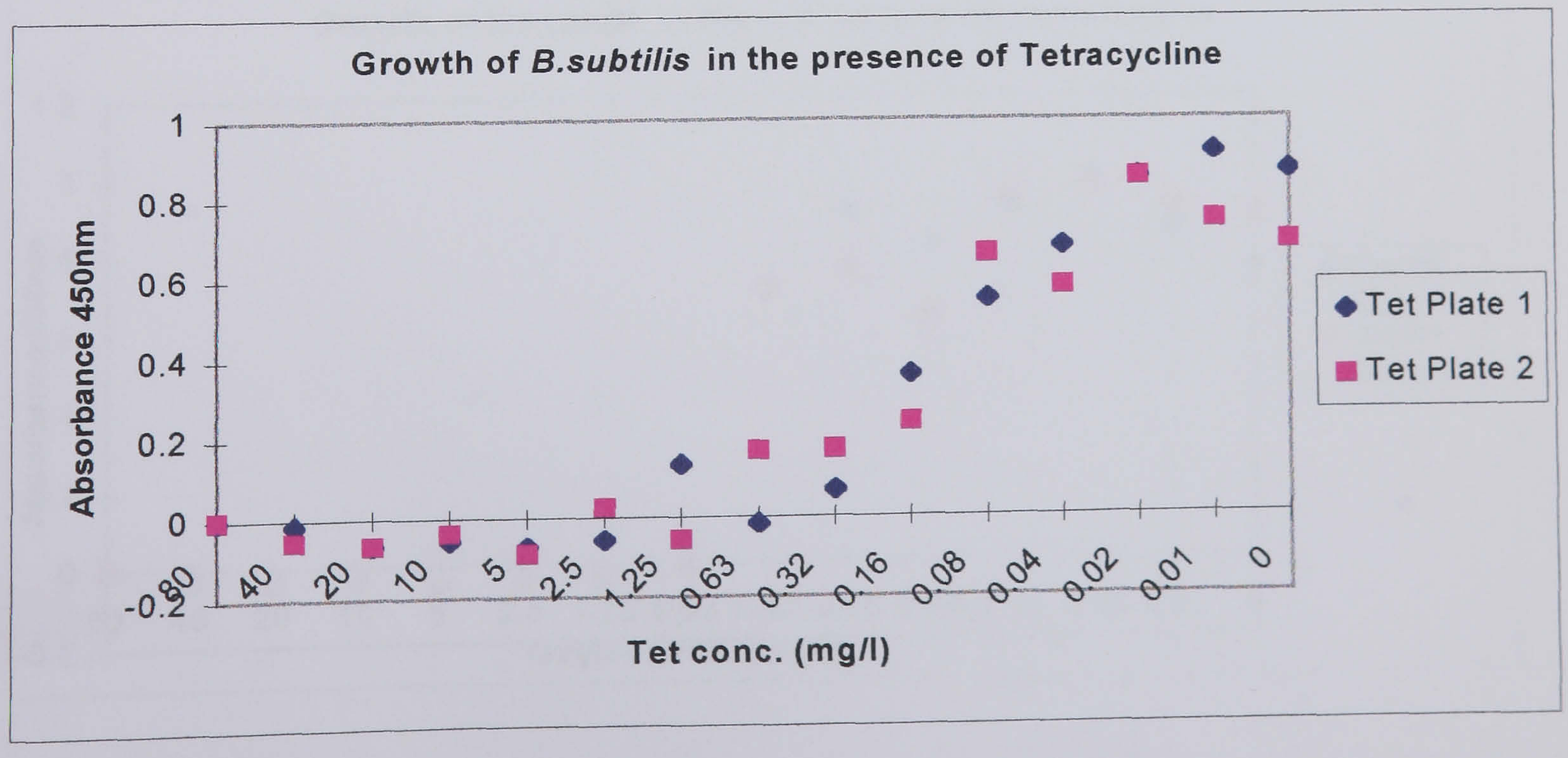
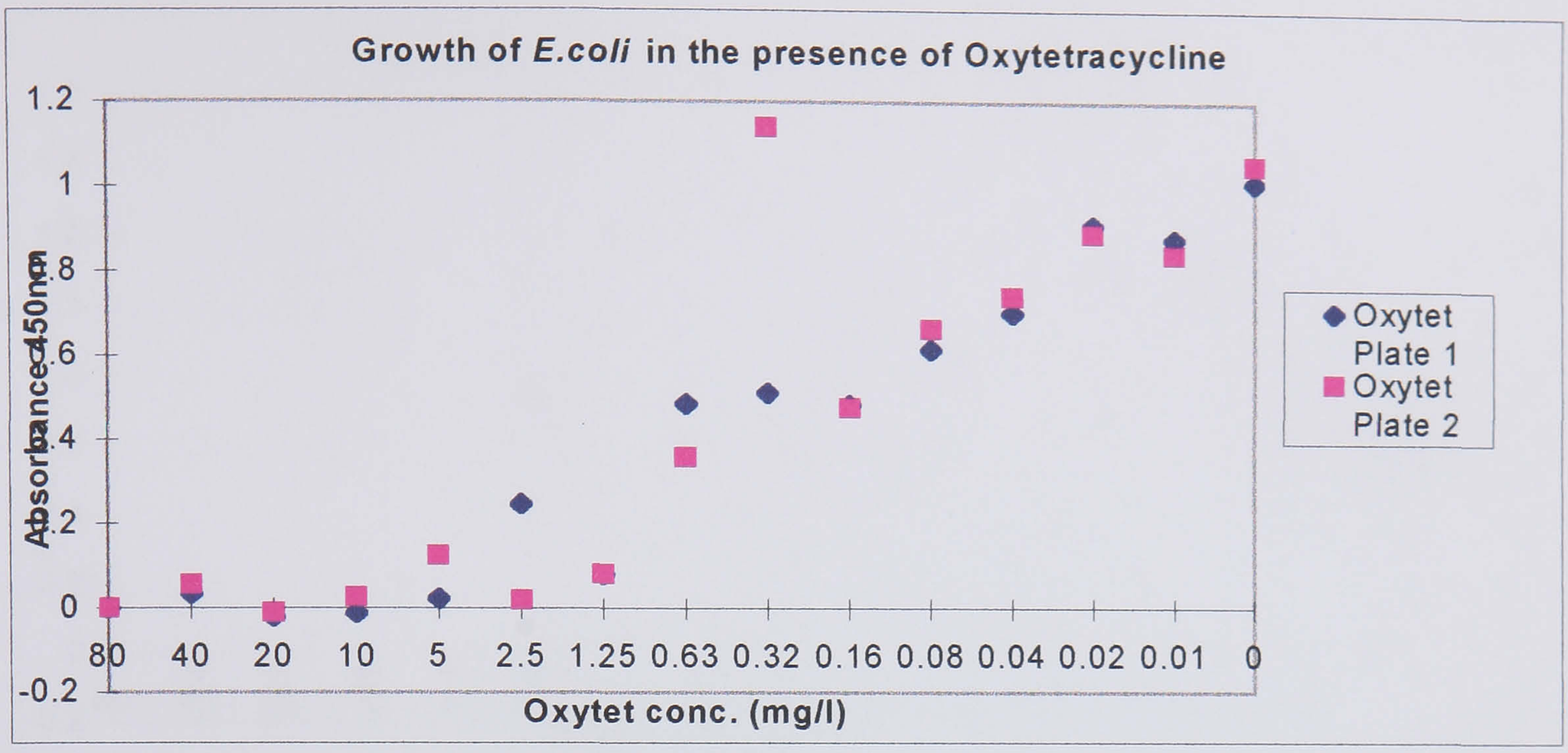
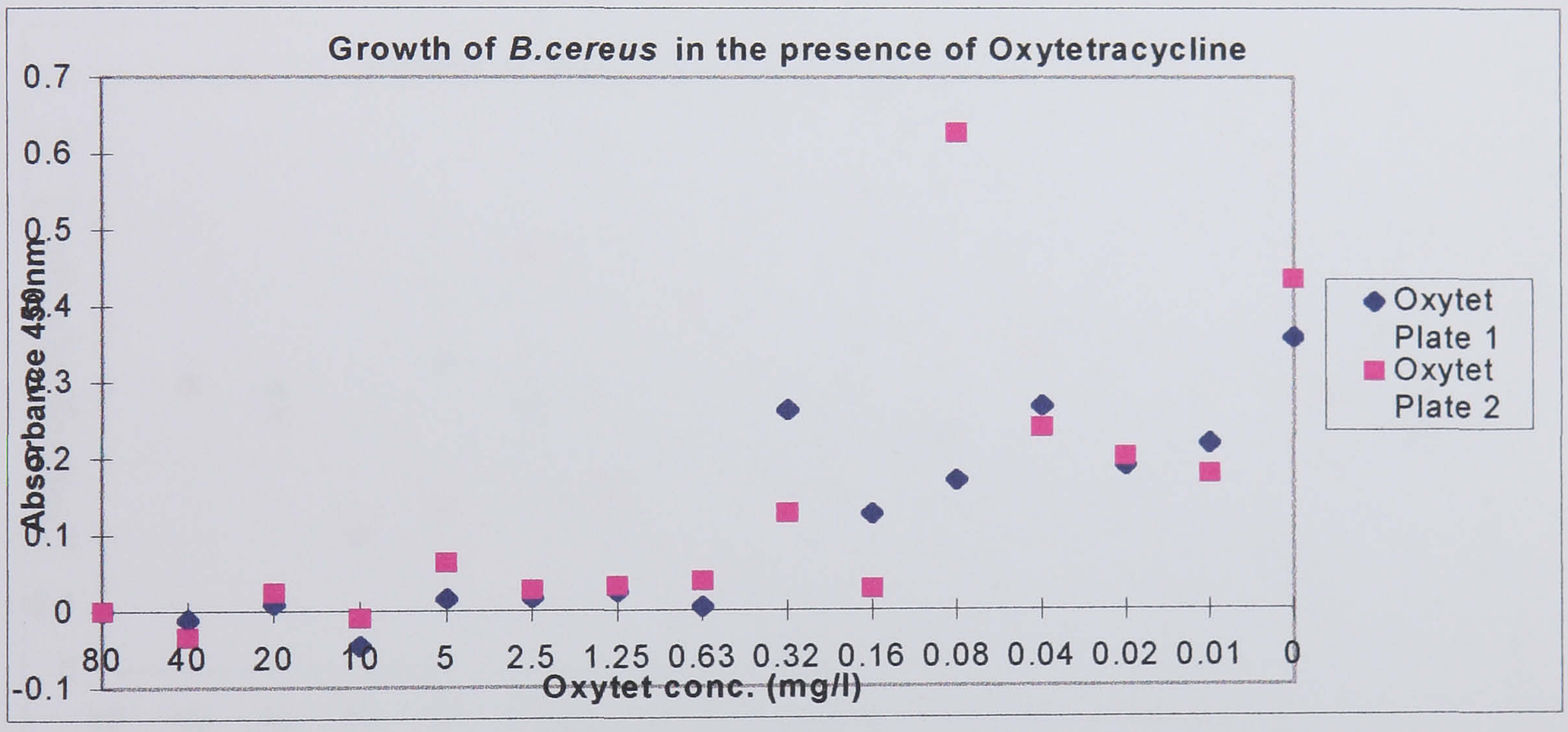


Figure 4.2: Growth of three bacteria in the presence of decreasing concentrations of oxytetracycline





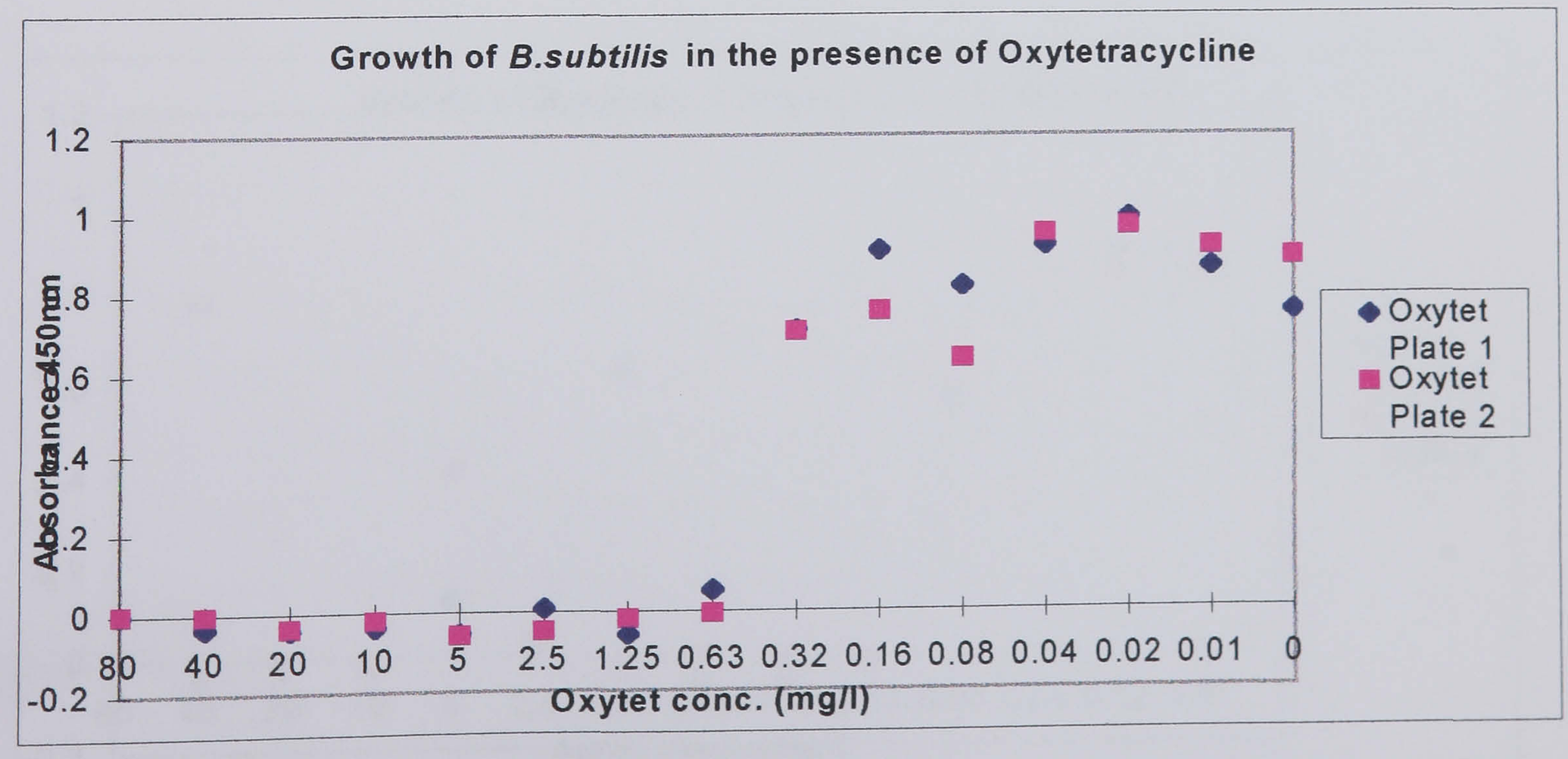
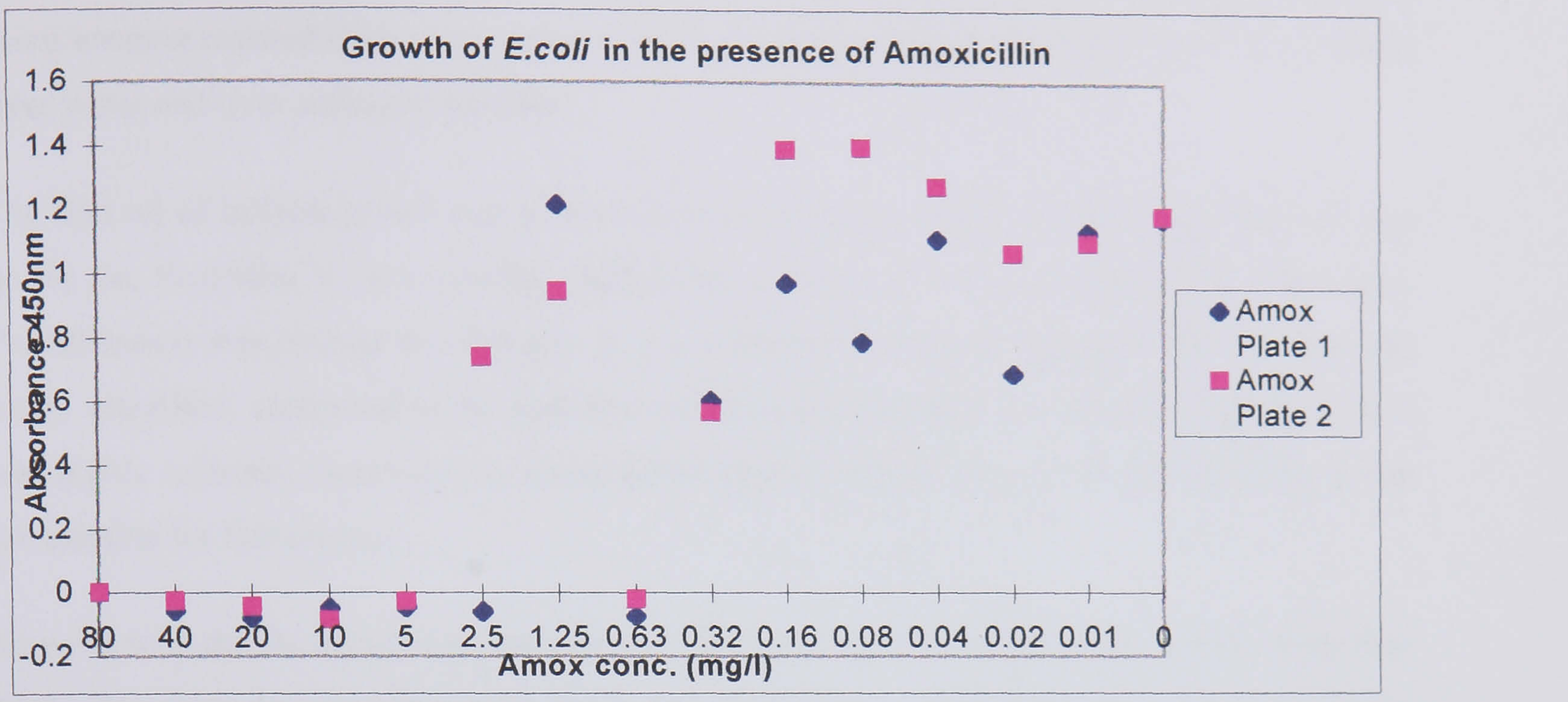
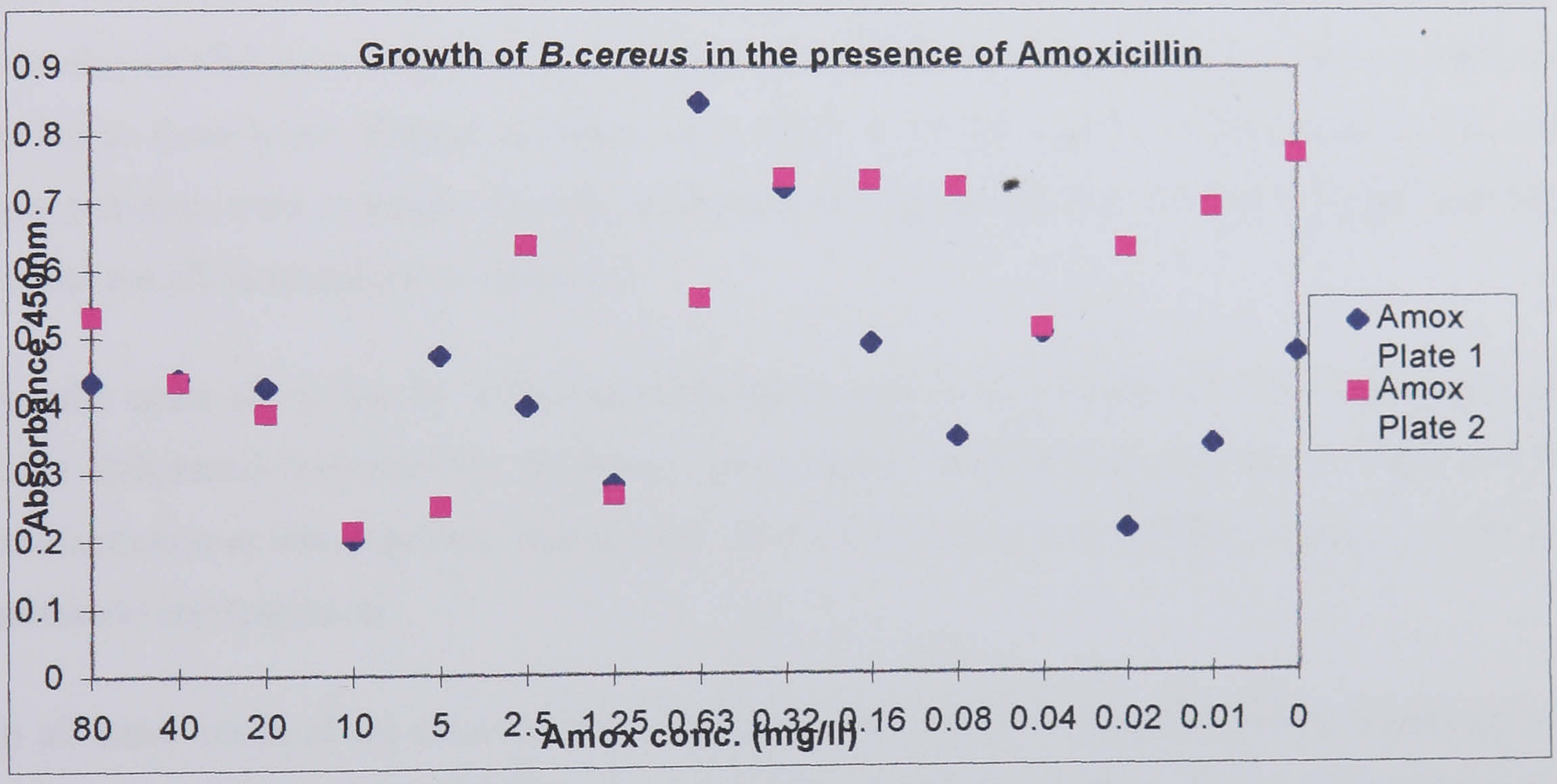
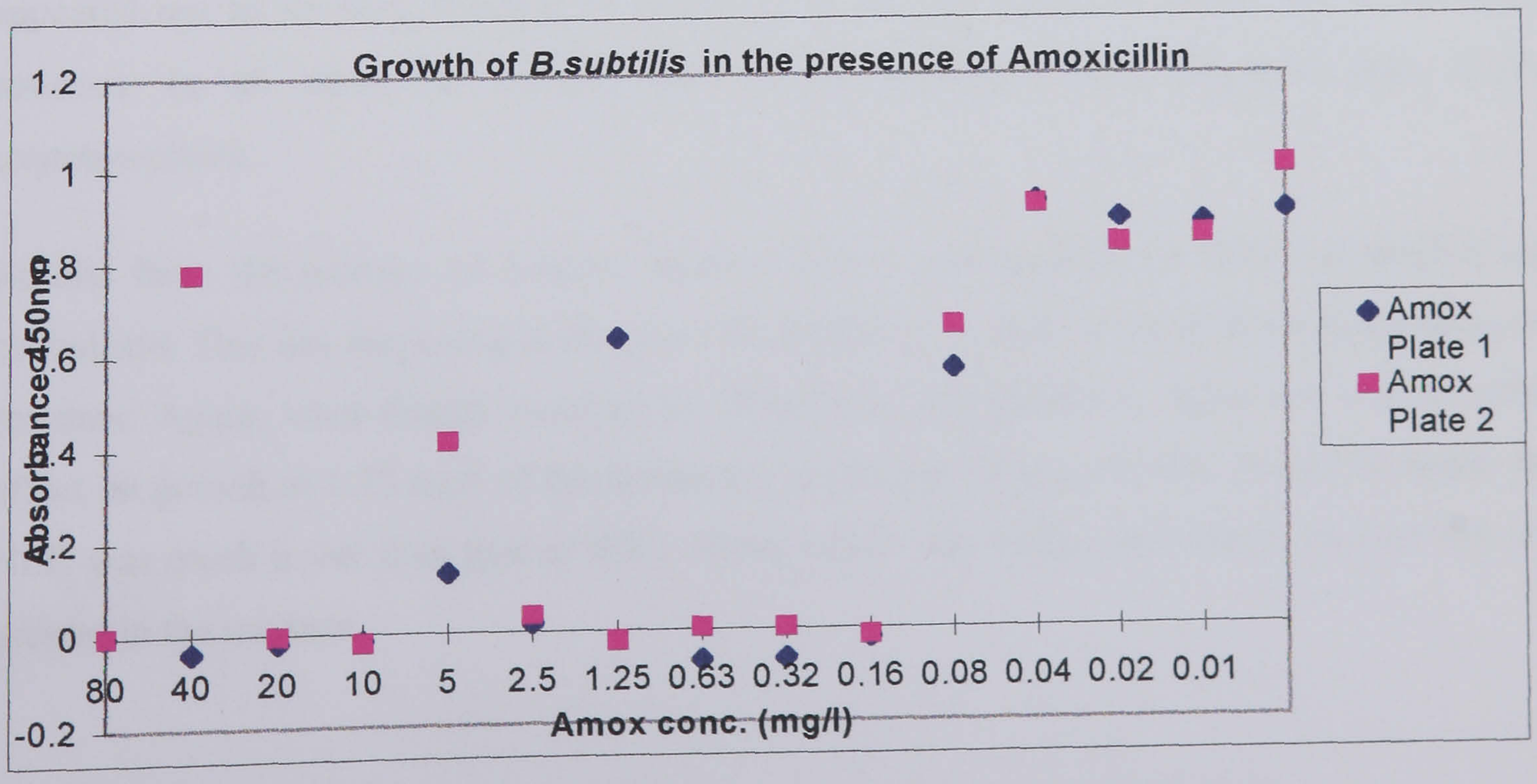


Figure 4.3: Growth of three bacteria in the presence of decreasing concentrations of amoxycillin







Having determined that this was a useful method for investigating the impact of low concentrations of antibiotics on bacteria, the next step was to carry out the same experiment using bacteria isolated from river samples. This was followed by experiments which used direct river water and river sediment samples.

The first set of isolates grown was a *Pseudomonas sp.* that was resistant to all antibiotics it was grown on, Penicillin V, amoxycillin, cephalexin, tetracycline and oxytetracycline. The reason *Pseudomonas* was chosen was because it is a common freshwater bacterium that is relatively easily identified, compared to the majority of the bacteria present in the water. However, as it was highly resistant, showing only some diminished growth at 100mg/l of tetracyclines, it was not suitable for this project.

As work with the *Pseudomonas* was unsuccessful, three unknown river water isolates were then used. The three species were used singly and as a mixture. Resistance was measured against three individual antibiotics, oxytetracycline, cephalexin and amoxycillin. The same experiment was also carried out using a mixture of the three antibiotics. Each antibiotic concentration was added to three wells. Repeat readings were taken at 24, 48 and 96 hours. Readings for each concentration were averaged. Results can be seen in Figures 4.4, 4.5, 4.6 and 4.7. MIC and NEC results are all summarised in Table 4.2.

Results again show that the effect of antibiotics is not an all or nothing one. There were very large differences between the antibiotic concentration at which no growth occurred and the concentration at which growth was normal. There was a dose related effect between growth and antibiotic concentration.

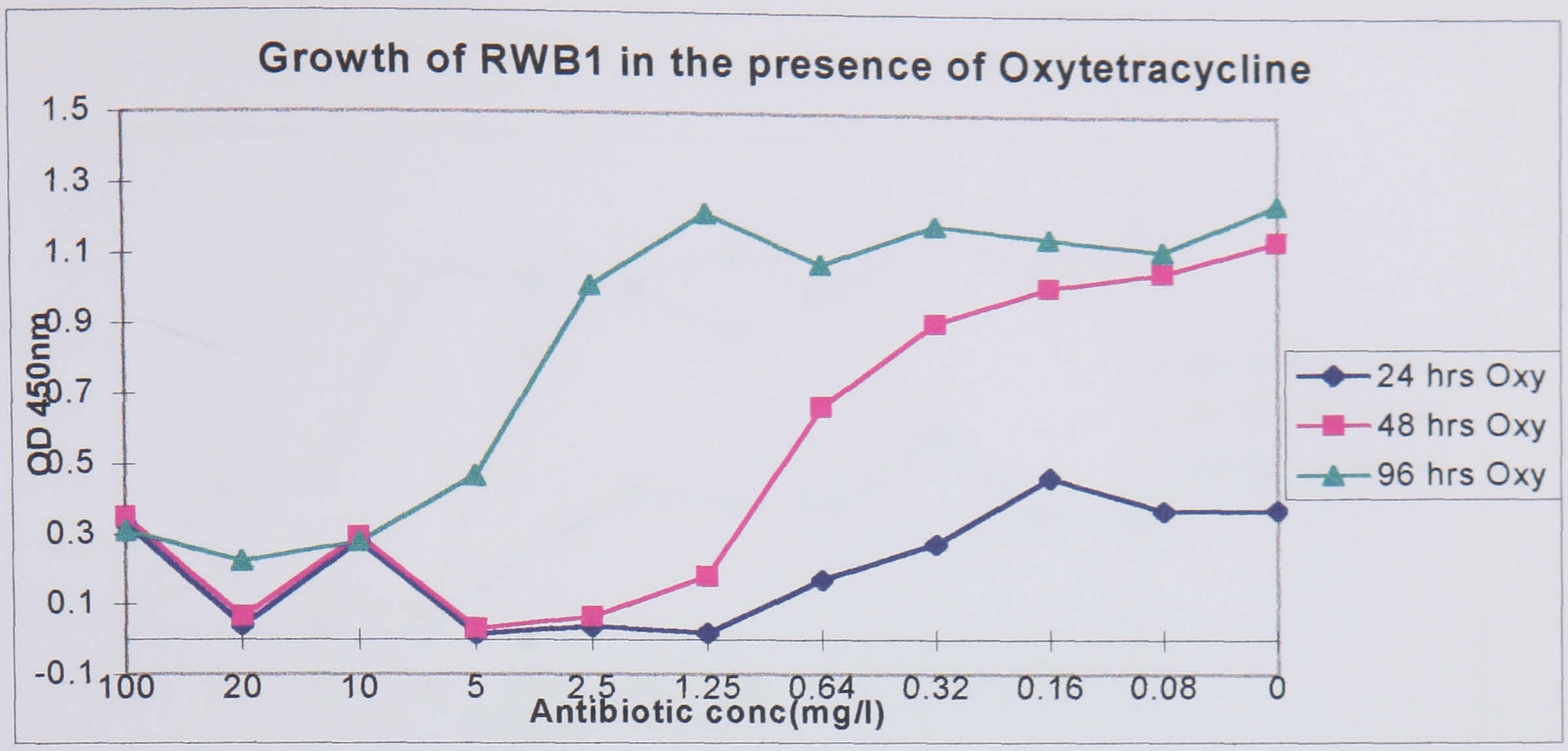
In all cases some of the concentrations that after the first 24 hours appeared to inhibit growth completely, did not do so after 96 hours. The MIC increased with time. In this way, bacteria that appeared not to be very resistant to antibiotic in the first instance turned out to be highly resistant. In all cases the bacteria were more resistant to the β-lactams than to the oxytetracycline.

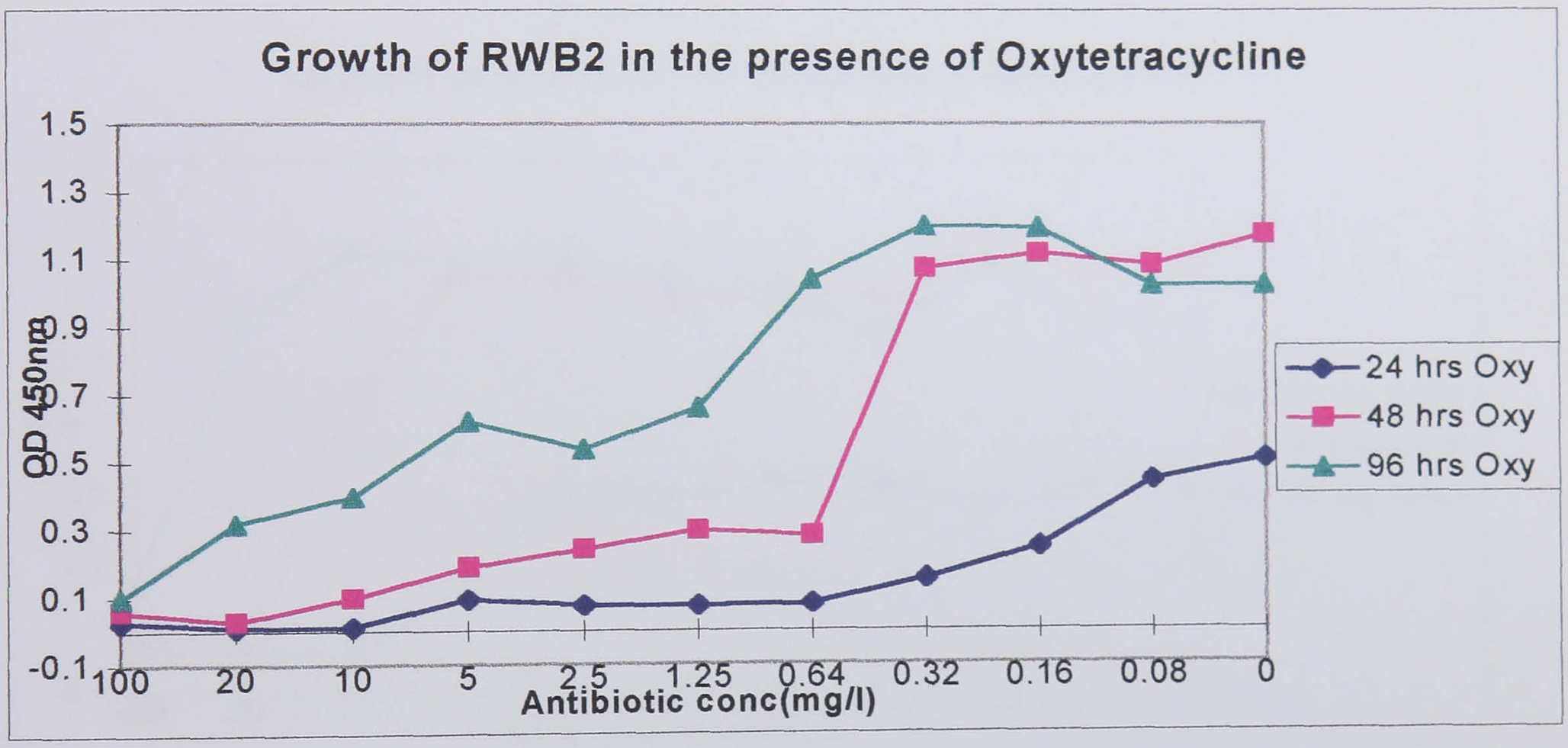
Results from the mixture of bacteria showed that it was resistant to both amoxycillin and cephalexin. This was surprising in the case of cephalexin, as none of the individual species were resistant. Again, even though resistant to 100mg/l of this antibiotic, there was still a visible effect on growth at 1.25 mg/l of the antibiotic. In the case of amoxycillin, though resistant, the NEC was much lower than that of RW3 alone, which was highly resistant to amoxycillin and present in the mixture.

Table 4.2 Microtitre plate growth experiment results. Three different bacteria grown in the presence of three different antibiotics

	RW1			RW2			RW3			RW1,2 +3		
	24hrs	**48hrs**	**96hrs**	**24hrs**	**48hrs**	**96hrs**	**24hrs**	**48hrs**	**96hrs**	**24hrs**	**48hrs**	**96hrs**
Amox (mg/l)												
MIC	5	100	100	10	20	100	Res	Res	Res	Res	Res	Res
NEC	1.25	1.25	5	2.5	5	10	100	100	100	2.5	0.32	2.5
Ceph (mg/l)												
MIC	5	20	20	5	20	Res	10	20	100	Res	Res	Res
NEC	1.25	1.25	10	2.5	2.5	2.5	1.25	0.64	10	1.25	1.25	1.25
Oxy (mg/l)												
MIC	1.25	2.5	10	10	20	100	5	5	10	2.5	10	20
NEC	0.16	0.08	1.25	0.08	0.32	0.32	1.25	2.5	2.5	0.08	0.08	0.08
Mixture (mg/l)												
(Total Ab)												
MIC	2.5	5	10	5	10	100	10	20	100	20	20	100
NEC	0.08	0.08	0.08	0.32	0.32	0.32	0.16	0.32	0.64	0.16	0.16	0.16
Mixture (mg/l)												
(Each Ab)												
MIC	0.83	1.67	3.33	1.67	3.33	33.33	3.33	6.67	33.33	6.67	6.67	33.33
NEC	0.03	0.03	0.03	0.11	0.11	0.11	0.05	0.11	0.21	0.05	0.05	0.05

Figure 4.4: Growth of three river water bacteria in the presence of decreasing concentrations of oxytetracycline





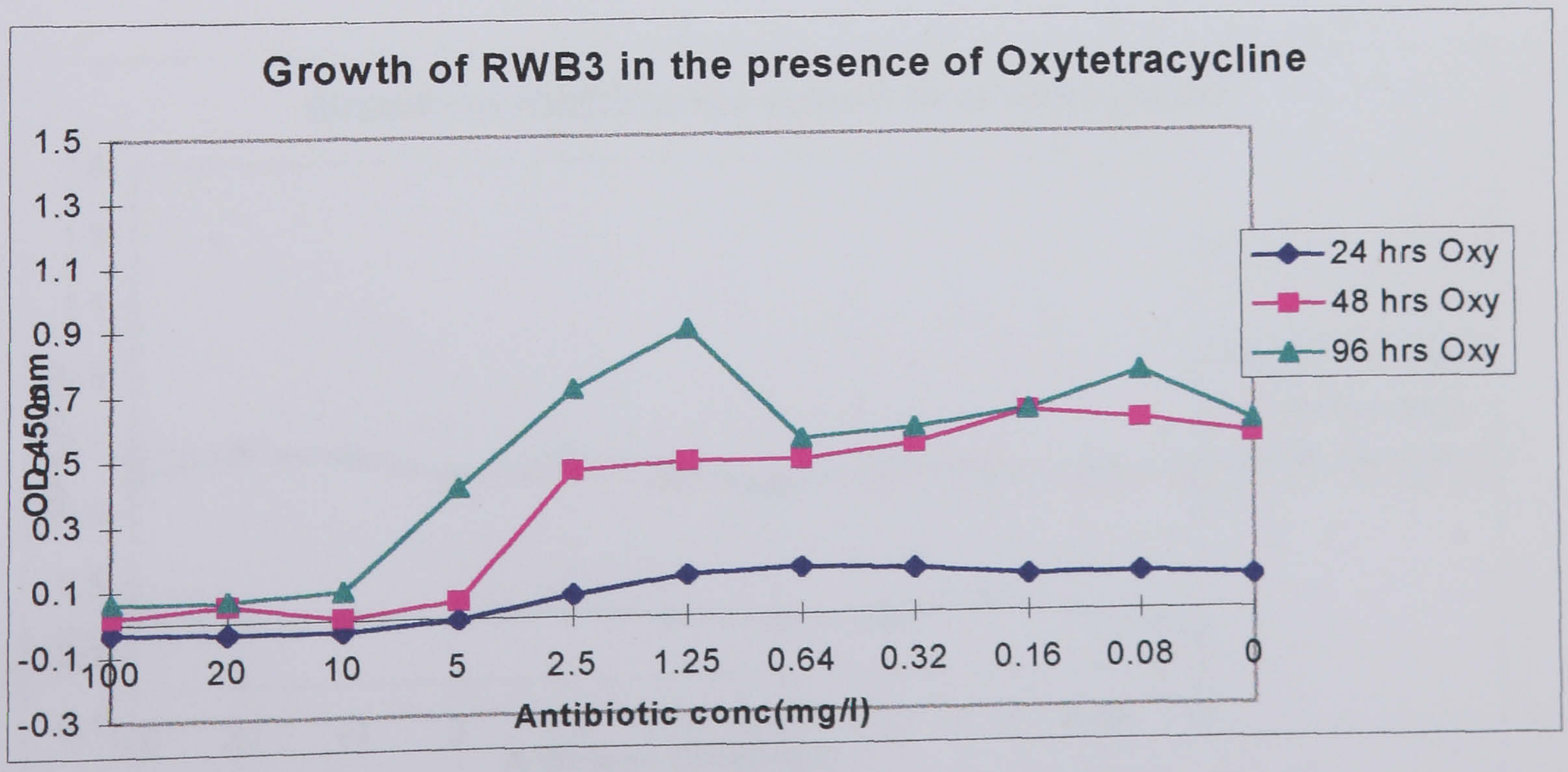
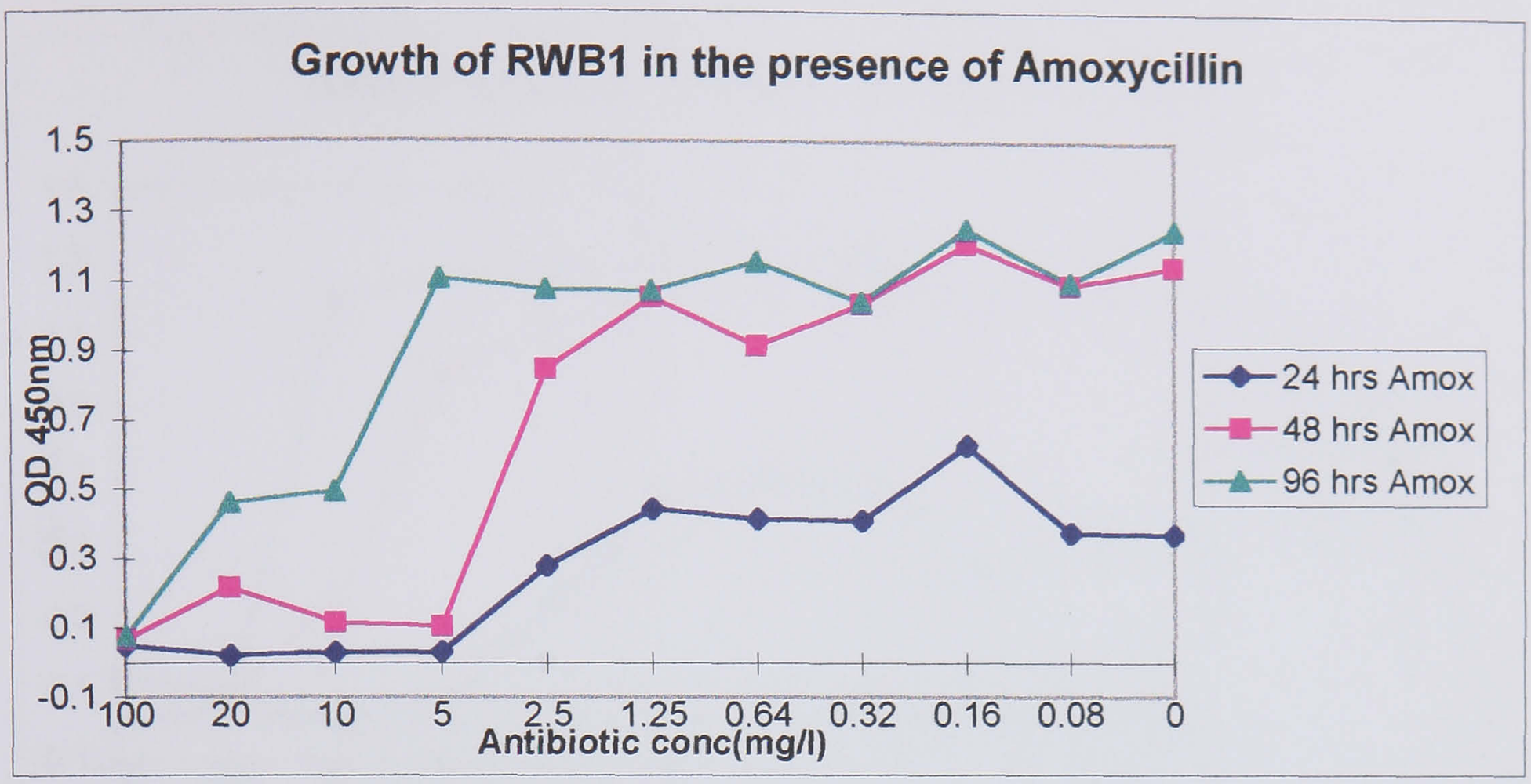
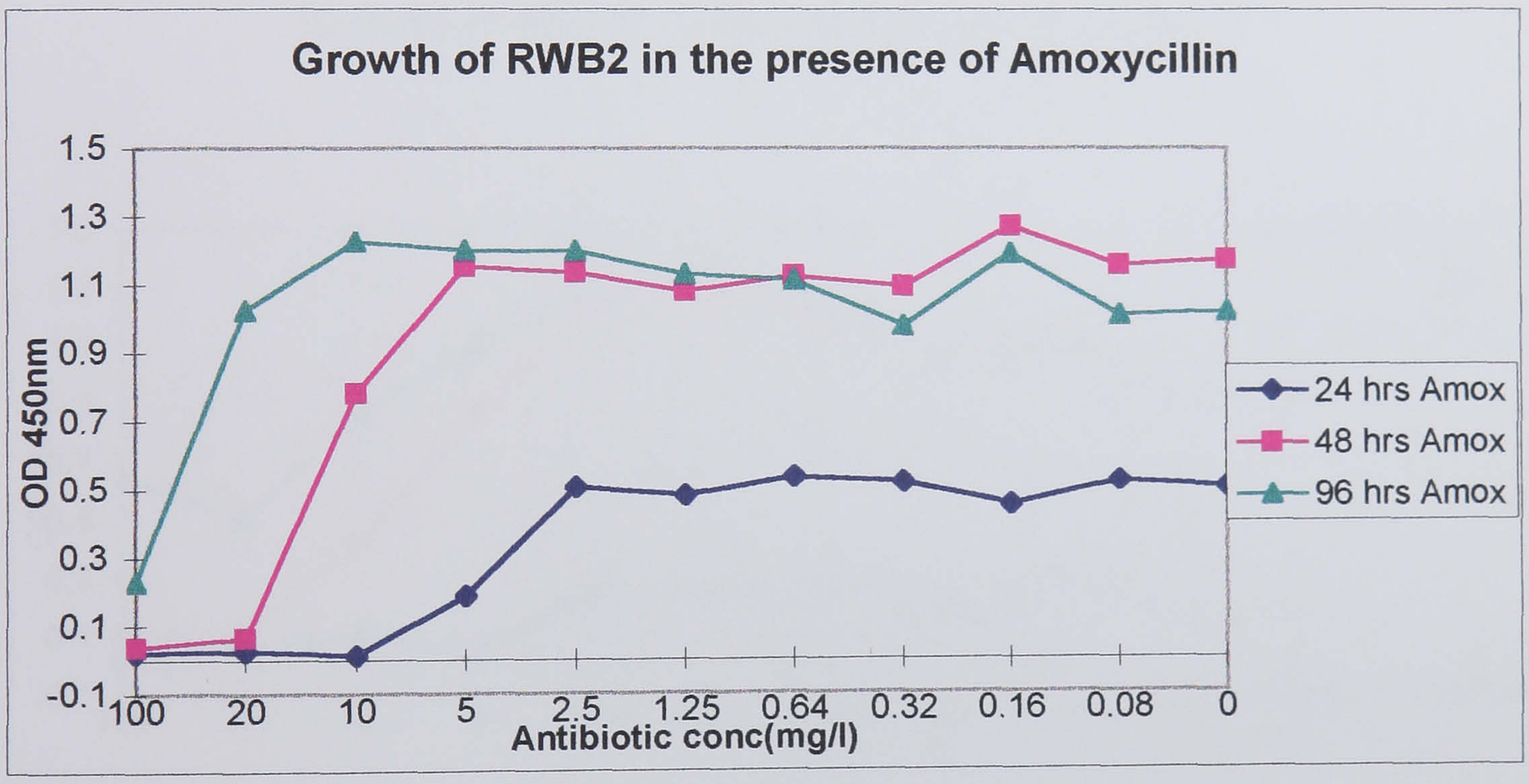


Figure 4.5: Growth of three river water bacteria in the presence of decreasing concentrations of amoxycillin





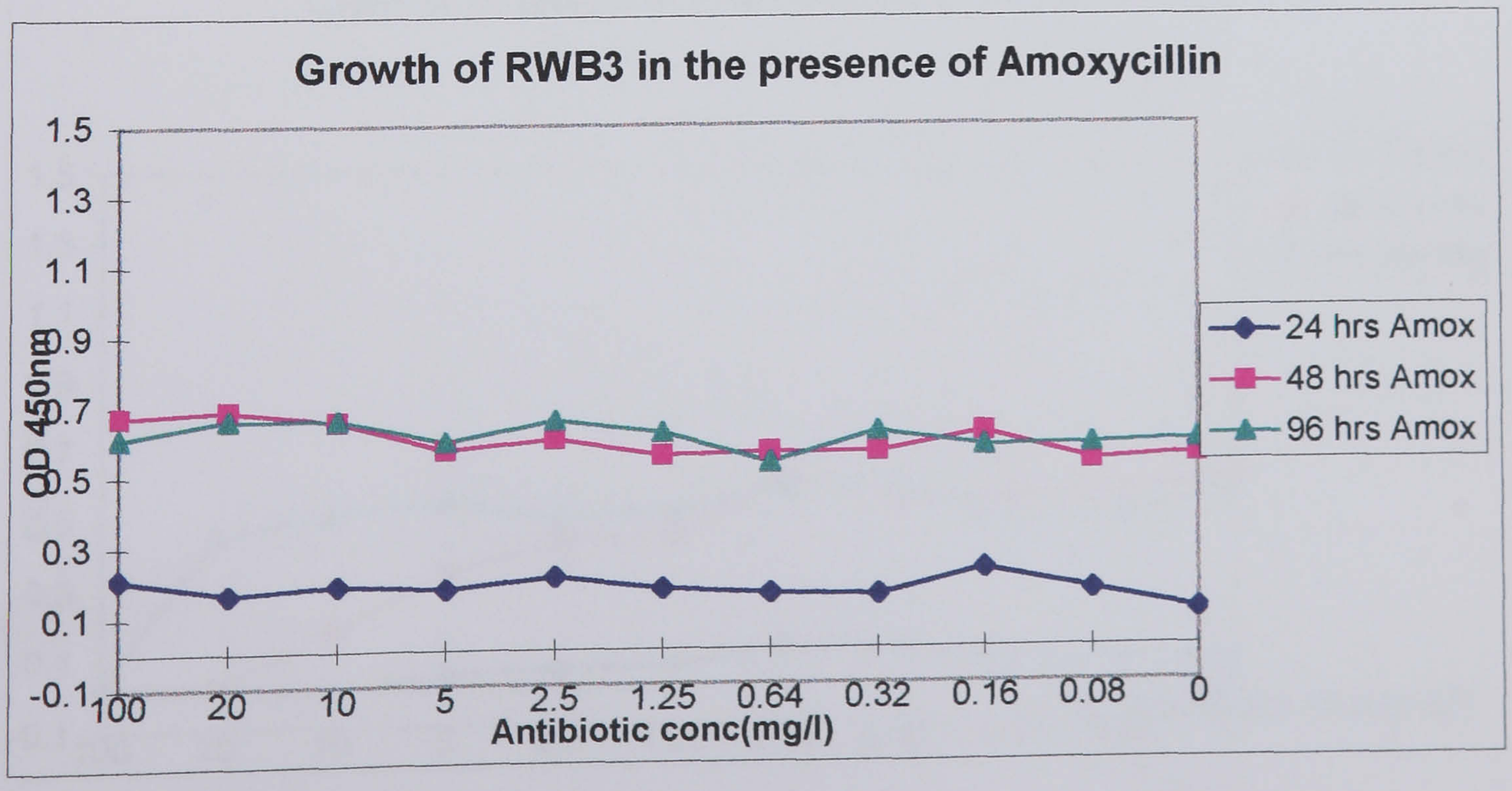
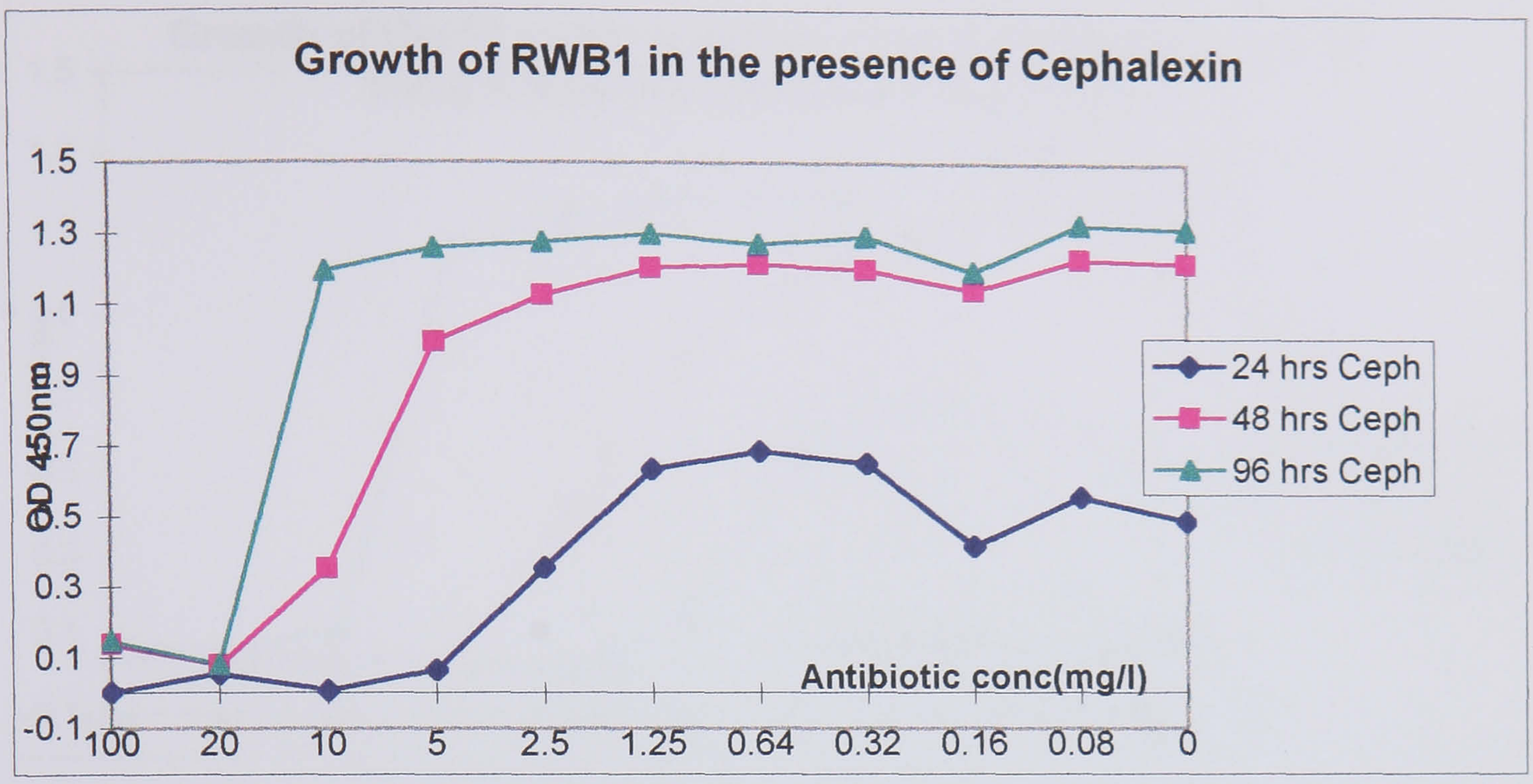
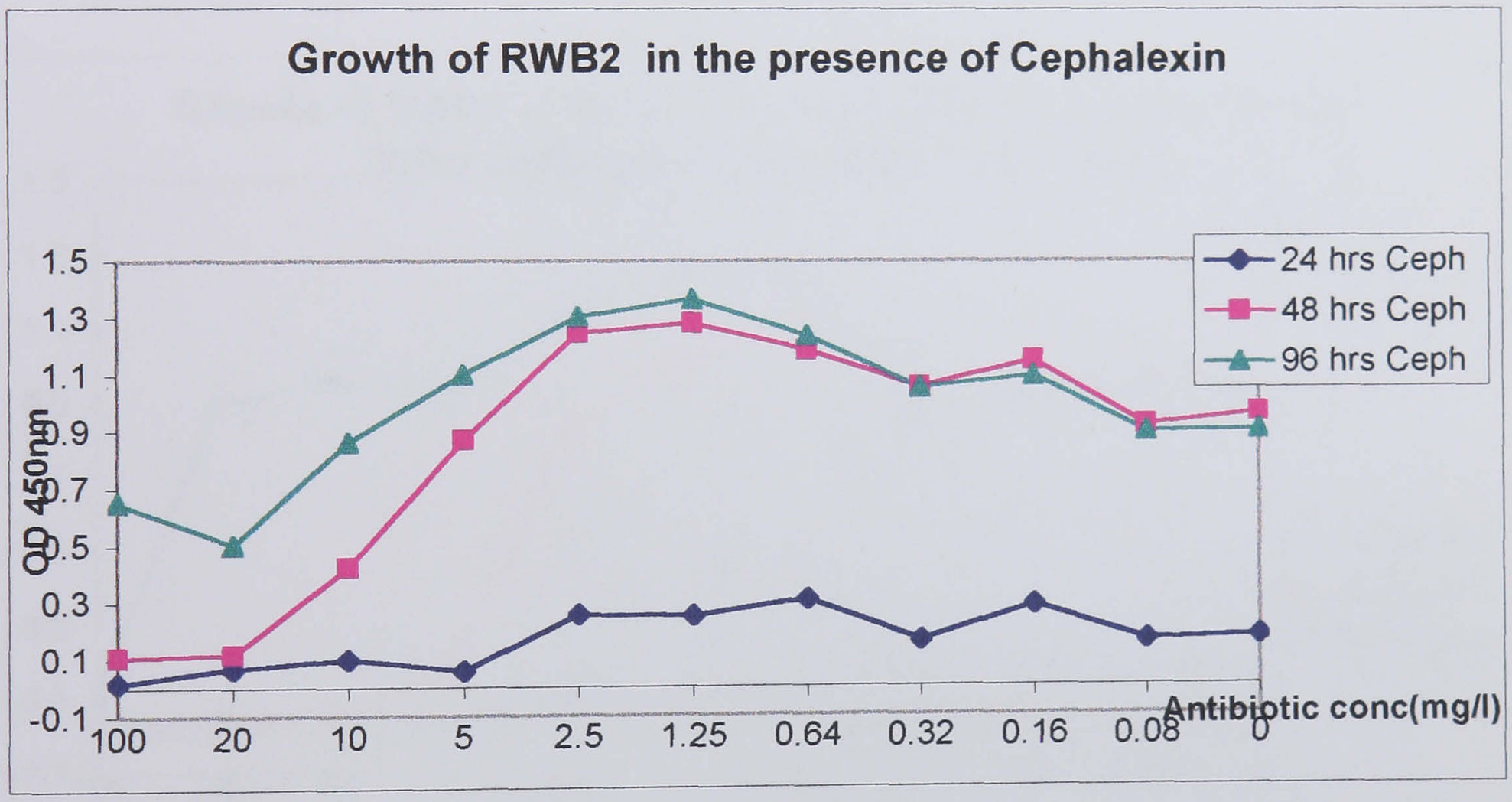


Figure 4.6: Growth of three river water bacteria in the presence of decreasing concentrations of cephalexin





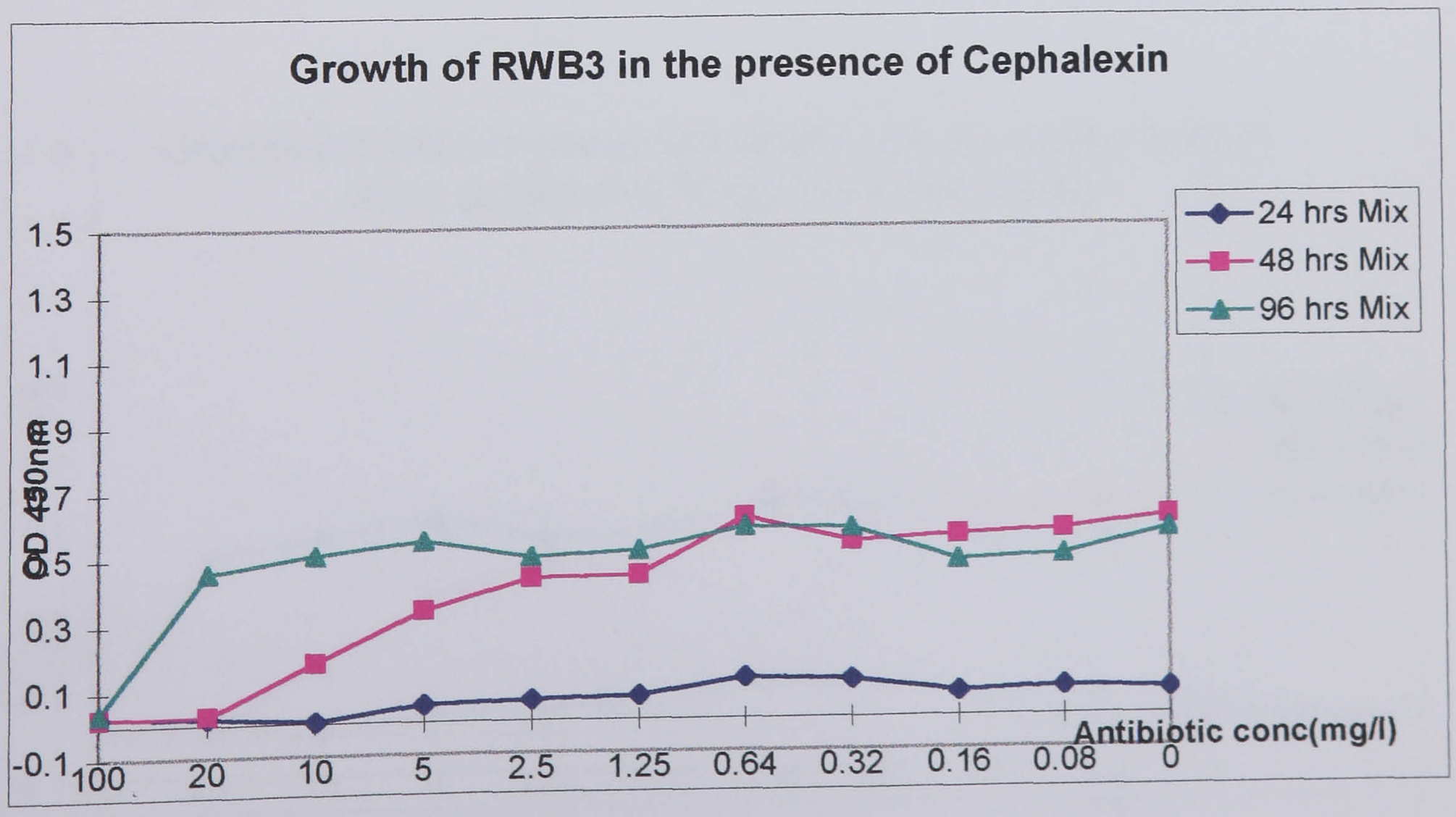
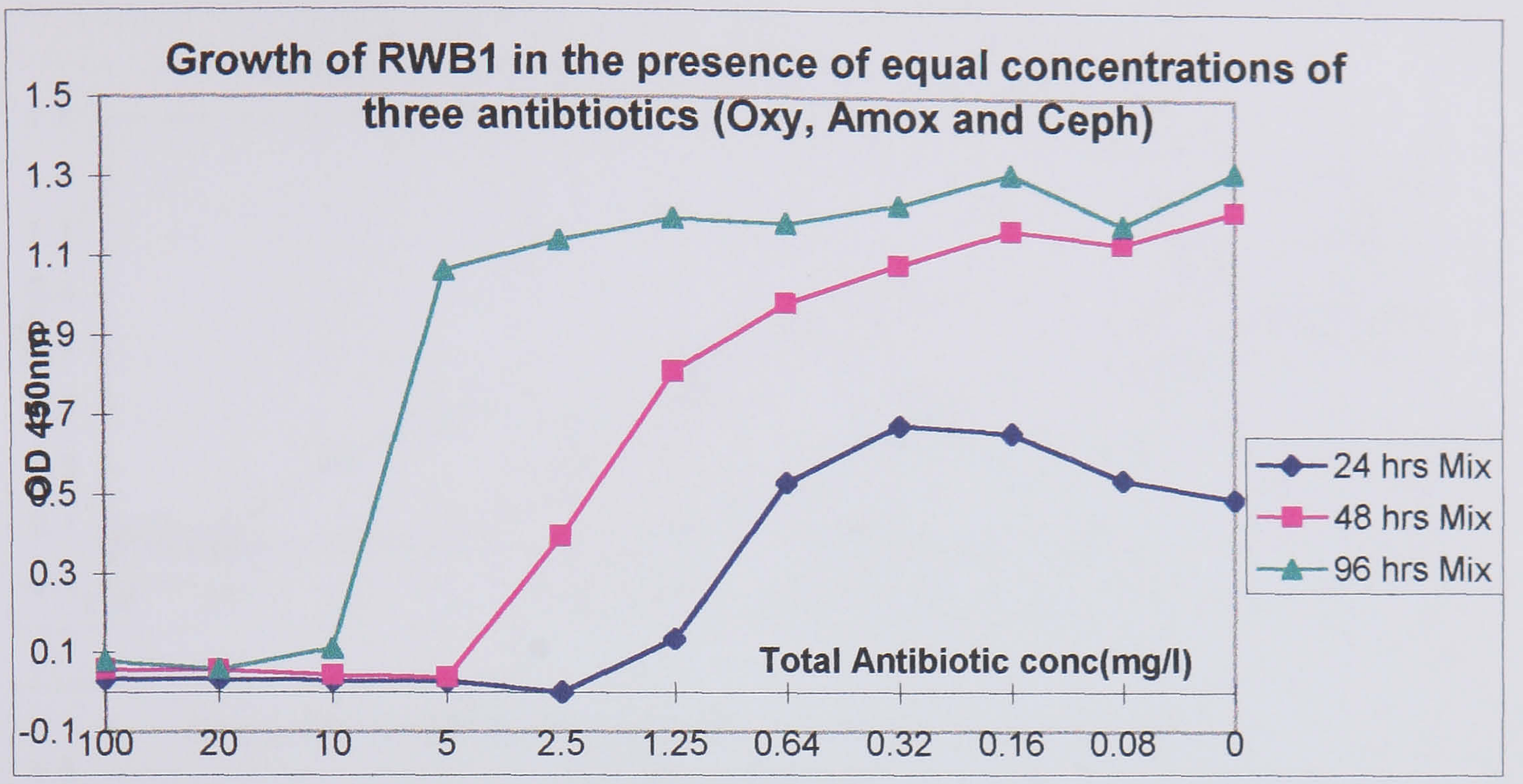
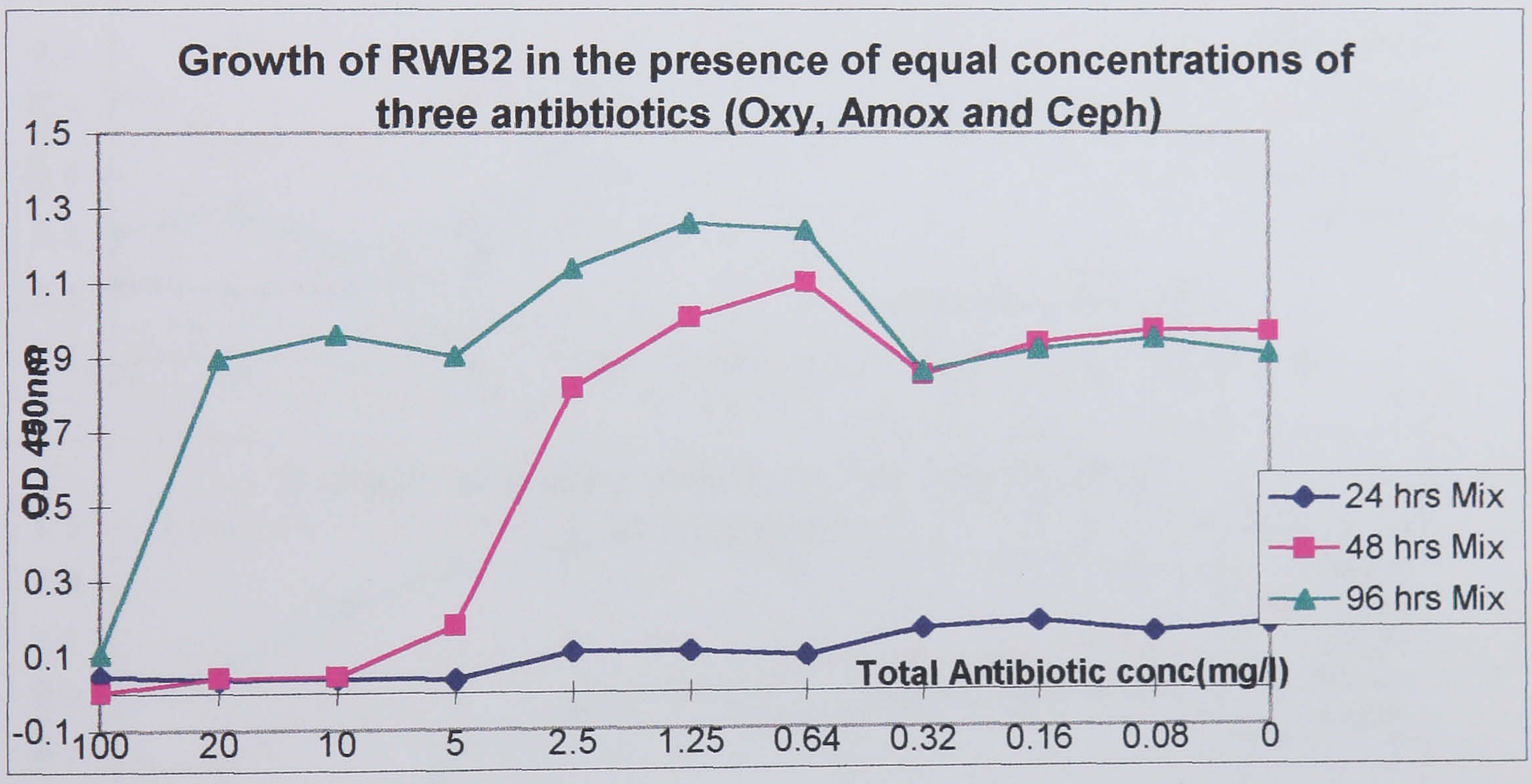


Figure 4.7: Growth of three river water bacteria in the presence of decreasing concentrations of a mixture of cephalexin. amoxycillin and oxytetracycline.





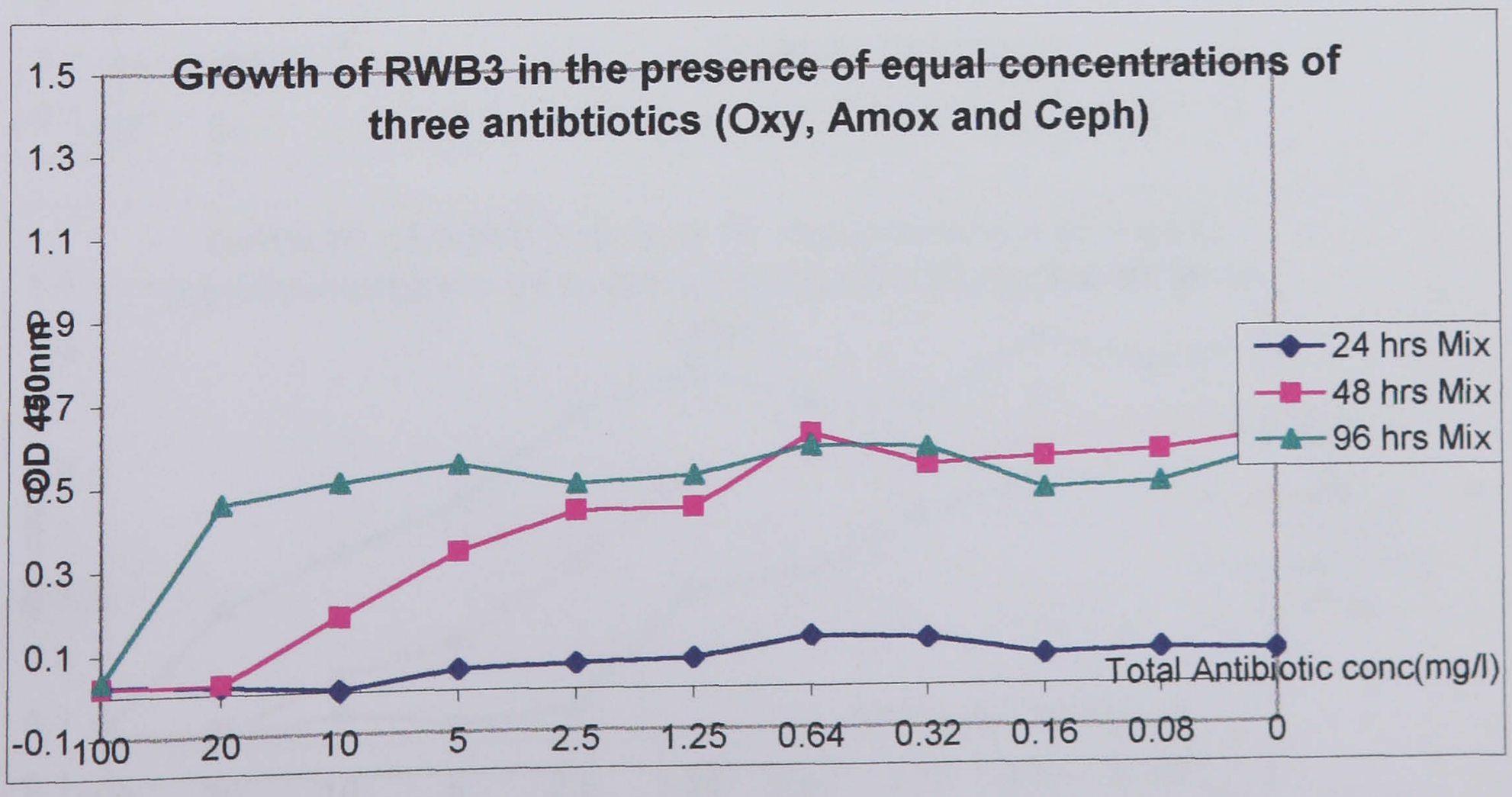
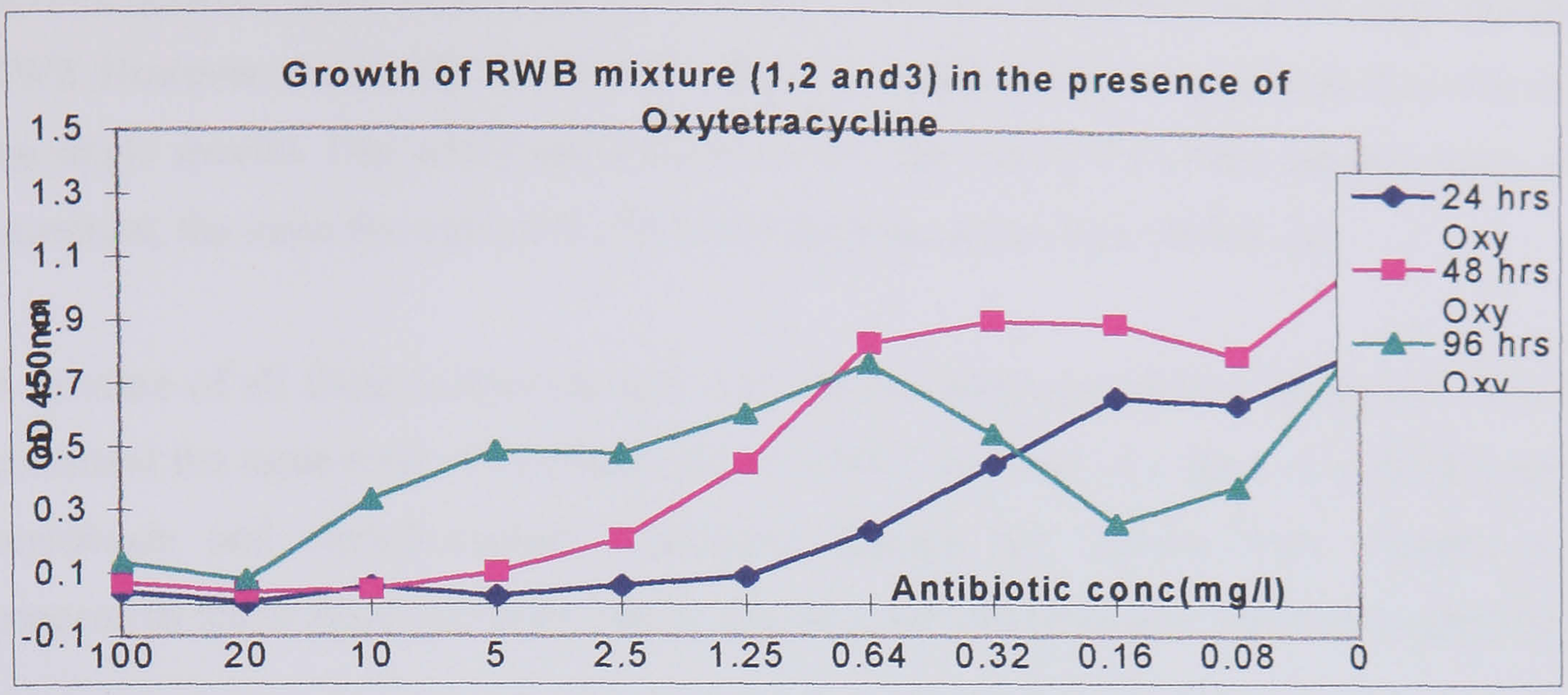
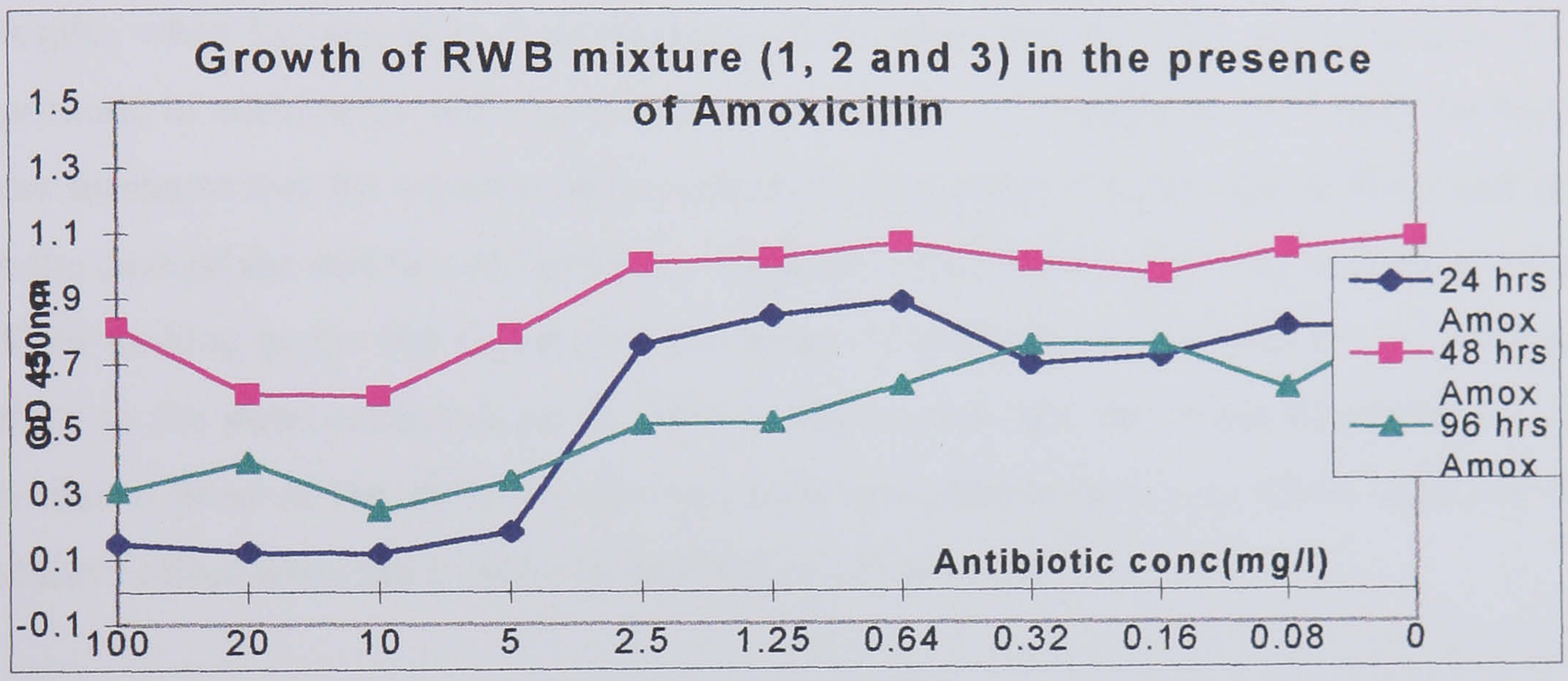
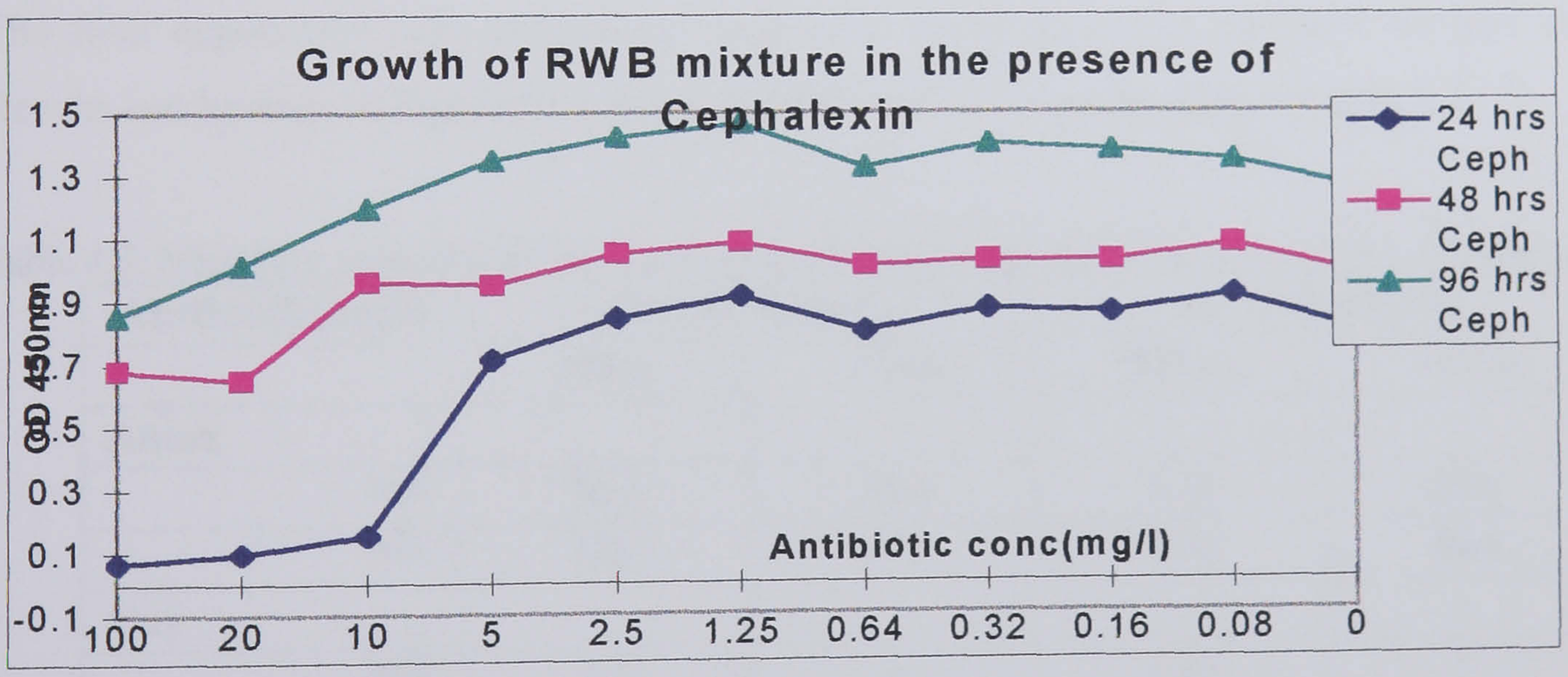
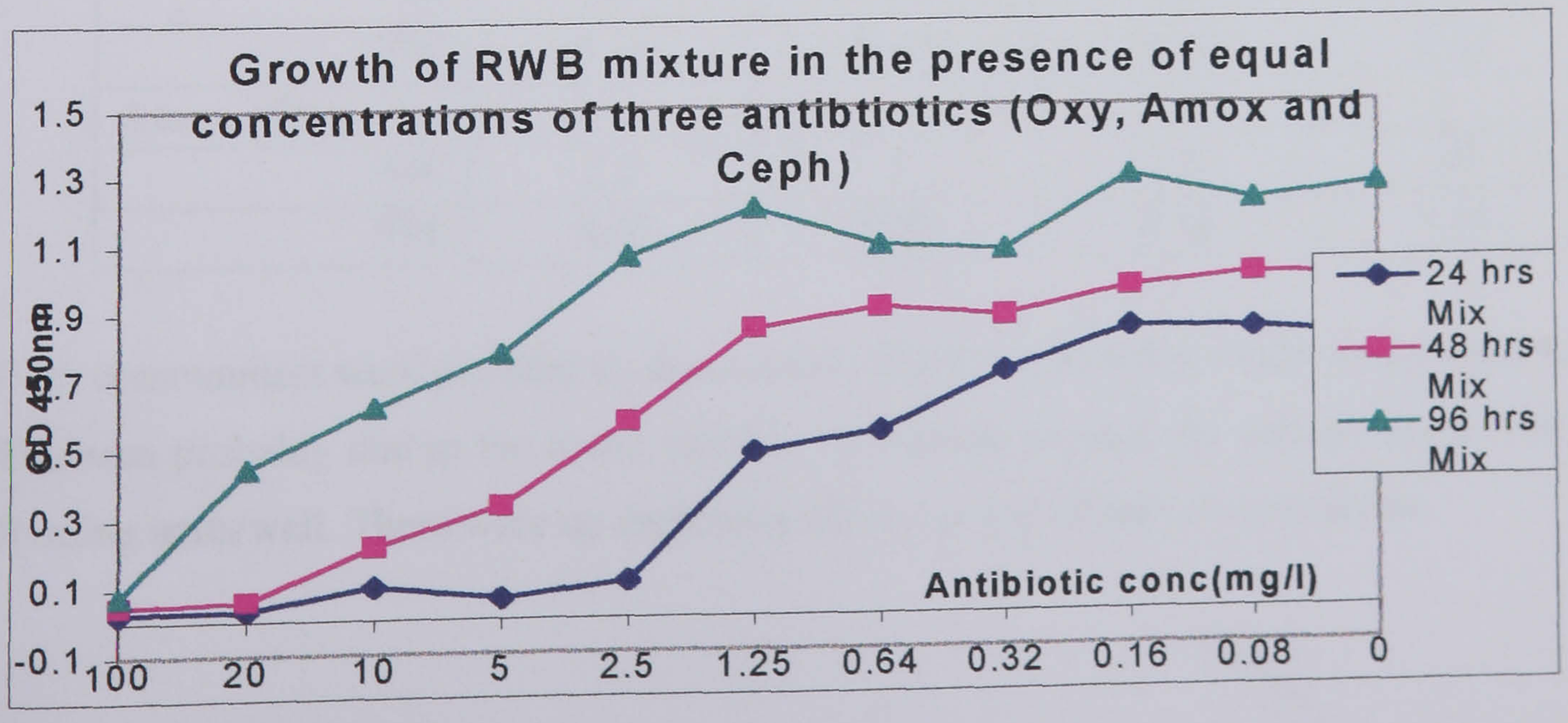


Figure 4.8: Growth of a mixture three river water bacteria in the presence of decreasing concentrations of cephalexin. amoxycillin and oxytetracycline, individually and as a mixture.









Effects of oxytetracycline on the mixture of bacteria showed resistance to antibiotic levels that were in between those of the less resistant species, RW1 and RW2, and the more resistant one, RW2. However, the results for the NEC showed a much lower concentration than that of any of the single species. One additional difference was that the NECs of the mixture were, with one exception, the same throughout the 96 hours the experiment was carried out.

A mixture of all three antibiotics was also used to determine effects on growth. The mixture contained the same total of antibiotic per millilitre, made up of a third of each of amoxycillin, cephalexin and oxytetracycline. Therefore, though the bacteria were exposed to lower concentrations of antibiotic individually, the total was the same as in the above experiments.

Results, when looking at total antibiotics present, show that the MIC was similar to that of the antibiotic to which each individual was most sensitive. For example, RW1 MIC for the mixture was similar to that for oxytetracycline. The NEC was reduced in the case of RW3 and increased in the case of the mixture of bacteria, compared to the lowest values for individual antibiotics. When looking at the individual concentrations of antibiotic in the mixture and comparing its effect to the same antibiotic on its own, it can be seen that the levels that have an effect are similar to those of the antibiotic the bacterium was most sensitive to. There appeared to be no additive effect when the mixture of antibiotics was present.

The final experiment was carried out used river water and river sediment to seed the agar. Results can be seen in Figures 4.9 and 4.10 MIC and NEC are detailed in Table 4.3.

Table 4.3: Microtitre experiments. MIC and NEC of bacteria present in river water and sediment samples

Antibiotic(mg/l)	**River Water**		**River Sediment**	
	48hrs	**72hrs**	**48hrs**	**72hrs**
Amox				
MIC	Res	Res	Res	Res
NEC	Res	Res	Res	Res
Oxy				
MIC	2.5	5	1.25	5
NEC	0.16	0.16	0.16	0.32
Amox +Oxy				
MIC	2.5	5	2.5	20
NEC	0.08	0.08	0.16	0.64

Both communities were resistant to amoxycillin. Results were more erratic in the water samples. This was probably due to the lower number of bacteria present, on average about 100 colony forming units/well. There were no detectable effects at any of the concentrations.

Figure 4.9: Results from river water microtitre growth experiments.

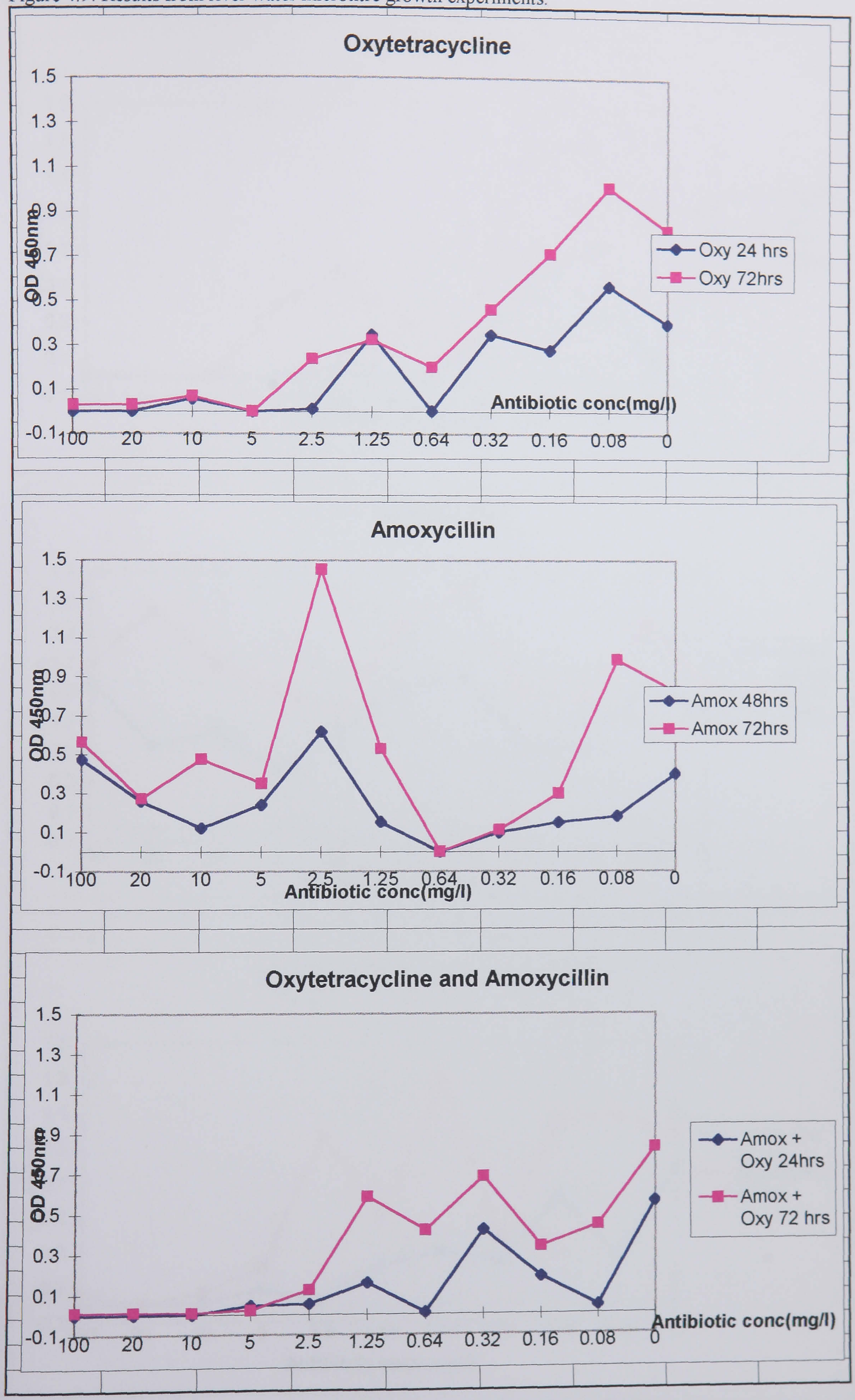
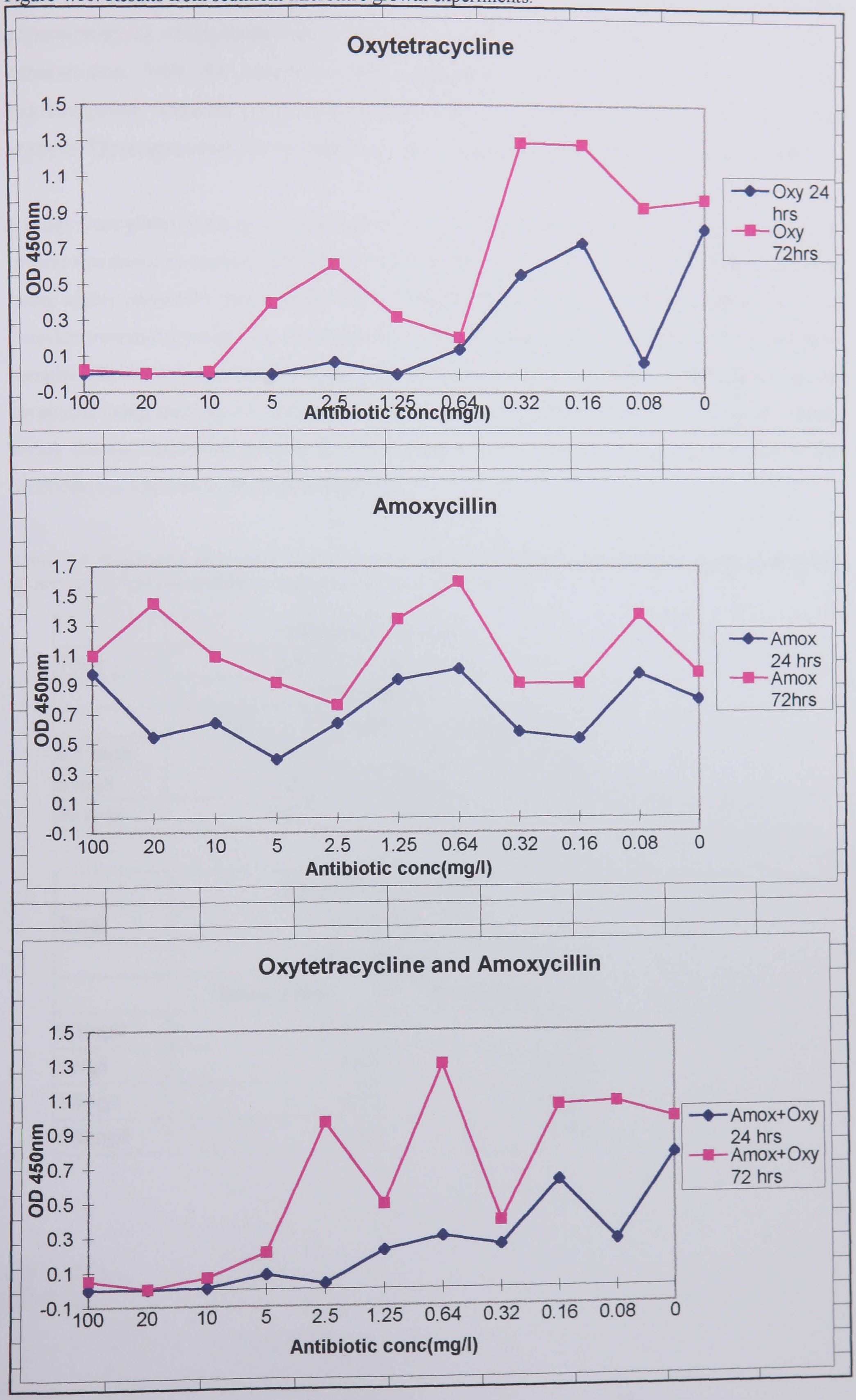


Figure 4.10. Results from sediment microtitre growth experiments.



In the case of oxytetracycline, the MIC and NEC were similar for both communities. Again, the concentration at which there was no effect was much lower, at least a tenth, of the MIC concentration. With the mixture of both antibiotics the results were similar to that for oxytetracycline, when the actual concentration (half of the total) in the mixture was taken into account. There appeared to be no impact due to the presence of the amoxycillin in the mixture.

Results from plate counts of water and sediment samples outlined in Table 4.4 show that there is greater resistance to amoxycillin in both bacterial communities with at least 10% of the bacteria being highly resistant to amoxycillin. In the case of tetracycline only 0.02% are highly resistant. Another interesting result was that resistance to amoxycillin and tetracycline seen in sediment samples (Table 4.4) showed no significant differences from resistance to tetracycline when compared using analysis of variance. This confirms results obtained using the microtitre plates which showed resistance patterns to the mixture of antibiotics to be the same as that of the antibiotic the bacteria were most sensitive to.

Table 4.4: Resistance of bacteria from sediment and water samples taken from the River Thames and grown on YEA plates containing various antibiotic concentrations.

	Sediment Samples		
Total	5.9×10^4/g (wet weight)		
	% Resistance		
	Tet.	**Amox.**	**Amox. + Tet.**
0.1 mg/l	60.84	73.99	55.28
5 mg/l	2.69	44.25	1.28
50 mg/l	0.02	16.27	0.01

	River Water Samples	
Total	2.91×10^4/ml	
	% Resistance	
	Tetracycline	**Amoxycillin**
0.1mg/l	109.37	60.39
1mg/l	4.65	102.20
10mg/l	0.46	41.65
100mg/l	0.02	10.84

4.3.2. MUVS of Bacteria

This work was based on the theory that the mid-ultraviolet spectrum (MUVS) of bacteria changes when they are exposed to stress. The absorbance of UV light by bacteria peaks at around 200nm. Baines (1993) reported that the MUVS of bacteria was strongly affected by the presence of toxins. Among other compounds, there was a detectable effect on bacteria of sulphamethazine, an antibiotic, at 100 times less than the MIC. Changes seen were reduction of peaks and changes of maximum absorbance wavelength. This method was thought to work due to the changes in haem oxidation in the oxidative phosphorylation pathway. Work was carried out to determine the suitability of this method as a measurement of the impact of sublethal levels of antibiotics on bacteria.

The first experiment was carried out using a National Type Culture Collection *E. coli* strain 10650. Following Bains' method the bacteria had antibiotic added after being grown up, and UV absorbance measured. In addition bacteria were grown in 0.1mg/l of either oxytetracycline or amoxycillin and their absorbance measured.

Figure 4.11 MUVS of E.coli exposed to oxytetracycline and amoxycillin. Antibiotic concentrations in mg/l.

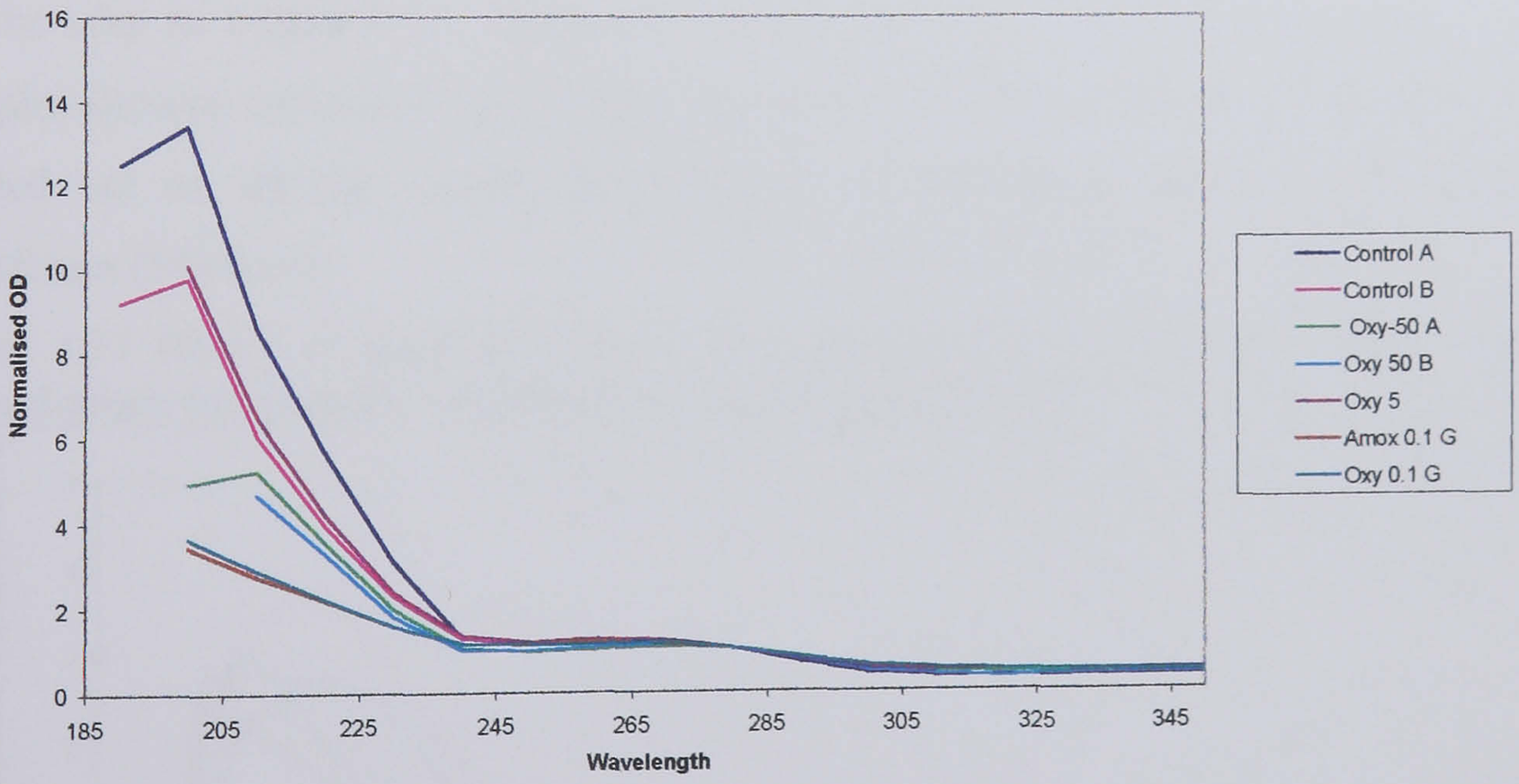
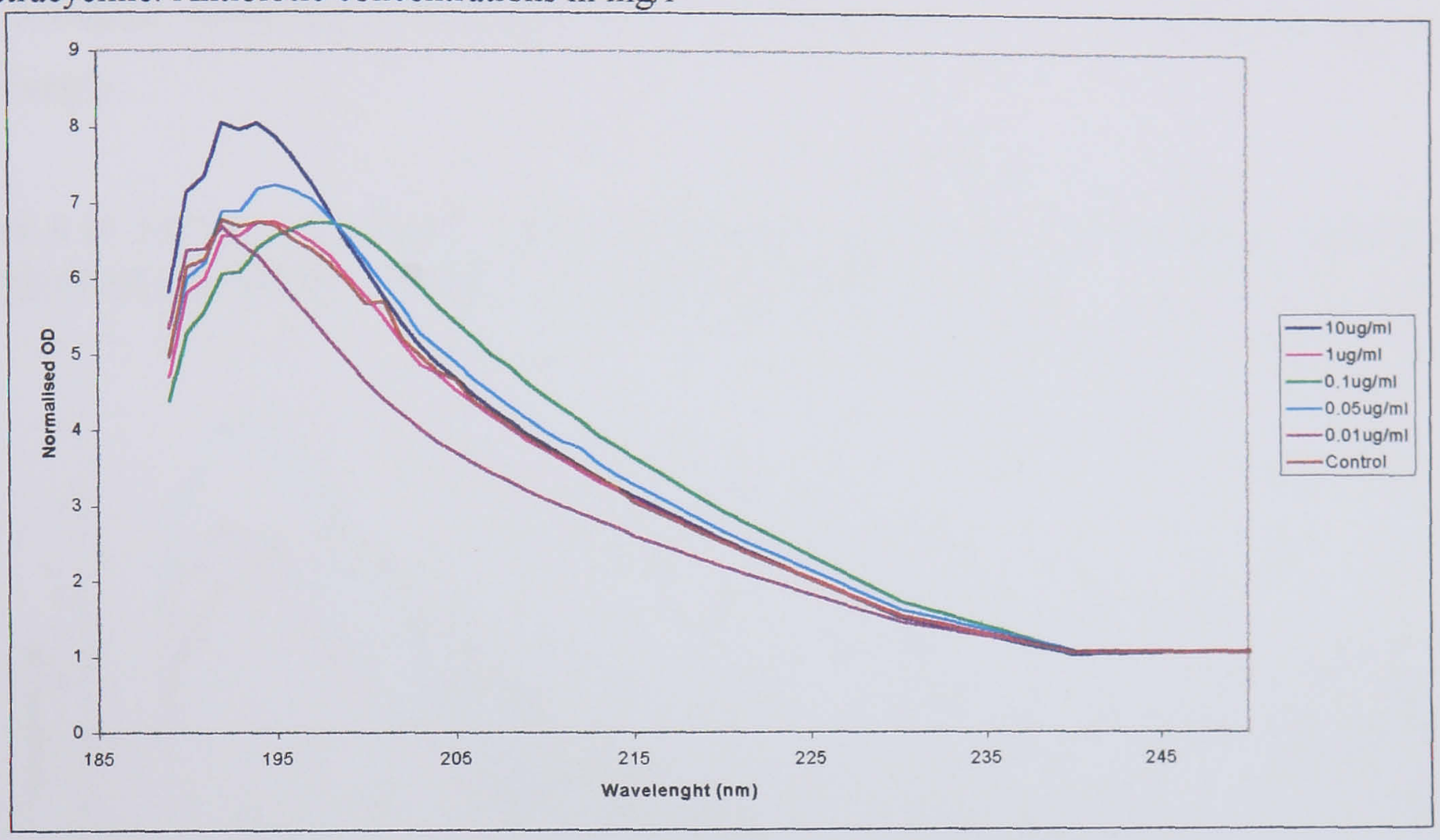


Figure 4.11 shows how reduced absorbance could be seen at 50mg/l but not at 5mg/l when bacteria were exposed to antibiotic after incubation. In the growth experiments, where antibiotic was added before incubation, a 50 times lower concentration had a much stronger impact. The effect of amoxycillin or oxytetracycline at 0.1mg/l was indistinguishable. Analysis of variance was carried out and differences were significant at the 5% level.

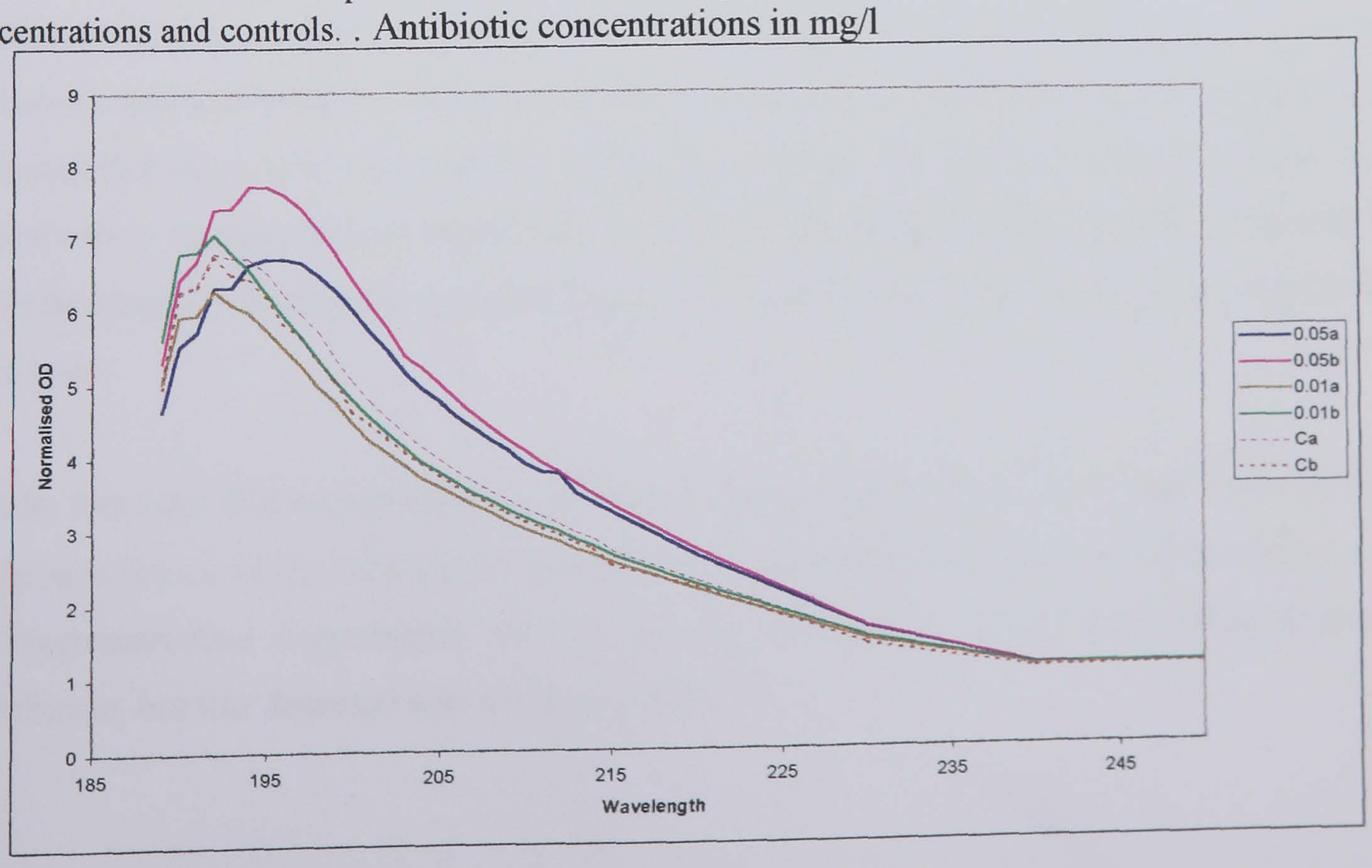
The above results appeared promising, so further experiments were carried out growing the same strain of *E.coli* in the presence of various concentrations of oxytetracycline. Duplicate samples with the same antibiotic concentrations were grown up separately and MUVS then measured. When looking at the relationship between MUVS changes and antibiotic concentration (Figure 4.12) no clear pattern emerged

Figure 4.12: Mean MUVS of duplicate samples of *E.coli* 10650 grown in various concentrations of oxytetracycline. Antibiotic concentrations in mg/l



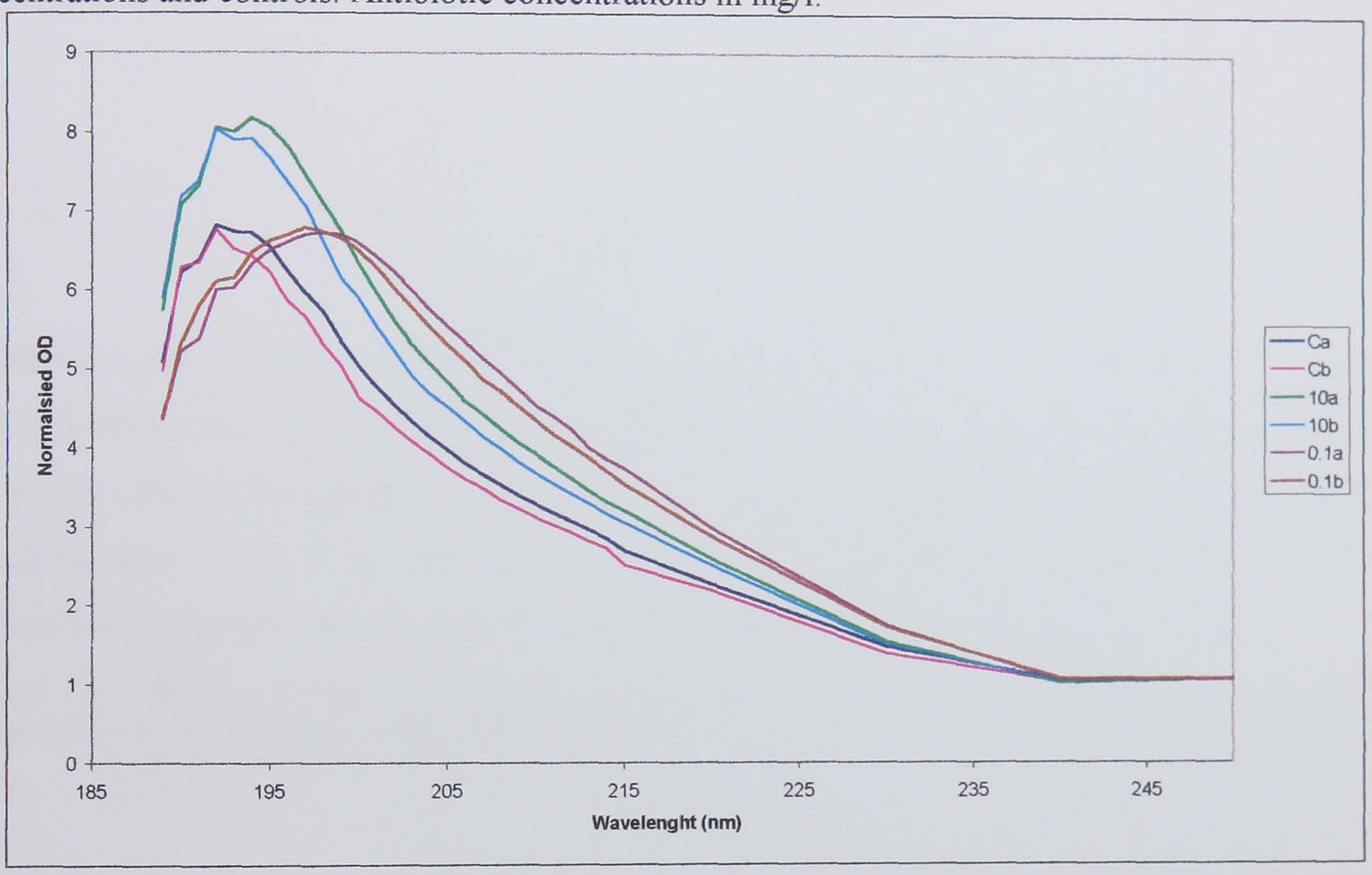
When comparisons were carried out between two concentrations only, differences became visible. The results from growing the culture in two different concentrations of oxytetracycline can be seen in Figure 4.13. There was a clear difference between the samples, and duplicate samples showed strikingly similar peak shapes. In addition, a two-way analysis of variance was carried out on all the results, this showed the differences between concentrations to be significant (5% level).

Figure 4.13 MUVS of duplicate E.coli 10650 samples grown up in two different oxytetracycline concentrations and controls. . Antibiotic concentrations in mg/l



The difficulty with these results was that the control samples, which were grown without any antibiotic, were difficult to distinguish from the other samples. They did not show the maximum or minimum absorbance of all samples, instead they were somewhere in the middle. There did not appear to be linear relationship between MUVS and antibiotic concentration, as can be seen in Figure 4.14. There were clear effects, which can be seen in this figure and the last one, at concentrations as low as 0.05mg/l, though this was not directly dose-related. Different concentrations increased or decreased absorbance and changed the maximum absorbance wavelength.

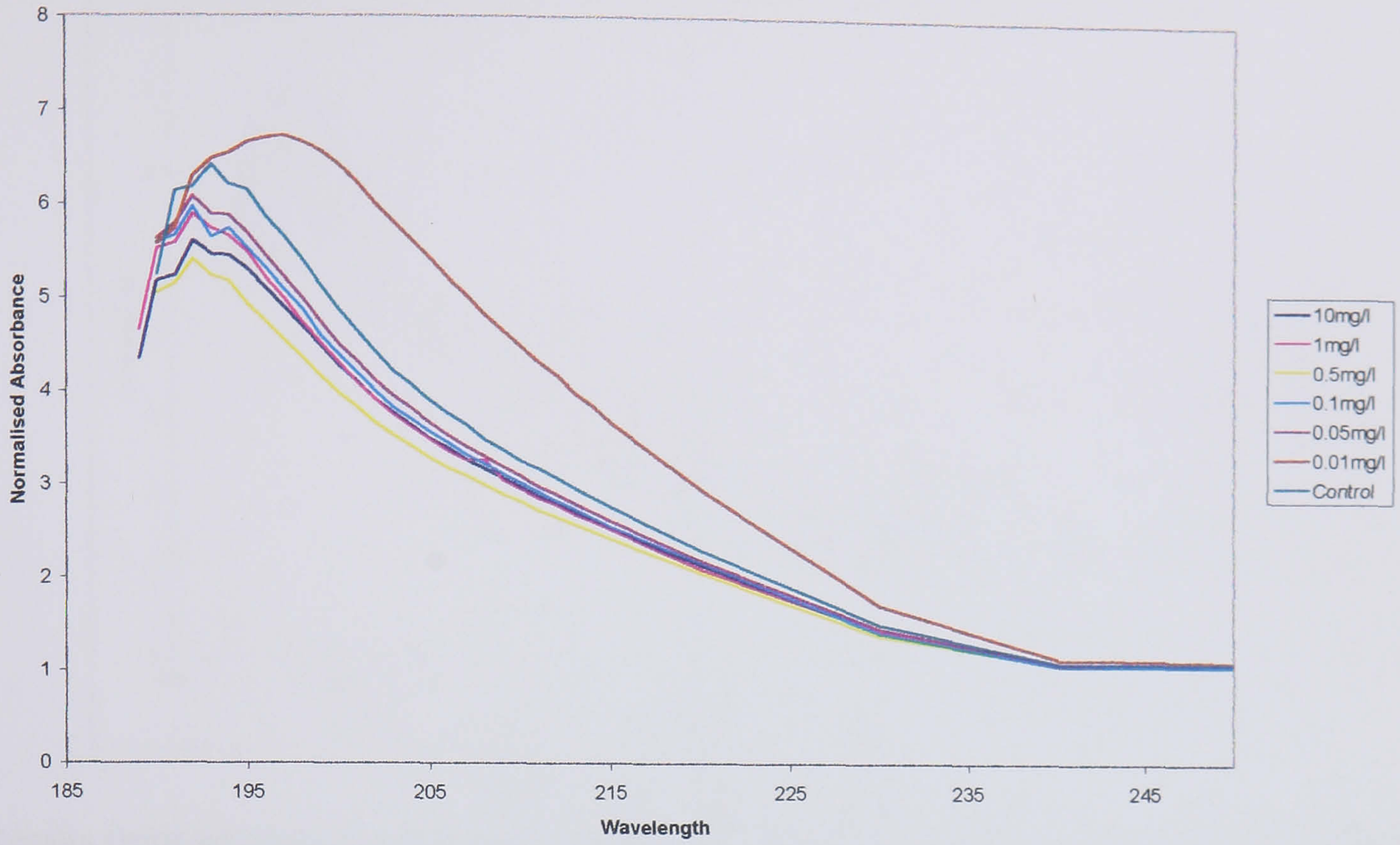
Figure 4.14 MUVS of duplicate *E.coli* 10650 samples grown up in two different oxytetracycline concentrations and controls. Antibiotic concentrations in mg/l.



Further work was carried out using the same bacterium and growing it up with amoxycillin, cephalexin and a mixture of oxytetracycline and amoxycillin. Results from all these experiments indicated that there was some kind of effect occurring, though again it was not a linear dose-related effect. Results proved statistically significant, but it was difficult to identify trends that could be related to changes in concentrations. The same difficulties as in the above experiments were seen.

Results from the first experiment can be seen in Figures 4.15. These show that when the mean of the absorbance of the various concentrations were plotted there were no visible trends. As in the oxytetracycline experiments all that can be determined is that amoxycillin decreased absorbance, but this decrease was not dose related.

Figure 4.15: Mean MUVS of duplicate samples of *E. coli* 10650 grown in various concentrations of amoxycillin. Antibiotic concentrations in mg/l.



Results for some of the concentrations can be seen in more detail in Figure 4.16. This shows that individual concentrations are not distinguishable from each other and that the controls span the whole range of absorbencies seen. The only exception to this is the 0.01mg/l concentration, which showed a shift of the absorbance peak to a higher wavelength. This is the clearest effect seen and it was at the lowest antibiotic concentration used in this experiment.

Figure 416: MUVS of duplicate *E.coli* 10650 samples grown up in three different amoxycillin concentrations and controls. Antibiotic concentrations in mg/l.

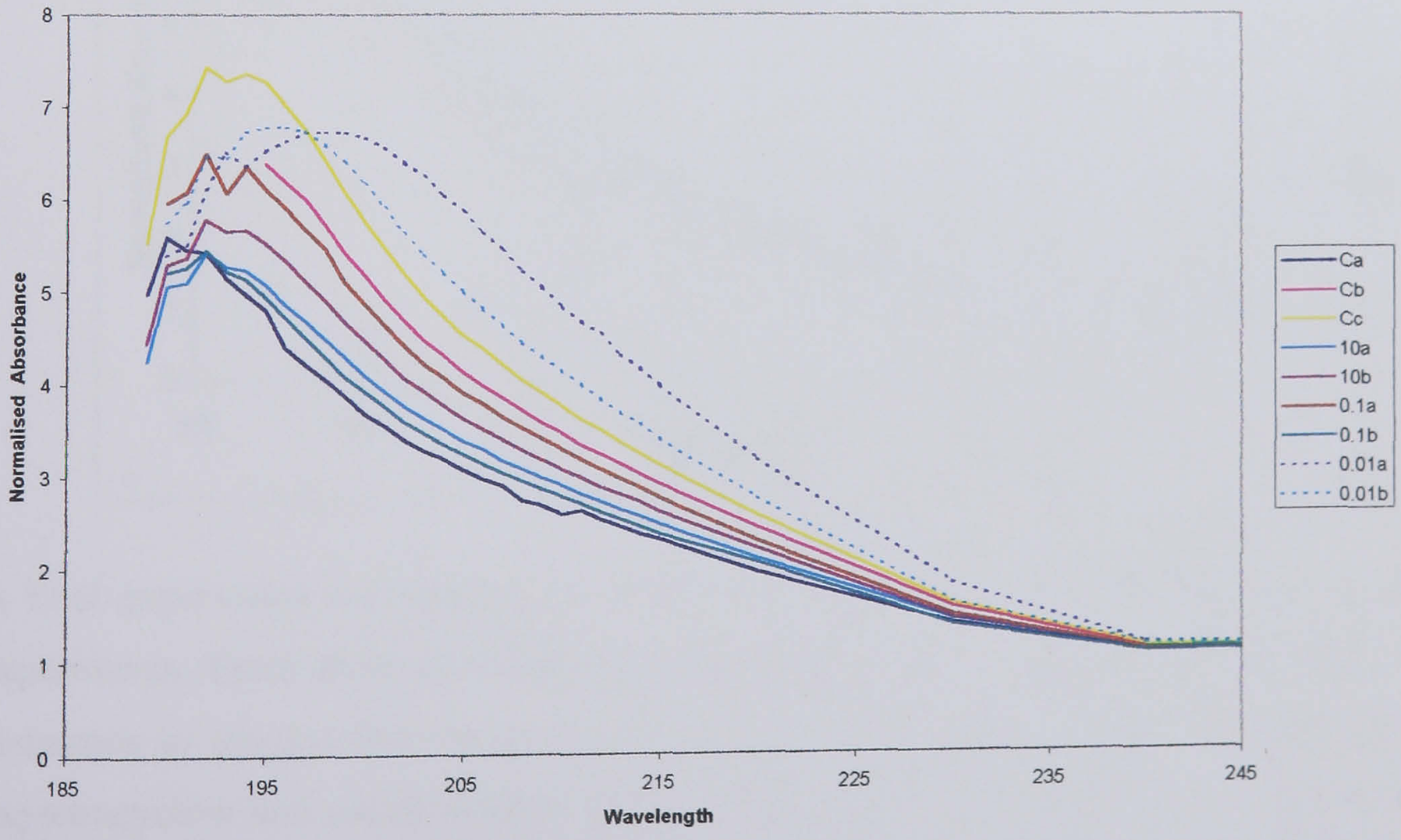
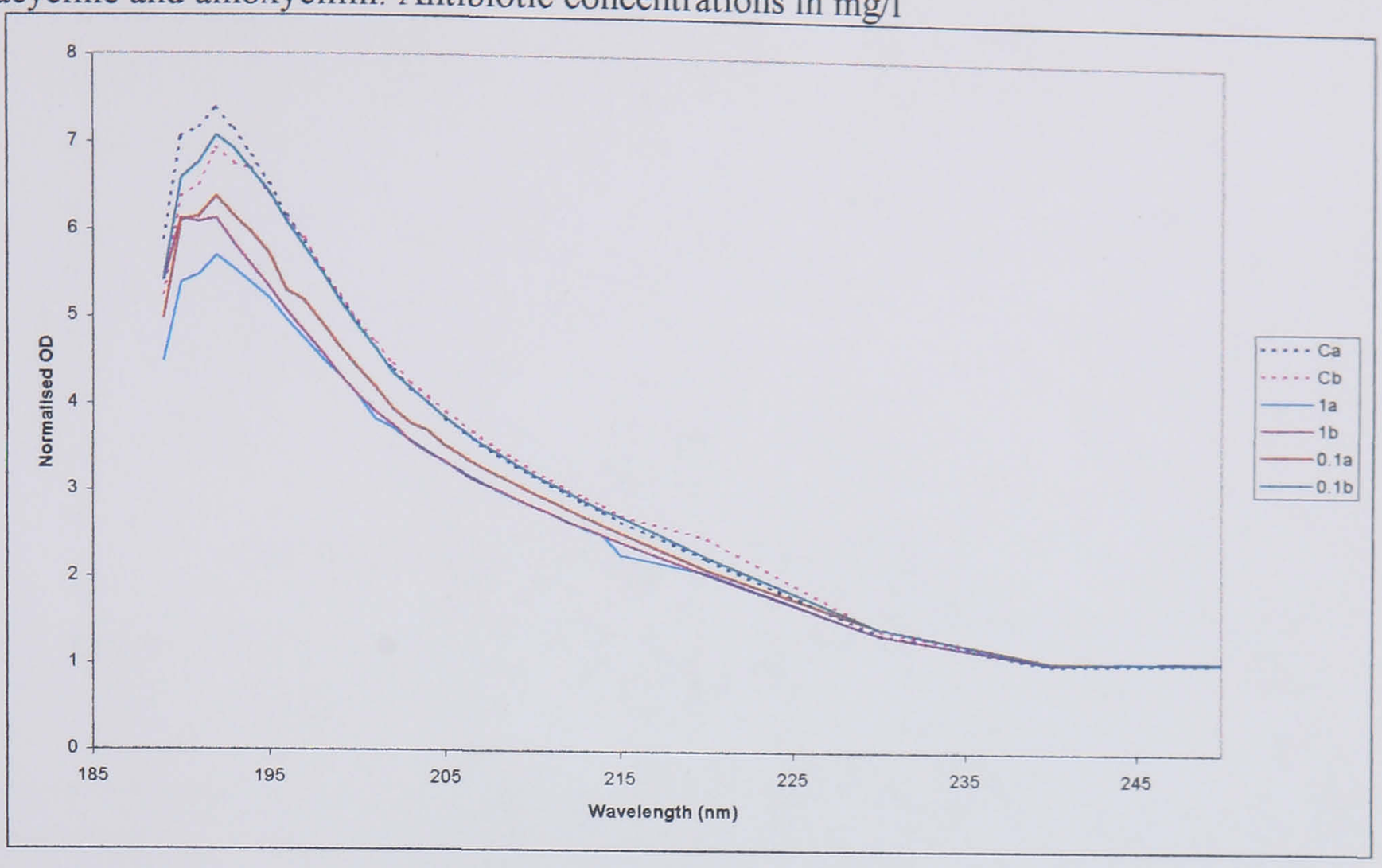


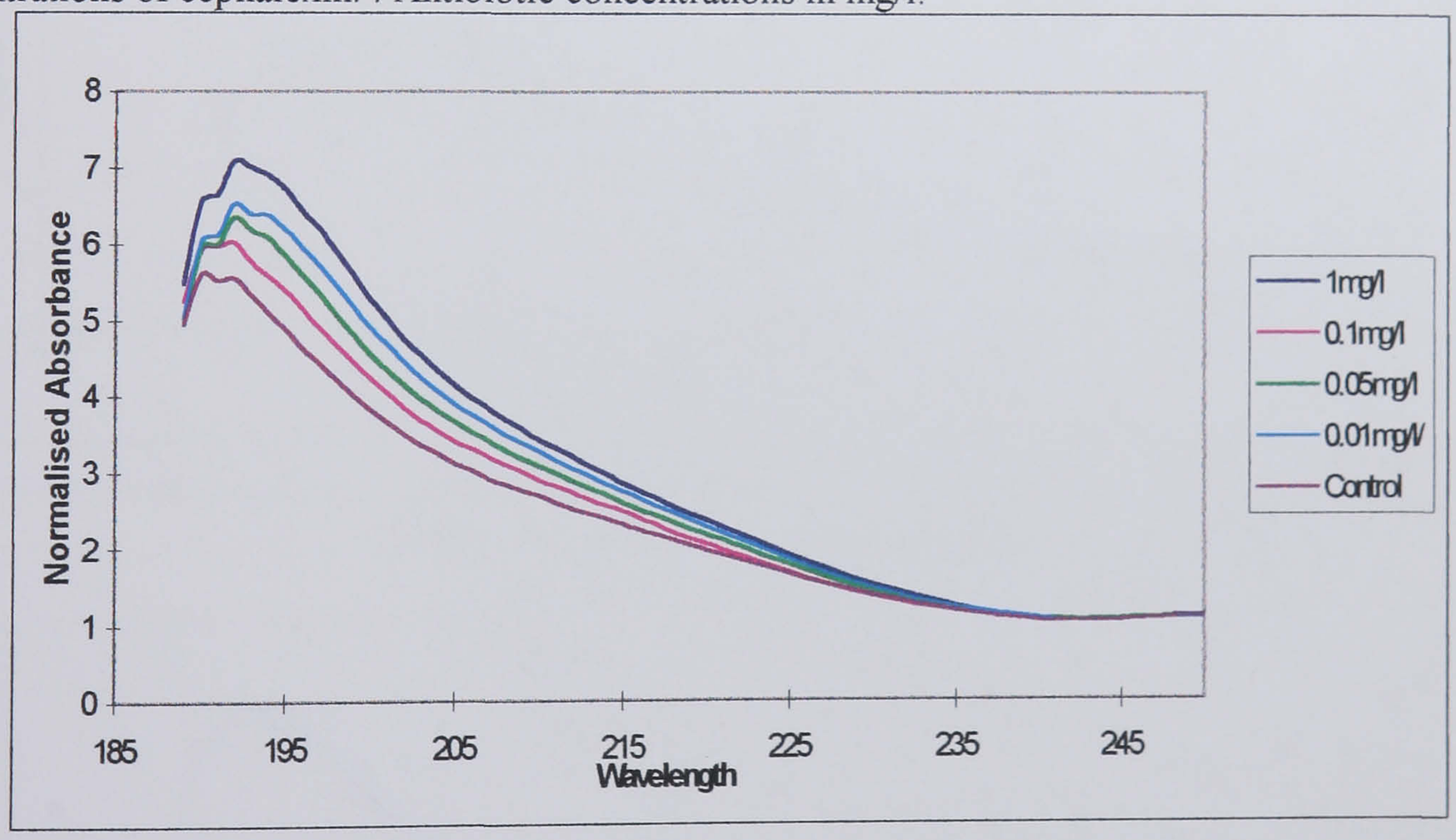
Figure 4.17 shows the effects of a mixture of oxytetracycline and amoxycillin on absorbance. There was reduced absorbance at 1mg/l each of amoxycillin and oxytetracycline. No clear effect was visible at the 0.1mg/l concentrations.

Figure 4.17: MUVS of duplicate samples of *E. coli* 10650 grown in various concentrations of oxytetracycline and amoxycillin. Antibiotic concentrations in mg/l



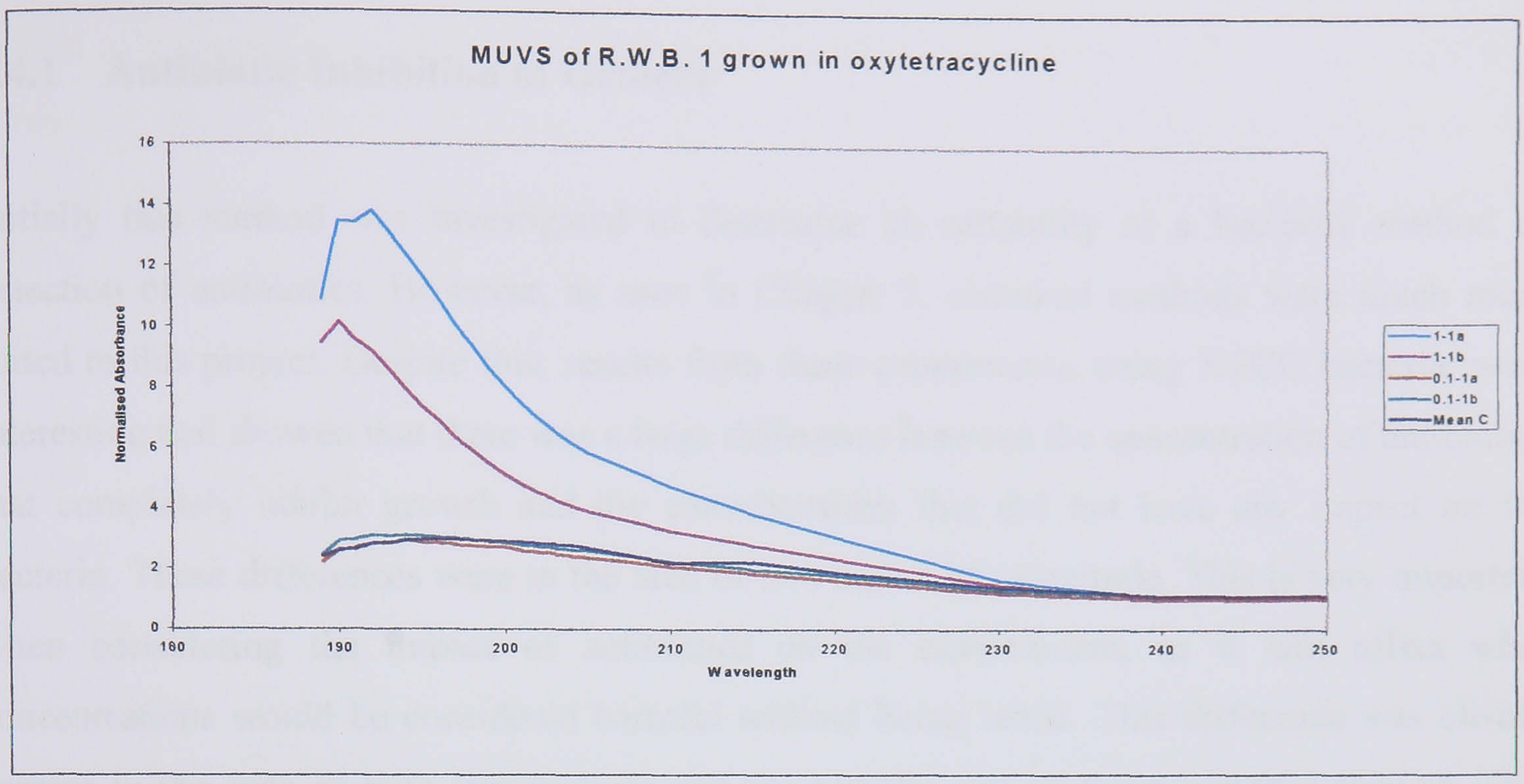
Results from growing *E.coli* in the presence of cephalexin can be seen in Figure 4.18. Exposure to the antibiotic in this case increased the MUVS of the bacterium, though again there was no linear relationship between the change and the antibiotic dose.

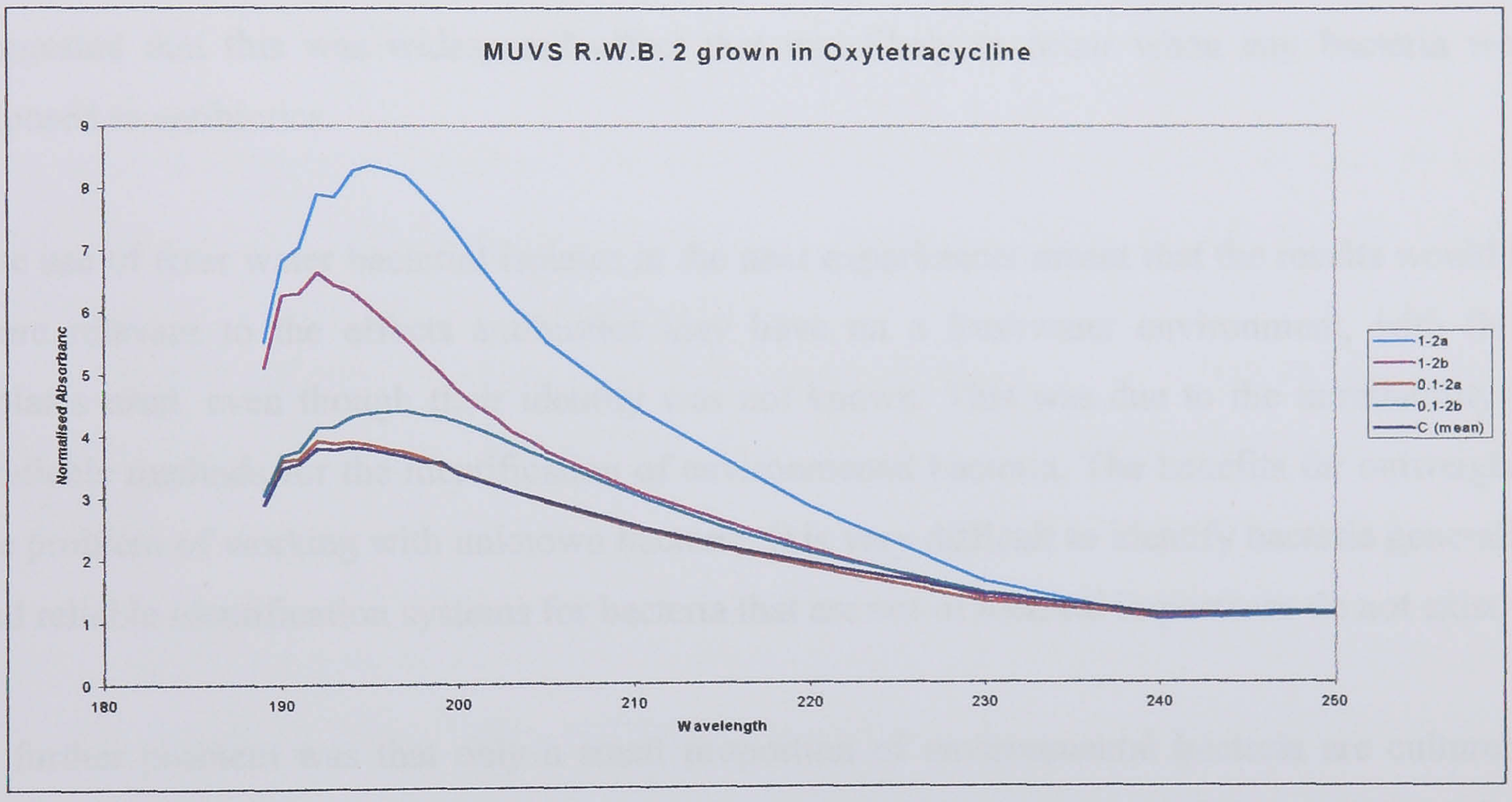
Figure 4.18: Mean of the MUVS of duplicate samples (triplicate controls) of *E.coli* grown in various concentrations of cephalexin. . Antibiotic concentrations in mg/l.

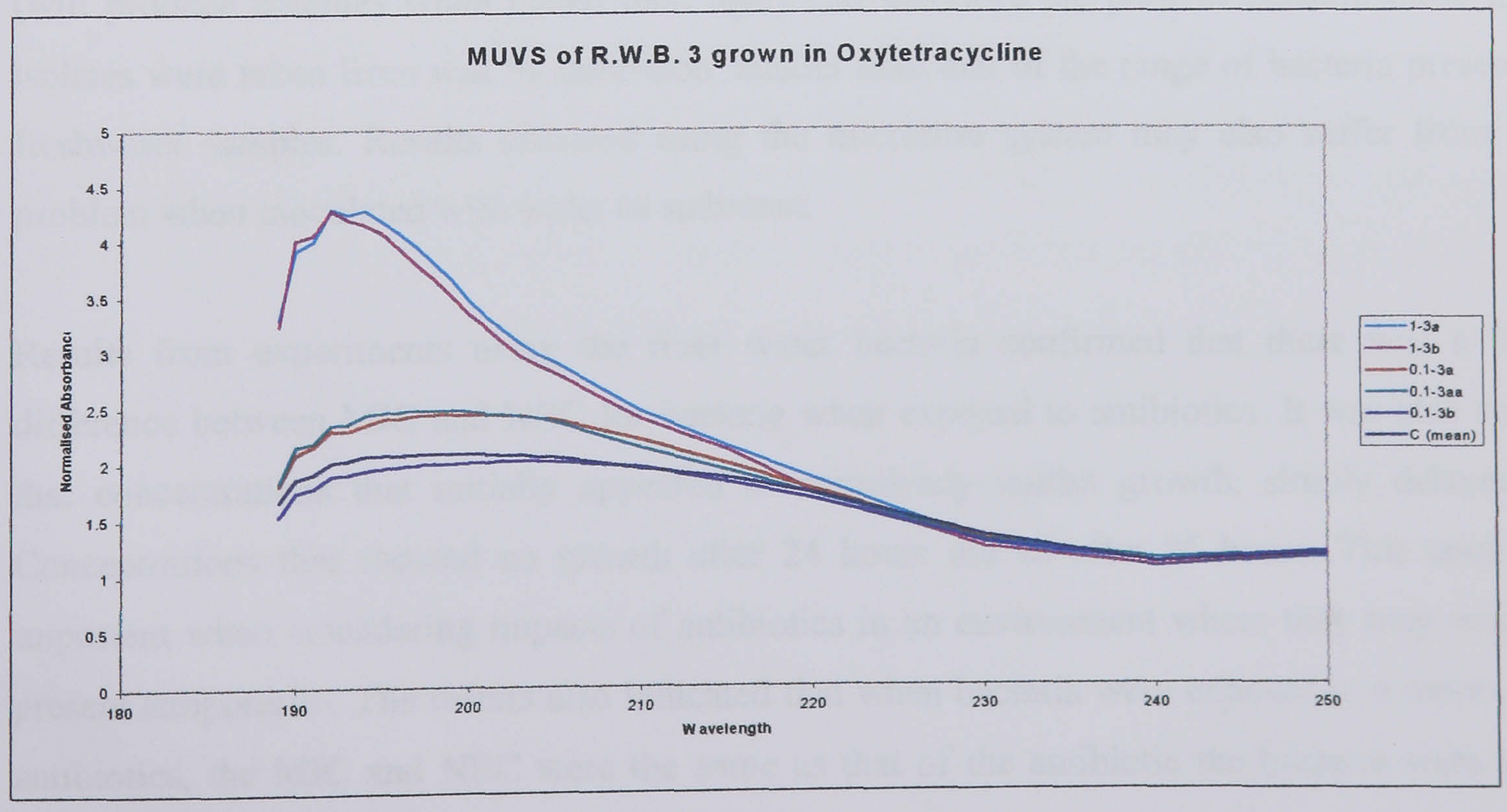


A final experiment was carried out using the same river water isolates as in the microtitre experiments. From these previous microtitre experiments, it was known that there was more resistance to amoxycillin, so oxytetracycline was used. All three isolates were grown up in oxytetracycline and measurements taken of the MUVS. Results show that there was a clear effect on the absorbance when the bacteria were grown in 1mg/l. Samples which had 0.1mg/l showed no differences from controls. This was the case with all three bacteria. Results can be seen in Figure 4.19

Figure 4.19: Effects of two concentrations of oxytetracycline on the MUVS of three different river water bacteria.. Antibiotic concentrations in mg/l.







4.4 Discussion

4.4.1 Antibiotic Inhibition of Growth

Initially this method was investigated to determine its suitability as a bacterial method of detection of antibiotics. However, as seen in Chapter 3, chemical methods were much more suited to this project. Despite this, results from these experiments, using NTCC bacteria, were interesting and showed that there was a large difference between the concentration of antibiotics that completely inhibit growth and the concentrations that did not have any impact on the bacteria. These differences were in the area of two orders of magnitude. This is very important when considering the impact of antibiotics on the environment, as it will affect what concentrations would be considered harmful without being lethal. This difference was clearly present in the case of both tetracyclines and amoxycillin and all three bacterial species. This suggested that this was widespread effect that was likely to occur when any bacteria were exposed to antibiotics.

The use of river water bacterial isolates in the next experiments meant that the results would be more relevant to the effects antibiotics may have on a freshwater environment, with three isolates used, even though their identity was not known. This was due to the unreliability of available methods for the identification of environmental bacteria. The benefits far outweighed the problem of working with unknown bacteria. It is very difficult to identify bacteria generally, and reliable identification systems for bacteria that are not of medical importance do not exist.

A further problem was that only a small proportion of environmental bacteria are culturable (will produce colonies when plated onto agar) and therefore the pool of bacteria these three isolates were taken from was by definition smaller than that of the range of bacteria present in freshwater samples. Results obtained using the microtitre system may also suffer from this problem when inoculated with water or sediment.

Results from experiments using the river water bacteria confirmed that there was a large difference between MIC and NEC for bacteria when exposed to antibiotics. It was also found that concentrations that initially appeared to completely inhibit growth, simply delayed it. Concentrations that showed no growth after 24 hours did so after 96 hours. This could be important when considering impacts of antibiotics in an environment where they may only be present temporarily. The results also indicated that when bacteria were exposed to a mixture of antibiotics, the MIC and NEC were the same as that of the antibiotic the bacteria were most sensitive to. No synergy or antagonism was observed.

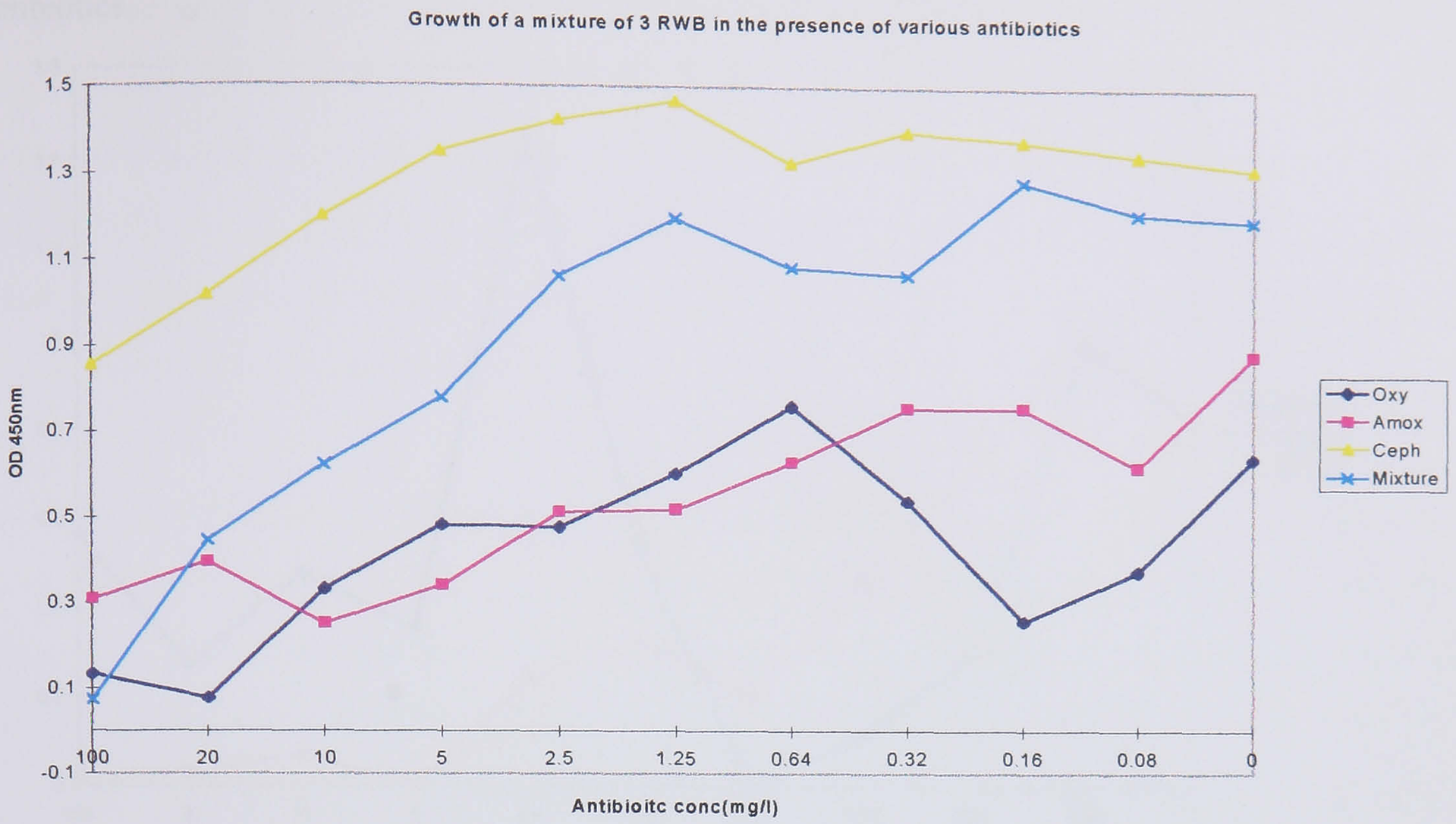
Effects of the antibiotics were not additive nor did the presence of any of the other antibiotics reduce the effect of oxytetracycline, which showed the most inhibition. This would suggest that in the environment the effects seen would not be additive, instead a similar impact would be seen to that of the individual antibiotic in the mixture that the river water and river sediment communities were most sensitive to.

Results from the mixture of bacteria showed that the combination of different species had a different resistance pattern from that of any of the individual bacteria present. There is a simple explanation for the resistance seen in the mixture when exposed to amoxycillin: as one of the species was resistant, there was visible growth, even if the other two species were not increasing in numbers. In the case of cephalexin, this is harder to explain as none of the individual bacteria appeared resistant until after 96 hours.

Comparing the resistance patterns of the mixture of bacteria to each antibiotic after 96 hours showed how the effect of the antibiotics varied. In Figure 4.17 it can be seen that the mixture was resistant to both cephalexin and amoxycillin, because there was growth in the presence of both of these at 100mg/l. However, there was a great difference in the effect these antibiotics had on the levels of growth. The NEC for amoxycillin was <0.08mg/l whilst for cephalexin it was 2.5mg/l.

Results from the mixture of antibiotics which contained a third of each of the antibiotics were, when this was taken into account, similar to those of oxytetracycline and amoxycillin, at the higher concentrations. There was no additive effect of the antibiotics on the mixture. When comparing the results it became apparent that the control wells on the different plates had different growth levels. This made it harder to compare the results as they showed different growth rates. This also highlighted the importance of environmental conditions on growth, and how what might just be small differences in conditions might affect growth.

Figure 4.20: Growth after 96 hours of the RWB mixture in the presence of various antibiotics



It can also be seen in Figure 4.20 that the final NECs were lower for the mixture of bacteria than for the individual bacterial species used in the mixture. This showed that, even when the population as a whole was resistant to a particular antibiotic, resistance alone was little indication of what effect the antibiotic had on bacterial growth long term. It appears that even though the mixture of bacteria was able to grow, because some of the species present were resistant, there was still an impact because the reduced growth of the less resistant bacteria reduced growth in the system as whole.

Results from the water (Figure 4.21) and sediment samples (Figure 4.22), which contained a large variety of bacteria, showed that both communities were highly resistant to amoxycillin, with neither showing any effect on growth at all, even with concentrations as high as 100mg/l. Not all components of the river communities are resistant to amoxycillin. In the case of the river water isolates, in Figure 4.20, resistance was seen, though with reduced growth. However, when the whole community is sampled there are likely to be more bacteria that can replace the growth of those bacteria inhibited. Therefore, overall, no differences in growth are seen from controls.

Results for the water samples were more erratic than for sediment samples, with variations in growth that were not related to concentration of antibiotic. This was likely to be because numbers in river water are not as high. Results from 3 wells were taken and averaged, which explains the variations seen. An even response in this assay would depend on a similar mix of bacteria being present in every well. In the case of water, with its lower numbers, results show that this is probably not always the case. Results from the sediment samples show similar variations, to a lesser degree, probably for the same reason.

Figure 4.21: Growth of river water samples after 96 hours in the presence of various concentrations of antibiotics

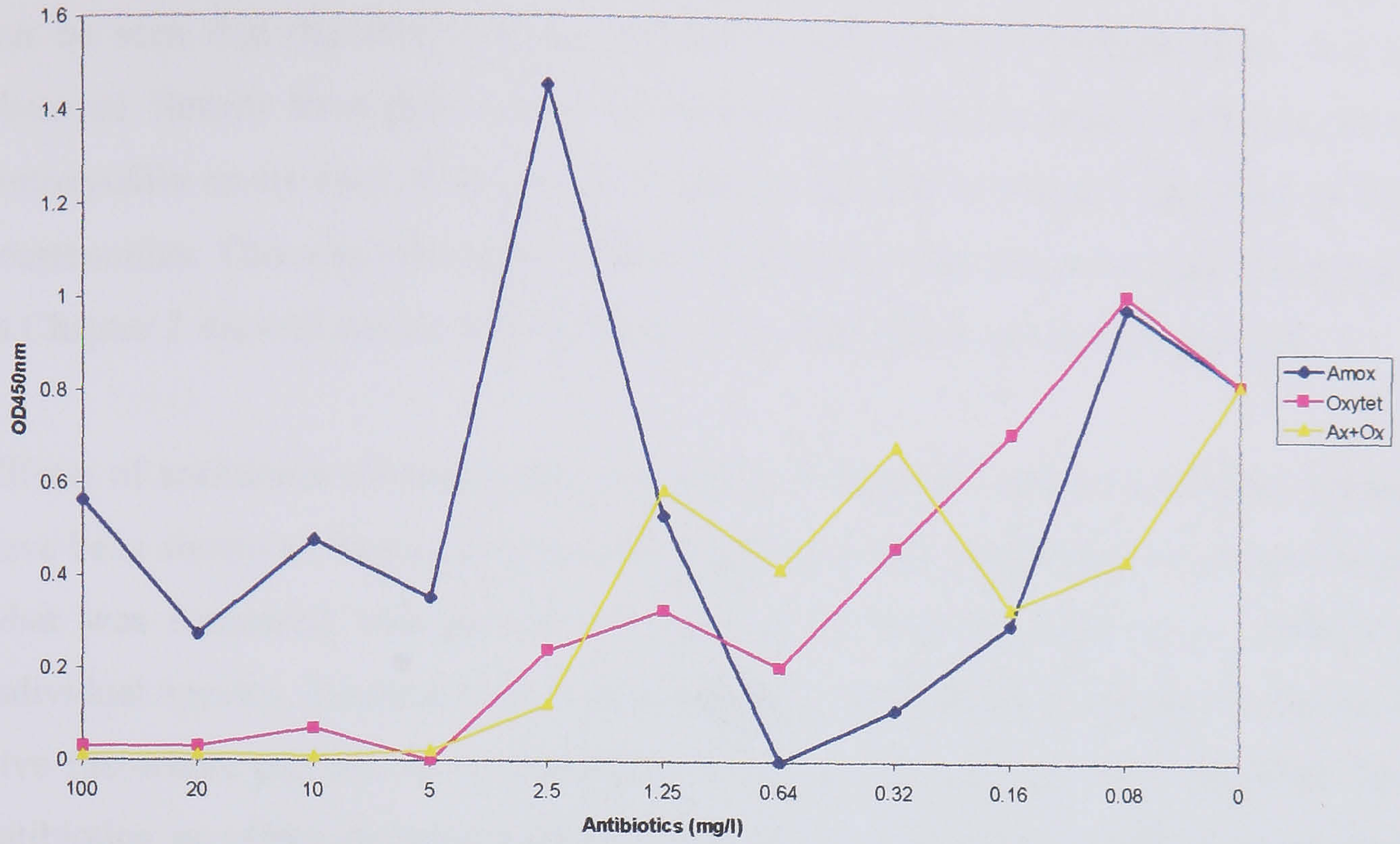
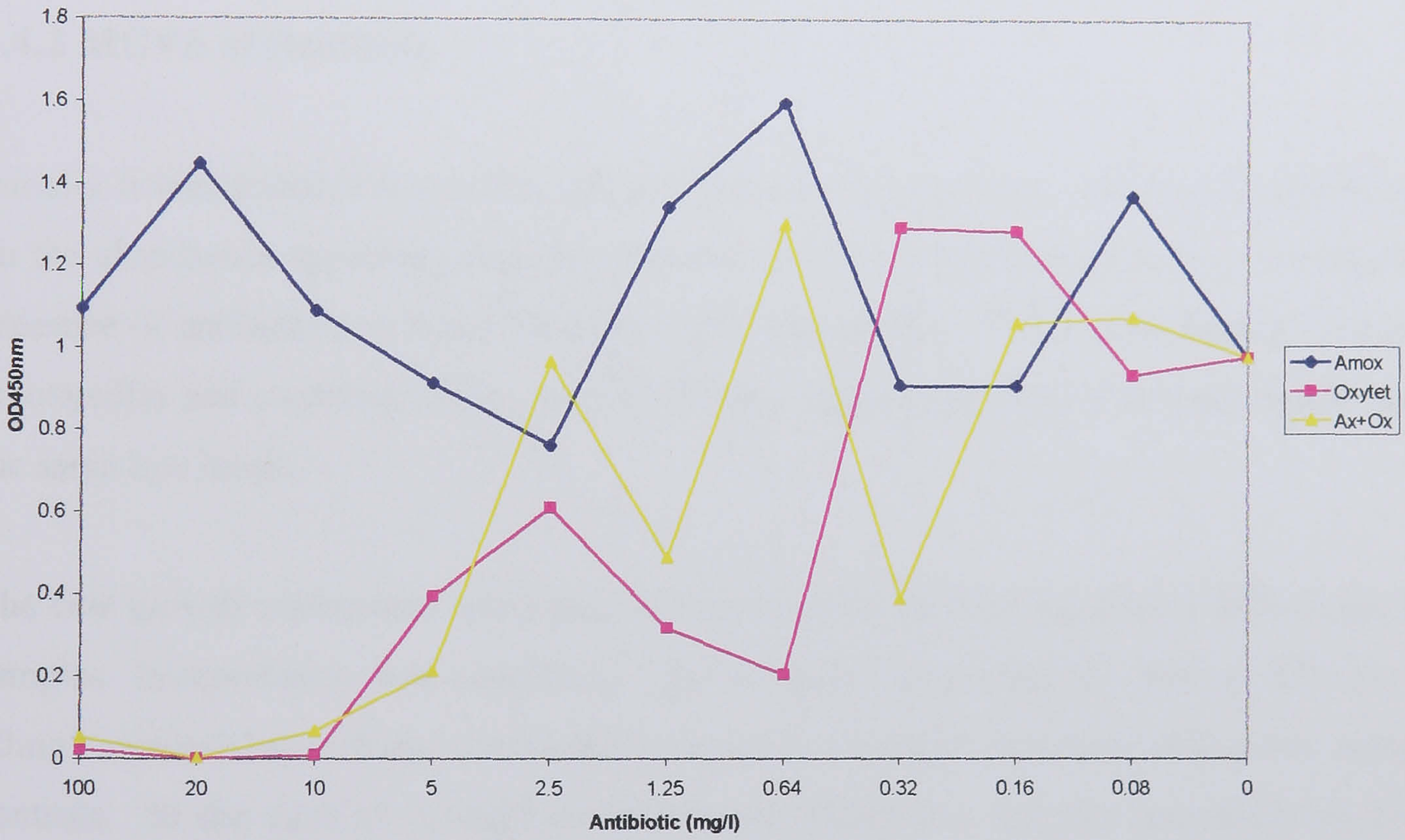


Figure 4.22: Growth of river sediment samples after 96 hours in the presence of various concentrations of antibiotics



Resistance to oxytetracycline was not present in the either sediment or water samples, with both communities sensitive to concentrations of 5mg/l of antibiotic. The NEC was again around an order of magnitude lower than this for both communities. These are similar results to those obtained for the river water bacterial isolates. Results from adding a mixture of both antibiotics to the samples showed that the resistance levels were similar to the oxytetracycline, which had the most impact on its own. The NEC for samples were similar to that of the oxytetracycline. These patterns of resistance are not surprising, as both communities were resistant to amoxycillin so any impact would be that of the oxytetracycline alone.

When comparing the response to the presence of the different antibiotics in each community it can be seen that the effect of the mixture is like that of oxytetracycline. No synergy was observed. Results from plate counts of sediment and water samples confirmed the effects seen. Amoxycillin on its own or as part of a mixture showed no impact on either of the freshwater communities. This was interesting as results from the research carried out into use of antibiotics in Chapter 2 showed amoxycillin to be the most prescribed antibiotic in the UK.

Effects of antibiotics on water and sediment communities, and on individual bacterial species, have been shown to be measurable and occurring at low concentrations of antibiotics. Because what was measured was growth overall and it was not possible to distinguish between individual species. Resistance or a measurement of no effect at certain concentrations did not give the whole picture. In a community some species will be more resistant than others to antibiotics, an effect on some bacteria which results in inhibition is likely to be masked, in part or completely, by growth of other competing bacteria that are less sensitive to the antibiotics.

4.4.2 MUVS of Bacteria

Initially it was possible to confirm that the presence of antibiotics did have a significant effect on the absorbance spectrum between 190 and 250nm. In addition growing the bacteria in the presence of antibiotics at lower concentrations reduced the MUVS even further. In both cases, amoxycillin and oxytetracycline, the MUVS was greatly reduced by 0.1mg/l concentrations to the same low levels.

The first growth experiment using only oxytetracycline showed significant differences between samples. Interpretation was complicated by different concentrations having different effects. 10mg/l increased absorbance compared to controls, 0.01mg/l decreased absorption compared to controls. In the case of 0.1mg/l and 0.05mg/l absorbance was not increased but maximum absorbance was shifted to higher wavelengths. In the case of 1mg/l absorbance was very similar to controls. This made it appear that there might not be any effect of the antibiotics, and that these changes were due to other factors. However, the similarities between the duplicate samples, with very similar shaped and positioned peaks suggest that there was an impact because of the presence of the antibiotic. A two-way analysis of variance (5%) on readings between 195 and 250nm showed there was a significant difference between antibiotic concentrations.

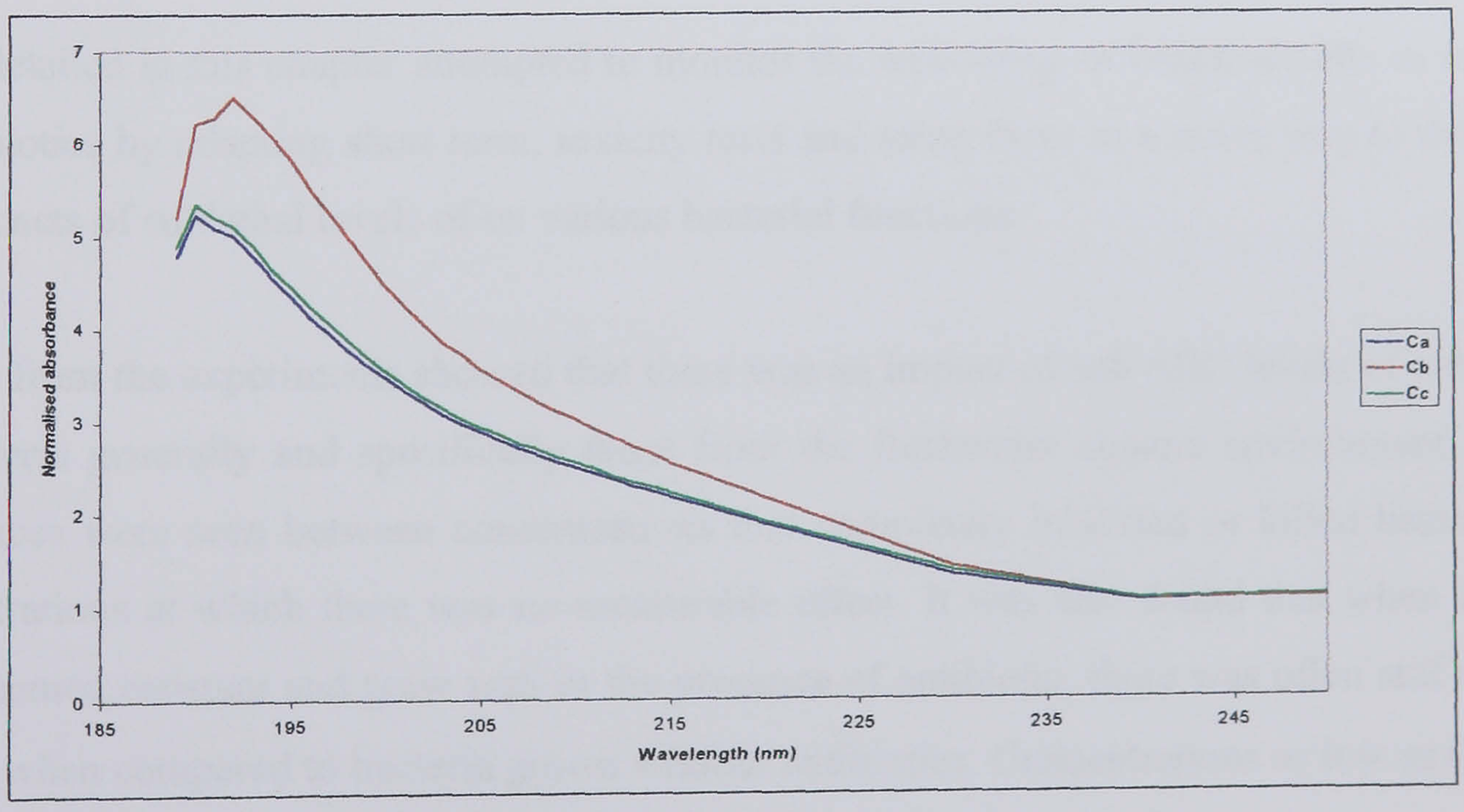
These results also showed that there were effects, but there was no direct linear relationship between antibiotic dose and a change in absorbance. Very high and very low concentrations had opposite effects, with medium concentrations showing no effect or simply changing absorbance patterns. Any possible explanation for this is difficult. Bains suggested that a change in the oxidative phosphorylation pathway, haem groups in particular, affected absorbance. However, all proteins and nucleic acids absorb strongly in the 180 to 220nm range, which is where the MUVS peaks are seen. Absorption by proteins in this region arises from electronic transitions in the polypeptide backbone (Mathews *et al.,* 1996). Small changes in this backbone are easily detected as changes in absorbance. Any number of proteins present in bacteria, which could be affected by antibiotics, could be responsible for changes in the MUVS. Effects by antibiotics on the nucleic acids could also be responsible for the changes in absorbance. Work by Lebrun *et al.* (1992) showed that in the case of *Pasteurealla multocida*, a pig pathogen, exposure to sub MIC of antibiotics, including tetracycline and a penicillin, did not change expression of outer membrane proteins and iron-regulated proteins. However, surface antigens were modified by the presence of sub-inhibitory concentrations of antibiotics. This could be important in pathogenic bacteria as it might affect virulence, but it is harder to determine what the effect of this might be on the fitness of environmental bacteria. It is possible that a change in surface structure, resulting in an increase or reduction of proteins, will affect UV absorbance patterns.

The effect of amoxycillin were again significant but difficult to interpret, with the strongest effect seen at the lowest antibiotic concentration. Results for the experiment where cultures were grown in equal concentrations of oxytetracycline and amoxycillin showed that there was an impact at higher concentrations of 1mg/l but no difference between controls and any of the lower concentrations. This was different from the results obtained using either amoxycillin or oxytetracycline alone. The results from the microtitre growth experiments suggested that one might expect to see, when two antibiotics are used, effects similar to that of the antibiotic bacteria are most sensitive to. This was not the case. The higher concentration of 1mg/ml decreased absorbance as did, though less, the 0.1mg/l concentration. The 0.05mg/l culture was not distinguishable from controls.

These results suggest various possibilities. There could have been some interaction between the two antibiotics, which resulted in a complete change in the impact these antibiotics had on the culture. Amoxycillin, though not impacting itself, may reverse the effect of oxytetracycline. It is possible that an antibiotic may not impact growth in any way, due to the presence of a more toxic antibiotic, and still have an effect on bacterial function which might be measurable in other ways, such as MUVS. Another possibility was that the results obtained were for some reason changing from one experiment to the next. It was possible that very small changes in the incubation conditions could affect the cultures and therefore the results. Bains (1993) also found that there were large variations, particularly when measuring the impact of the same chemical on different batches of the same bacterium, for which there was no known explanation.

The experiment in which the *E.coli* culture was grown up with cephalexin again gave results that were difficult to interpret. This was because of the MUVS obtained for the three controls. Two of the controls gave curves that were nearly identical and much lower than any of the samples containing antibiotics. However, one of the controls gave much higher readings, as can be seen in Figure 4.23. The differences seen in the controls alone exemplify the difficulties in interpreting the results obtained using this method. This problem was also seen in the amoxycillin experiment (Figure 4.16).

Figure 4.23:MUVS of E.coli. Control samples for cephalexin experiment.



The final experiment, which used the same river water bacteria as the microtitre growth experiments, showed significant differences between samples. When looking at the absorbance curves it can be seen that these differences can be attributed to the samples that contained 1mg/l of oxytetracycline. The 0.1mg/l samples were very similar to controls in all three cases. When these results were compared to those of the growth assay results for 14 hours, it was found that, except for the case of RWB3, the growth assay showed effects at lower antibiotic concentrations and was therefore much more sensitive to the presence of antibiotics.

No further work was carried out with this method as results did not seem promising and time was limited. Overall, the results were difficult to interpret. The method initially showed promise, because the absorbance in the mid-ultraviolet spectrum is a universal characteristic of all bacteria. The MUVS of any bacterium was easily measured. However changes in the MUVS due to external stresses proved harder to determine. Simply looking at maximum peak height as an indication of an antibiotic impact gave no clear results. Differences seen were highly significant when analysed, in all cases. Antibiotics did appear to be having some kind of impact. The similarity in the shape of the peaks for samples that contained similar amounts of antibiotics showed that it might well be possible, with further work, to establish a relationship between antibiotics and the effect they have on the MUVS of bacteria. What could be concluded from the work carried out so far was that there was no linear correlation between dose and effect.

4.5 Conclusions

Work detailed in this chapter attempted to monitor the well-being of bacterial cells in response to antibiotics by adapting short term, toxicity tests and using them in a novel way to determine any impacts of sublethal levels of on various bacterial functions.

Results from the experiments showed that there was an impact of sub-MIC levels of antibiotics on bacteria generally and specifically those from the freshwater aquatic environment. Large differences were seen between concentrations that completely inhibited or killed bacteria and concentrations at which there was no measurable effect. It was also found that when bacteria were deemed resistant and grew well in the presence of antibiotic, there was often still reduced growth when compared to bacteria grown without antibiotics. Concentrations as low as 0.08μg/l of antibiotic inhibited growth. In addition, the impact of multiple antibiotics was not additive. Lack of synergy between antibiotics would suggest that there is less of a likelihood of mixtures of antibiotics present in the environment having adverse effects. From all this it can be concluded that there is a strong possibility that, at least locally, any antibiotics entering the environment could impact bacteria. In addition, it was shown that when bacterial communities were exposed to antibiotics, the presence of resistant bacteria could mask the impact of

antibiotics on the less resistant parts of the communities. The only way of distinguishing changes in what appears to be a completely resistant community would be to attempt to measure changes in the structure of the community due to exposure to antibiotics.

The results from this method highlight the existence of a concentration range where bacteria are able to grow, though not as efficiently as when antibiotics are not present at all. It is this area that is of interest when examining the impacts of antibiotics in the environment, as the amounts found are very low. Long term adverse impacts are more likely if there is this gradual effect rather than an all or nothing inhibition of growth.

Results from measuring impacts of antibiotics on the MUVS showed that there appeared to be effect. Results were difficult to interpret and concentrations that appeared to show detectable effects were higher than those seen in the growth assay when the same bacterium was used. This made this assay less sensitive than the growth assay. However, an impact on MUVS could be an indication of an effect on proteins and nucleic acids, which if further explored might help to understand the mechanism of sub-MIC effects of antibiotics and their likely environmental impact. The method initially showed promise, because the absorbance in the mid-ultraviolet spectrum is a characteristic of all bacteria, however the results were difficult to interpret.

The MUVS of any bacterium was easily measured. However changes in the MUVS due to external stresses proved harder to determine. Simply looking at maximum peak height as an indication of an antibiotic impact gave no clear results. The additional disadvantage of this method was that the mechanism by which the antibiotics could be having an effect on the MUVS of bacteria was not understood, which made it a lot harder to interpret the results obtained. If more work were carried out on this area to determine this, it could make this method a very important tool in determining the impacts of antibiotics on bacteria, and whether a wide range of compounds are toxic to them.

In conclusion, it can be said that some sub-MIC effects were found for freshwater bacteria and communities. These effects were difficult to measure, particularly where whole bacterial communities are concerned and these methods were not the most appropriate for determining the impacts these effects might have on the aquatic ecosystems as a whole.

Chapter 5

River water microcosm experiments using radioactively labelled carbon compounds to investigate the impact of sublethal doses of antibiotics on freshwater microbial ecosystems.

5.1 Introduction

Laboratory experiments that attempt in some form or other to mimic environmental conditions are an important part of an assessment of the impact of pollutants in the environment. Experiments in small laboratory microcosms can provide information on general ecosystem behaviour and give quantative information indexes on biodegradability of chemical substances, ecological magnification etc (Lungdren, 1985). The validity of this approach for determining the ecological impacts of pollutants has been considered by various authors (Cairns and Pratt, 1989; Calow, 1989 and Lungdren, 1985). Experiments, using river water microcosms are vital in helping to determine the fate and impact of pollutants and provide data in a way which single organism experiments do not. Ecosystems are dynamic and adaptable structures that are often capable of absorbing impacts without change (Bianchi and Bianchi 1995), therefore chemical information or single species experiments alone can be misleading. A possible alternative is that minimal effects on a vital part of an ecosystem could have devastating effects on the ecosystem as a whole. Microcosm experiments help to determine if these kinds of impacts and inter relations exist.

It was considered important for this project to determine whether sublethal concentrations of antibiotics could have an impact on the freshwater ecosystems they might be reaching. The results from this work could then be combined with the data obtained on antibiotic consumption, chemical data, the calculations on amounts reaching rivers, together with results from laboratory work to determine whether an impact is likely in the environment.

As pointed out previously bacteria, are vital components of aquatic ecosystems (Sherr and Sherr, 1988; Meyer, 1994). For this reason the work has focused on the impacts antibiotics would have on the bacteria contained in an ecosystem. The vital role of bacteria in the microbial loop, in particular how they take up and break down organic carbon, is a function that could be affected by the presence of sublethal levels of antibiotics. This vital role controls the input of energy into the ecosystem. As stated by Kaplan *et al.* (1992), 'Organic carbon dynamics are central to stream ecosystem studies because they link energy flow and nutrient cycling. Dissolved Organic Carbon may contribute 30-70% of the total energy input into stream ecosystems'.

Radiolabelled compounds have, in the past, been used extensively to look at nutrient cycling in aquatic ecosystem studies. Both [^{14}C] (Boon, 1992; Neely and Wetzel, 1995) and [^{3}H] (Neely and Wetzel, 1995; Kristiansen *et al.*, 1992; Sander and Kaliff, 1993) labelled compounds have previously been used to study bacterial and fungal growth.

There is only one example in the literature of using labelled compounds to look at the impact of antibiotics on aquatic bacteria (Boon, 1992). This was a very short term experiment (24 hours). Its main concern was whether the use of antibiotics in selectively inhibiting bacteria was a satisfactory method of limnological research. The experiment used [^{14}C] in two forms, both containing uniformly labelled carbon, firstly glucose and secondly a mixture of uniformly labelled amino acids, to see if exposure to various antibiotics (amongst them oxytetracycline) had an impact on the uptake of these. Uptake of both these sources was measured and the measurable effect on [^{14}C] uptake was found to be greater in the case of amino acids, although effects were seen in both cases

Previous work by Bealing *et al.* (1993) used a closed system to determine the fate of carbon labelled pollutants in a river water microcosm. This system gave information about how quickly a particular pollutant was taken up, broken down by bacteria and respired as CO_2. This method was adopted so that it could be used to determine the impacts of low levels of antibiotics on the uptake and breakdown of uniformly [^{14}C] labelled sodium acetate and a uniformly labelled [^{14}C] mixture of amino acids.

5.2 Materials and Methods

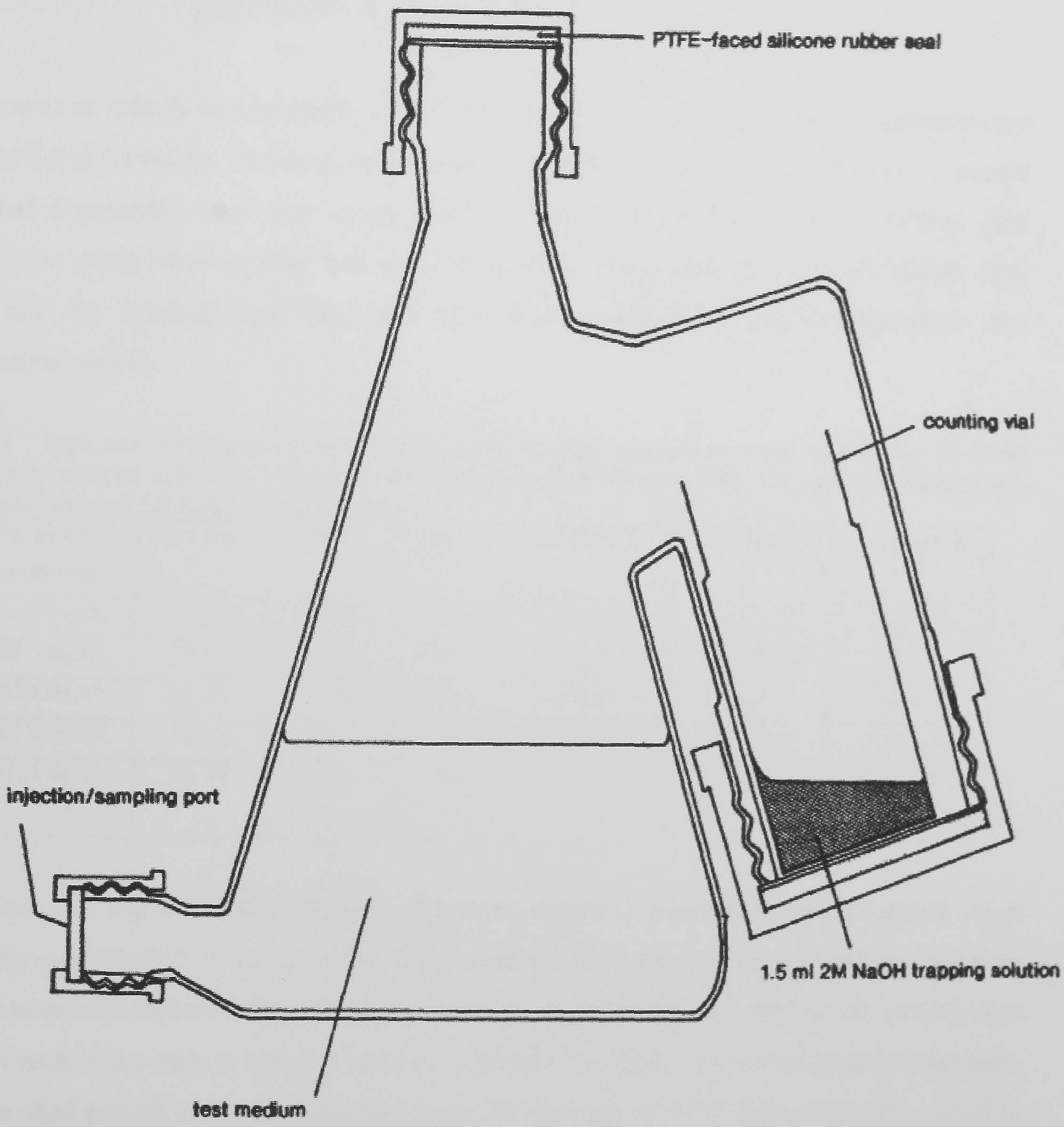
The method used was based on that of Bealing *et al.* (1993). This involved using radiolabelled compounds in a closed system. Two different compounds were used, both available from Amersham Pharmaceuticals, UK. [U^{14}C] Acetic Acid Sodium Salt and [U^{14}C] Protein Hydrosilate[1].

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[1] Typical analysis (by % of activity):

L-Alanine	9.5%	L-Lysine	4.8%
L-Arganine	6.9%	L-Methionine	0.6%
L-Aspartic acid	10.0%	L-Phenylalanine	7.4%
L-Glutamic acid	9.0%	L-Proline	5.3%
Glycine	5.8%	L-Serine	3.2%
L-Histidine	1.6%	L-Threonine	5.7%
L-Leucine	12.7%	L-Tyrosine	5.8%
L-isoLeucine	5.8%	L-Valine	5.9%

Figure 5.1 Modified flask used in carbon cycling experiments using uniformly labelled [^{14}C] compounds. (from Beling *et al.*, 1993).



The system used conical flasks which were adapted by adding a trap compartment on the side (see Figure 5.1) and which had a screw top lid. Flasks and all equipment were sterilised before beginning the experiments to prevent contamination.

Flasks were set up as follows

Controls: 200ml of freshly collected Thames river water taken from Medmenham.

Abiotic controls: 190 ml of freshly collected river water to which 10 ml of a 37% Formaldehyde solution was added.

Experimental flasks: Each containing 200ml of river water plus varying concentrations of oxytetracycline, amoxycillin or both.

The amounts of activity and antibiotic added at the beginning of each individual experiment are set out in Table 5.1 below. Duplicate flasks were set up for the first four experiments. In the case of the last experiment, three live controls and one abiotic control were set up instead. This decision was made because there was little information being obtained from the abiotic jars, except that the systems were dead and all activity remained in solution throughout the experimental period.

Table 5.1 : Type and concentration of antibiotics added to jars during experiment 1 to 5. No Ab (river water control with no antibiotic added); O (Oxytetracycline); A (Amoxycillin), SA (Sodium acetate) AA (amino acid mixture) * 0.5mg/l of each antibiotic.

Exp No	^{14}C Activity Added/ ml	Jar 1 and 2	Jar 3	Jar 4	Jar 5 and 6	Jar 7 and 8	Jar 9 and 10
1	927.2 Bq SA	No Ab	Abiotic	Abiotic	0.01 mg/l **O**	0.1 mg/l **O**	5 mg/l **O**
2	462.5 Bq AA	No Ab	Abiotic	Abiotic	0.01 mg/l **O**	0.1 mg/l **O**	5 mg/l **O**
3	462.5 Bq AA	No Ab	Abiotic	Abiotic	0.01 mg/l **O**	0.1 mg/l **O**	5 mg/l **O**
4	462.5 Bq AA	No Ab	Abiotic	Abiotic	0.01 mg/l **A**	0.1 mg/l **A**	5 mg/l **A**
5	462.5 Bq AA	No Ab	No Ab	Abiotic	0.5 mg/l **O**	0.5mg/l **A**	0.5mg/l **O** + 0.5mg/l **A***

Every flask then had a calculated amount of sodium acetate or amino acid mixture added. After adding the radiolabelled compounds, the flasks were stirred vigorously for half an hour and then sampled to determine the initial activity. Scintillation vials containing 1.5ml of 2M NaOH were placed in each of the traps in order to catch all respired CO_2. Flasks were then placed in the dark, to reduce algal growth and protect bacteria from UV damage, at 15°C and continually stirred in between samplings, using a multiple magnetic stirrer.

The first two experiments compared the fate of sodium acetate and amino acid in the systems when oxytetracycline was present. Experiment 3 duplicated the second oxytetracycline experiment using labelled amino acid. The fourth experiment was identical to 2 and 3, except for the usage of amoxycillin as the antibiotic. The last experiment looked at the most significant concentrations of both antibiotics as well as a mixture of these. Had time been available the last two experiments would have been repeated. Results which were in Disintegrations per Minute (DPM) were then calculated.

5.2.1 Sampling

The following samples were taken at regular intervals during incubation. Although regular sampling of between 24 and 72 hours was attempted, this was not always possible due to the very limited access that was allowed to the laboratory because of security regulations.

1. Headspace trap counts

   Vials in the flask trap were removed and replaced by fresh vials containing 1.5ml of 2M NaOH. 10ml of Optiphase Hisafe 3 scintillation cocktail were added and vials placed in a scintillation counter. The counter was an LKB 149 RakBeta, made by Wallak UK.

2. Filter counts:

   10ml of sample from each flask were withdrawn and passed through a 25mm diameter filter capsule containing a 0.2µm cellulose nitrate filter. Filter were then washed with 5ml of 0.5M HCl followed by 5ml of distilled water and then 5ml of air to remove excess water. Membranes were then removed and placed in scintillation vials. 10ml of Filter Count scintillation cocktail were then added. This dissolved the filters and allowed any activity present on the membrane to be counted without interference. Once the filters had dissolved the vials were placed in the counter.

3. Wash counts

   1ml of filtrate from each flask (from 2 above) was collected and placed in a fresh scintillation vial containing 1.5ml of 2M NaOH. 10ml of Optiphase Hisafe 3 scintillation cocktail was added and the vials placed in the counter.

4. Total counts

   1ml of river water was removed from each flask using a syringe via the spiking sampling septum. Each sample was placed in a scintillation vial to which 1.5ml of 2M NaOH solution and 10ml of Optiphase Hisafe 3 scintillation cocktail were added and vials were placed in the counter.

Each of the above counts gives some insight into the fate of the radiolabelled carbon added, and therefore, of the compound of which it formed part.

Headspace counts: Carbon taken up, broken down and respired as CO_2.

Filter counts: Carbon taken up by bacteria and present inside them (adsorbed labelled carbon is washed off before counting).

Wash counts: Free carbon still remaining in solution, either as the original compound or having being taken up, broken down and returned to solution.

Total counts: Total carbon still remaining in solution, including that taken up by bacteria.

Results are presented in graphs which show the mean readings, in DPM per millilitre of river water in the jars at the time of sampling. The volume of river water decreased by 11ml after each sampling. In the case of the headspace counts results are presented as cumulative total DPM because, unlike the rest of the readings, which show the levels of labelled carbon at a particular moment in time in each part of the system, these counts are the amount of labelled carbon respired over a period of time and as such are time dependent. Full results can be seen in Appendix 2. This contains all counts obtained for all experiments, in DPM/ml of sample remaining in the flasks

A two way analysis of variance with replication was carried out on results using Microsoft Excel 7.0.data analysis tool.

5.3 Results

5.3.1 Experiment 1

Antibiotic: Oxytetracycline

Carbon Source: [$U^{14}C$] Sodium Acetate.

Duration 356 hours (15 days)

Activity added: 927.2 Bq/ml (55632 DPM/ml)

Results from Experiment 1 can be seen in Figure 5.2.

A two way analysis of variance was carried out on all results (5% level)

Carbon Respired as CO_2

A significant reduction of evolved CO_2 was seen in the system containing 5mg/l of oxytetracycline from the moment the experiment was set up. The other treatments showed no difference from controls. One of the 0.01mg/l treatments showed a sudden decrease in CO^2 production half way through the experiment, but it is unlikely that this was related to the antibiotic concentration because the other replicate showed no difference from controls.

Bacterial Uptake

Differences seen in filter counts were significant. In the first hour all three treatments showed increased uptake of the sodium acetate compared to the controls. This effect was stronger in the 0.1 and 0.01 mg/l jars. However, within 24 hours the 5mg/l treatments had the bacteria which contained the highest level of labelled carbon and continued to do so throughout the rest of the experiment, with levels beginning to be reduced over the last few days. After the initial difference the other two treatments again followed the same pattern as the controls. There was an initial peak over the first two days after which the amount of labelled carbon present was greatly reduced and reached a steady state after 10 days.

Free Carbon in Solution

Results seen in this experiment were significant. In this case the controls showed an initial low level of carbon in solution which mirrored the peak in the bacterial carbon, after which there was a rise to a level which is still lower than the initial spike (this would be expected as there was a continuous loss from the system due to respiration of carbon by bacteria as CO_2). From that point there was a continuous steady decrease of free carbon in solution. Both the 0.1 and 0.01mg/l treatments followed the same pattern as the controls, except for the fact that they showed the biggest initial reduction in free carbon after the first few hours.

Figure 5.2: DPM measurements for Experiment 1: River water with oxytetracycline and uniformly labelled [^{14}C] Sodium Acetate

Figure 2a: Carbon respired as CO2 (Cumulative headspace counts)

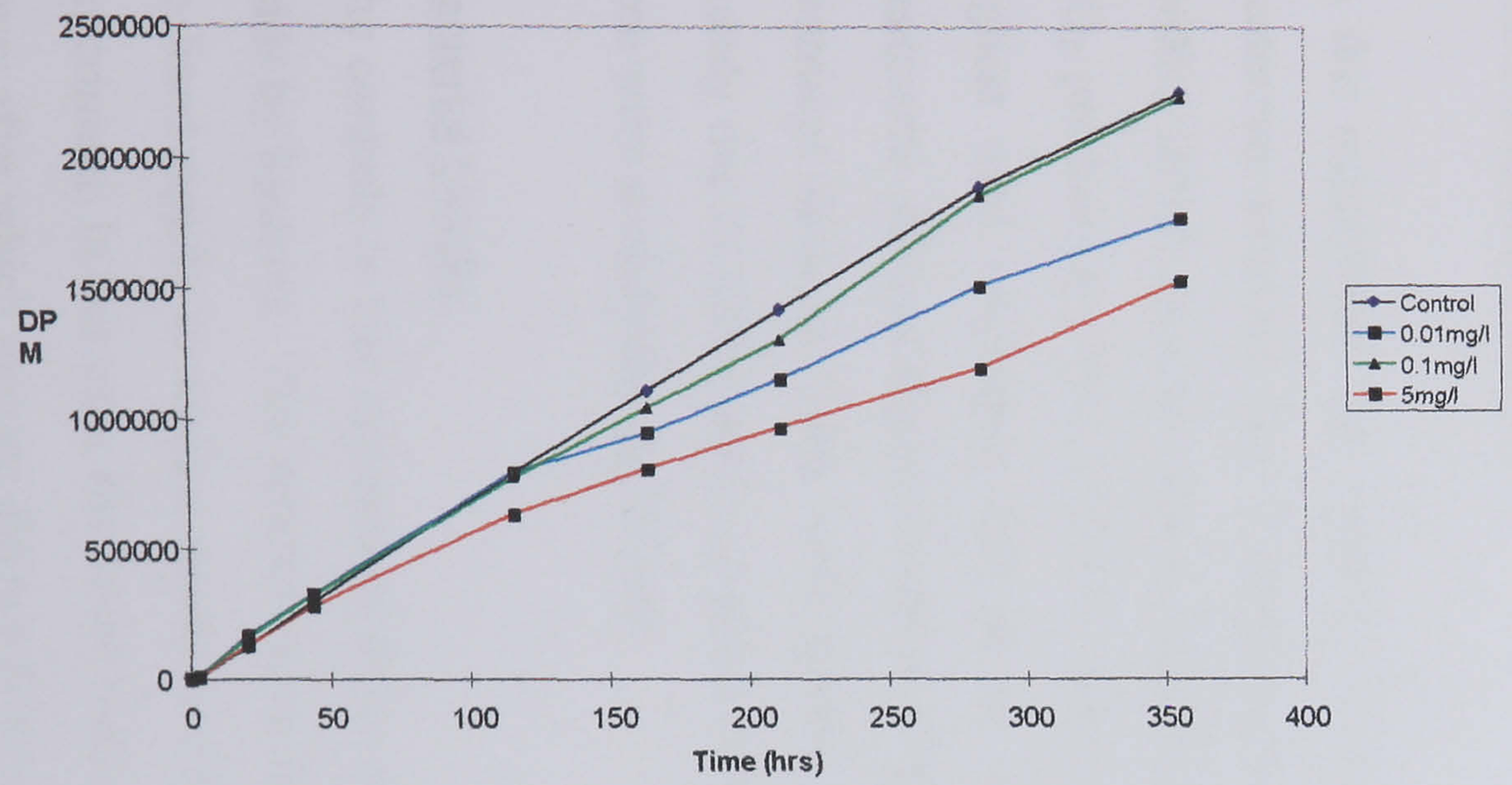


Figure 2b: Carbon Uptake by Bacteria (Filter counts)

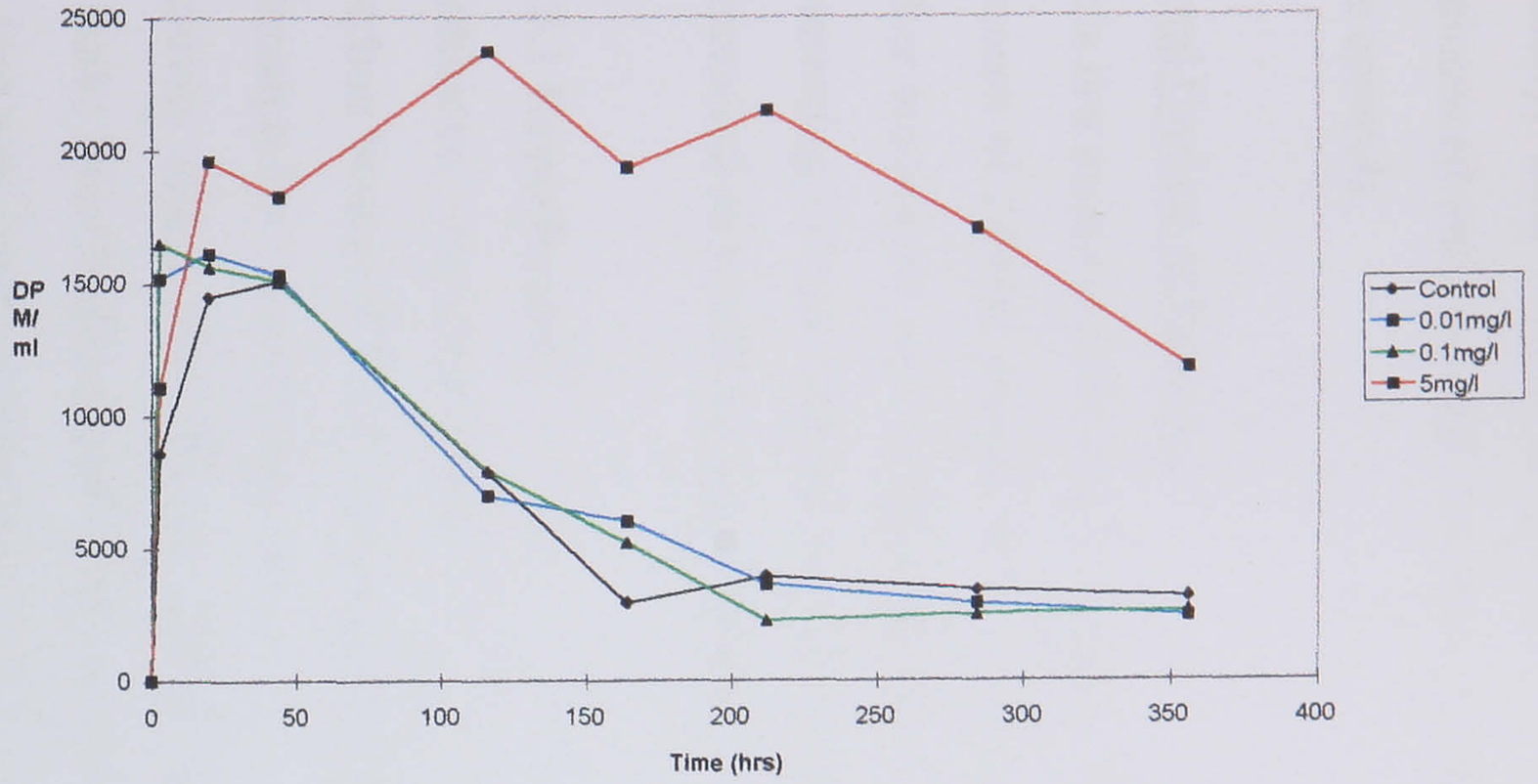


Figure 2c: Free Carbon in Solution (Wash counts)

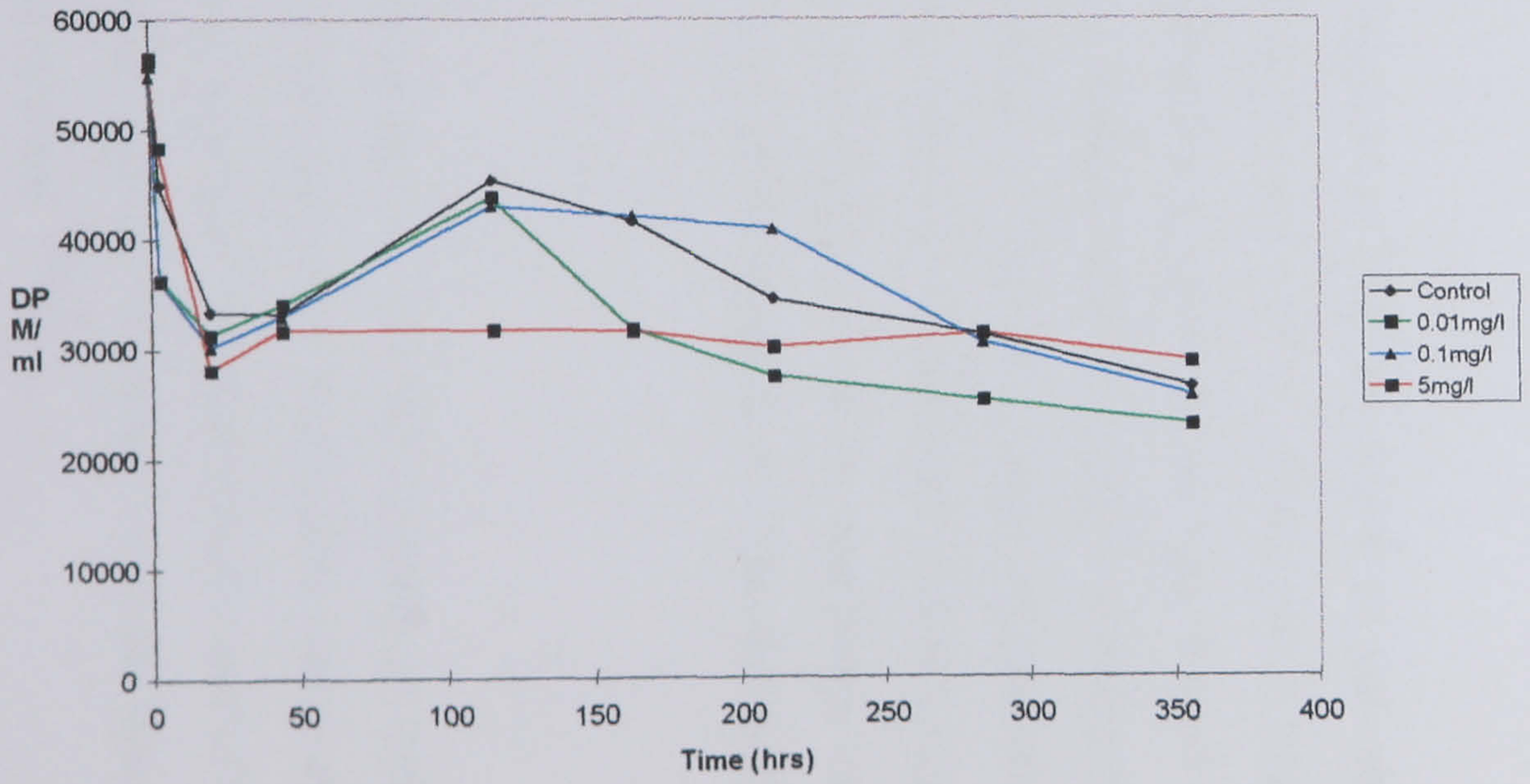
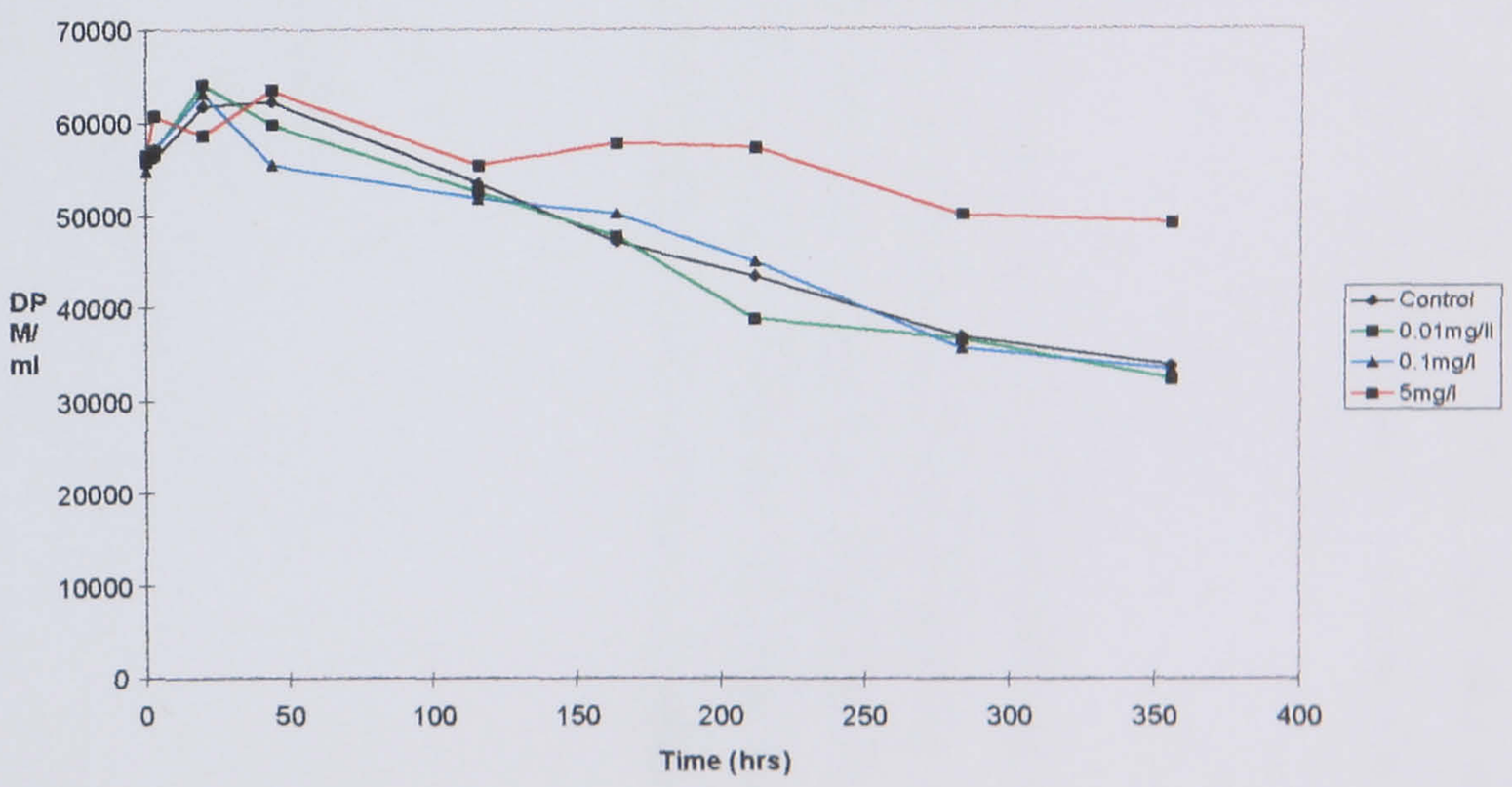


Figure 2d: Total Carbon in Solution (Total counts)



In the case of the 5mg/l treatments, there was a smaller reduction in free carbon than in controls initially, after which there was reduced free carbon compared to controls and this was maintained until the last few days of the experiments when results became much more similar to the controls.

Total Carbon in Solution

This is a measurement that is a sum of free and bacterial carbon. In this case it confirmed that the amount of carbon present in the system was much greater for the 5mg/l treatment than for the other treatments which again all show similar patterns. This was a significant difference. It is interesting to note that the total and free carbon readings were very similar for the other three treatments as would be expected due to the reduced level of labelled carbon present in bacteria.

5.3.2 Experiment 2

Antibiotic: Oxytetracycline

Carbon Source: [$U^{14}C$] Protein Hydrosilate (amino acid mixture)

Duration 356 hours (15 days)

Activity added: 426.5 Bq/ml (22590 DPM/ml)

Results from Experiment 2 can be seen in Figure 5.3.

A two way analysis of variance was carried out on all results (5%).

Carbon Respired as CO_2

In this experiment, the controls showed the lowest outputs of CO_2 compared to the other treatments over the first 4 hours (61889 DPM). The 0.01 mg/l (70505 DPM) and 0.1mg/l (66885 DPM) showed intermediate levels and the 5mg/l treatment showed the highest level of CO_2 production (79622 DPM). After this, there was a change, with the control showing the highest total respiration rate at the end of the experiment. The two lower concentration treatments showed slightly reduced CO_2 evolution at the end of the experiment with the 5mg/l treatment showing much more greatly reduced levels. The differences in the treatments were mainly due to differences occurring in the middle ten days of the experiment. The differences seen were statistically significant.

Bacterial Uptake

The controls in this experiment showed, as in the previous one, a rapid initial uptake of amino acids by bacteria. This resulted in an increased level of labelled carbon in the cells, which then declined rapidly to reach a steady low level which in turn remained constant until the end of the experiment. In this case, the 5mg/l treatment showed reduced uptake by bacteria over the first 48 hours after which the rate did not decrease but remained constant.

Figure 5.3: DPM measurements for Experiment 2: River water with oxytetracycline and uniformly labelled [^{14}C] Amino Acid Mixture

Figure 3a: Carbon respired as CO2 (Cumulative headspace counts)

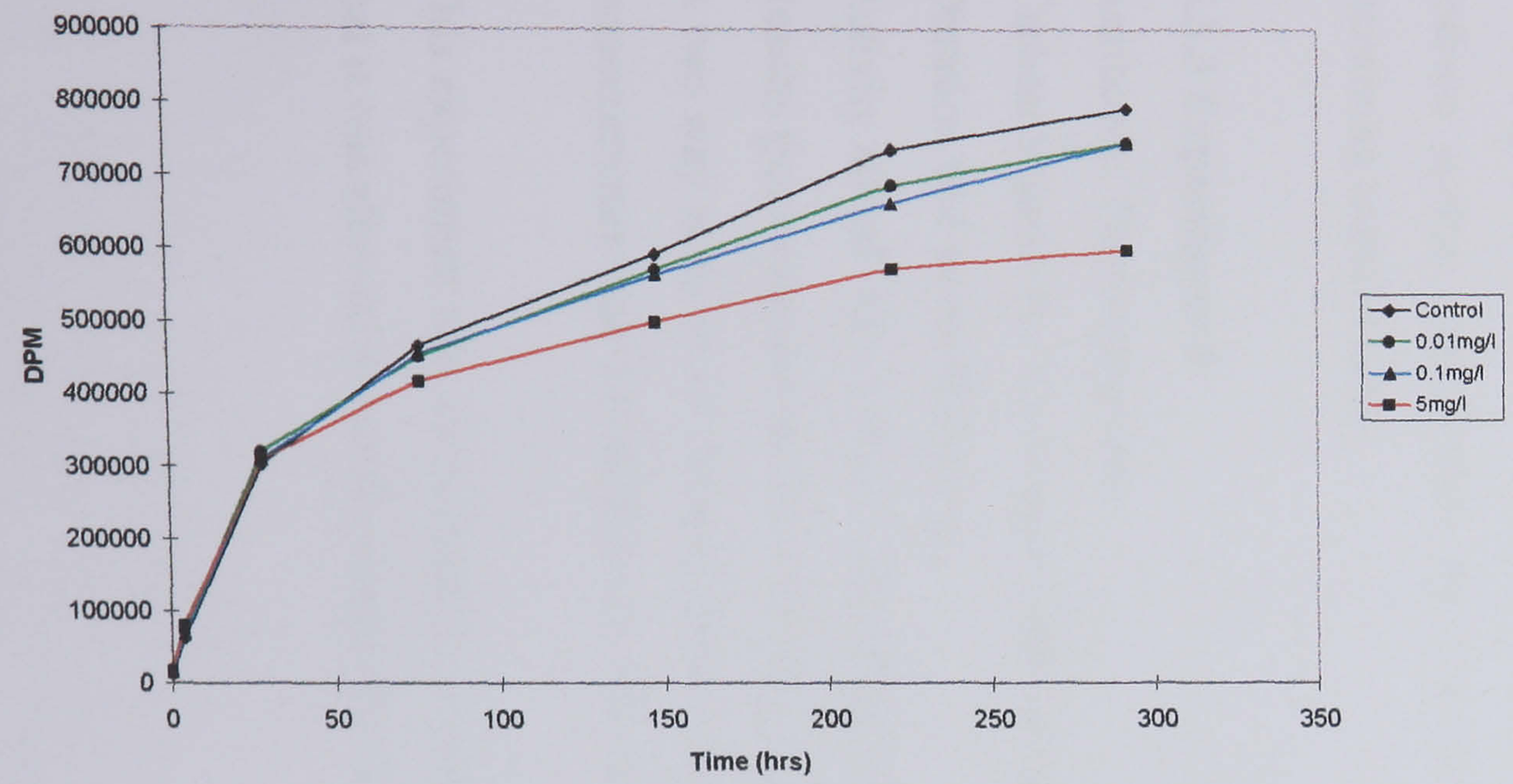


Figure 3b:Carbon Uptake by Bacteria (Filter Counts)

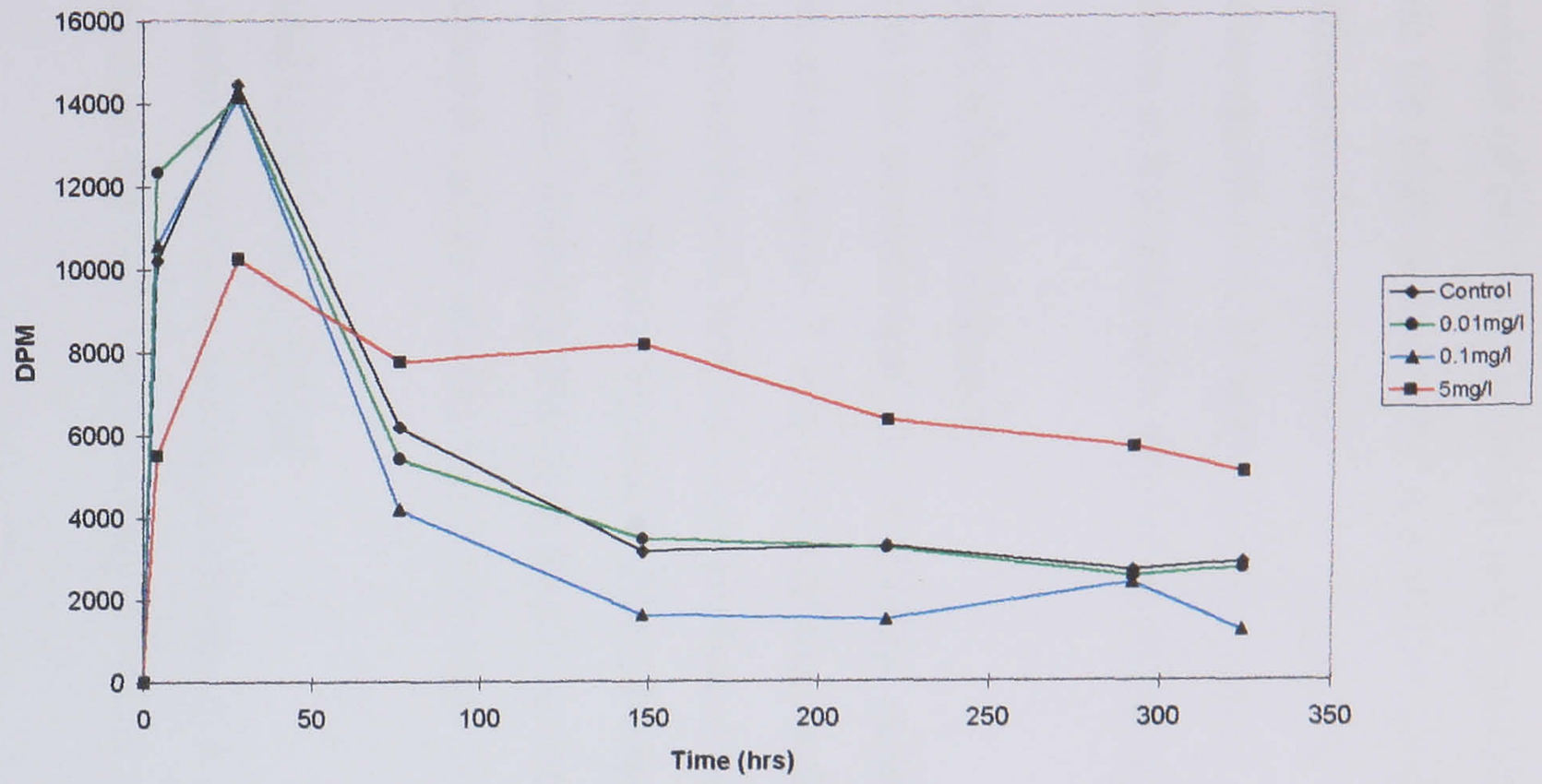


Figure 3c: Free Carbon in Solution (Wash counts)

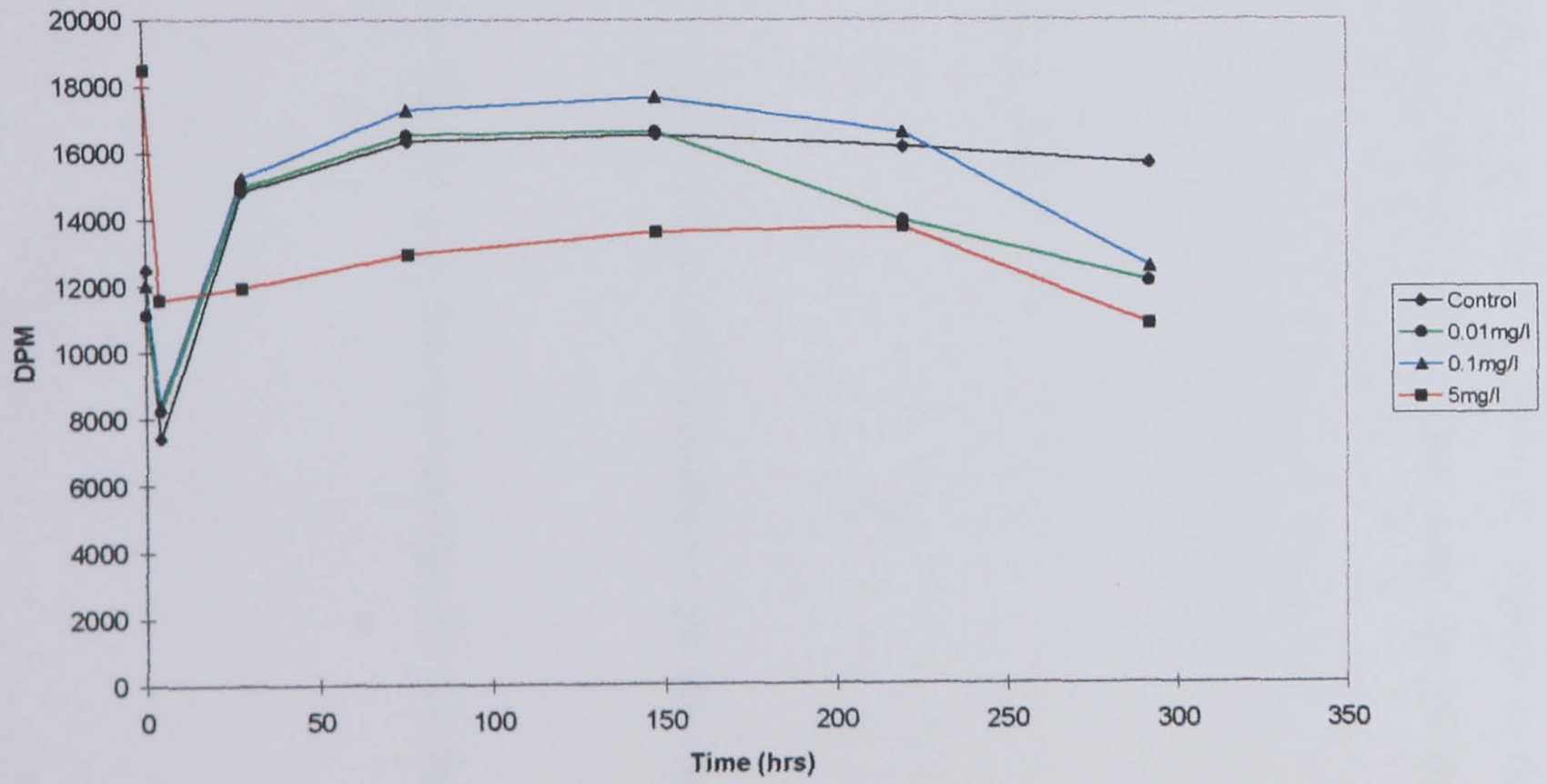
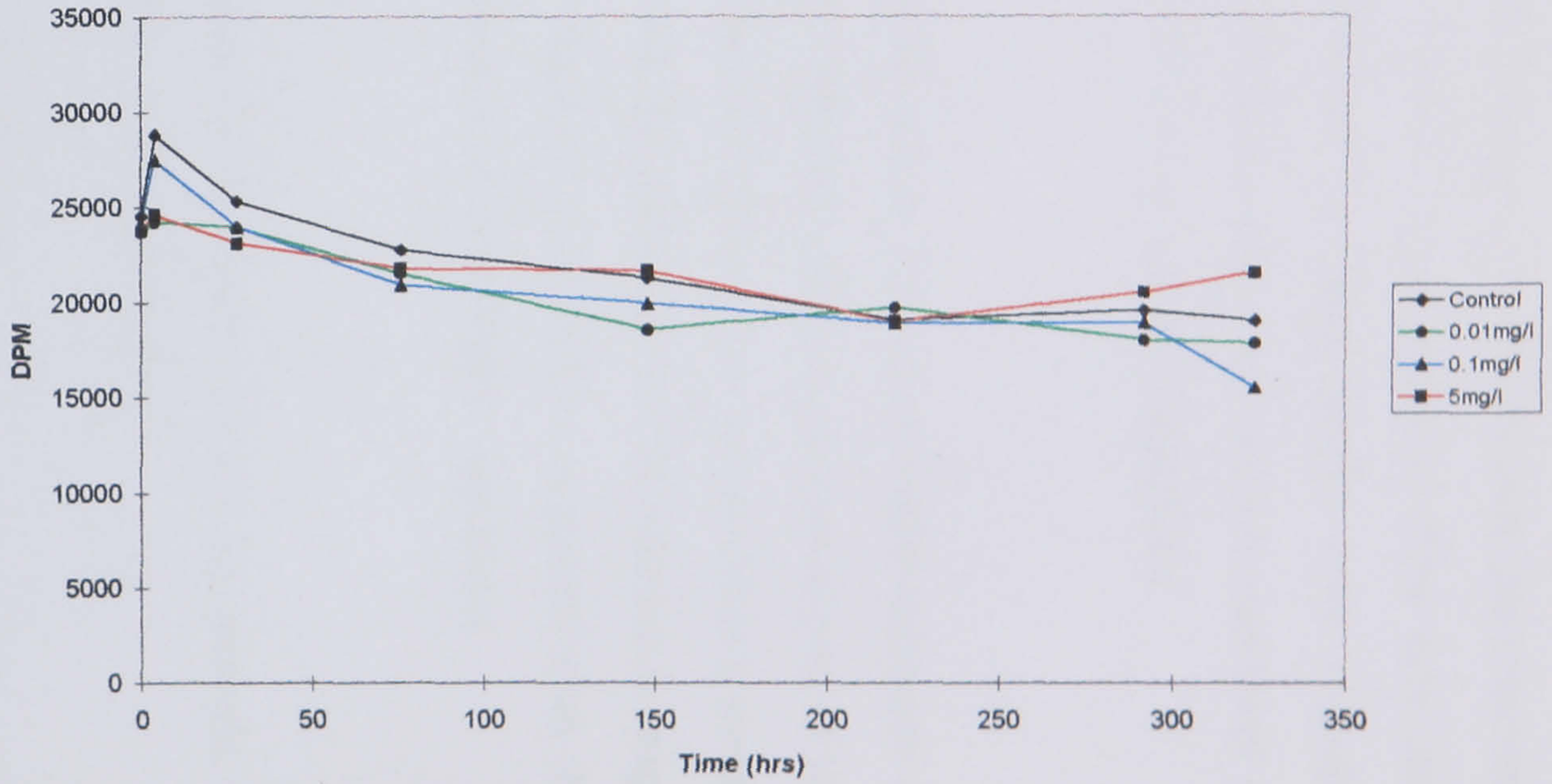


Figure 3d: Total Carbon in Solution (Total Counts)



Because of this (as the levels in the rest of the treatments decreased) it then became the treatment with the highest bacterial content of labelled carbon. The 0.01 mg/l treatment show very little difference from controls, so much so that after the first 48 hours results were virtually indistinguishable. However, in the case of the 0.1mg/l treatment there was a decreased level of carbon in bacteria after the initial 48 hour peak. This difference is real and significant.

Free Carbon in Solution

With the exception of the 5mg/l jars, results from the wash counts showed an initial reduction in free amino acids. It was assumed that in the initial stages labelled carbon is present as part of the amino acids and had not been broken down. Levels of labelled carbon then increased to a steady level. Again the control and 0.01mg/l treatment were virtually indistinguishable with the 0.1mg/l treatments showing elevated levels of free carbon in solution, as would be expected from the bacterial carbon counts. However none of these differences were statistically significant.

Total Carbon in Solution

Results from these counts were very similar to each other for all treatments and the controls. If this were the only measurement taken the results would be indistinguishable.

Results from these two experiments showed the use of amino acids to be a better method for detecting effects at the lower concentrations of oxytetracycline, which showed no impacts in the sodium acetate experiment. As a result of this labelled amino acids were used in all the remaining experiments.

5.3.3 Experiment 3

Antibiotic: Oxytetracycline

Carbon Source: [$U^{14}C$] Protein Hydrosilate (amino acid mixture).

Duration 664 hours (28 days)

Activity added: 426.5 Bq/ml (22590 DPM/ml)

Results from experiment 3 can be seen in Figure 5.4.

A two way analysis of variance was carried out on all results (5% level): Results for all the four measurements were found to be significant.

This experiment was carried out in exactly the same way as the previous one except for the fact that it was allowed to run for double the time.

Figure 5.4: DPM measurements for Experiment 3: River water with oxytetracycline and uniformly labelled [^{14}C] Amino Acid Mixture

Figure 4a: Carbon respired as CO2 (Cumulative headspace counts)

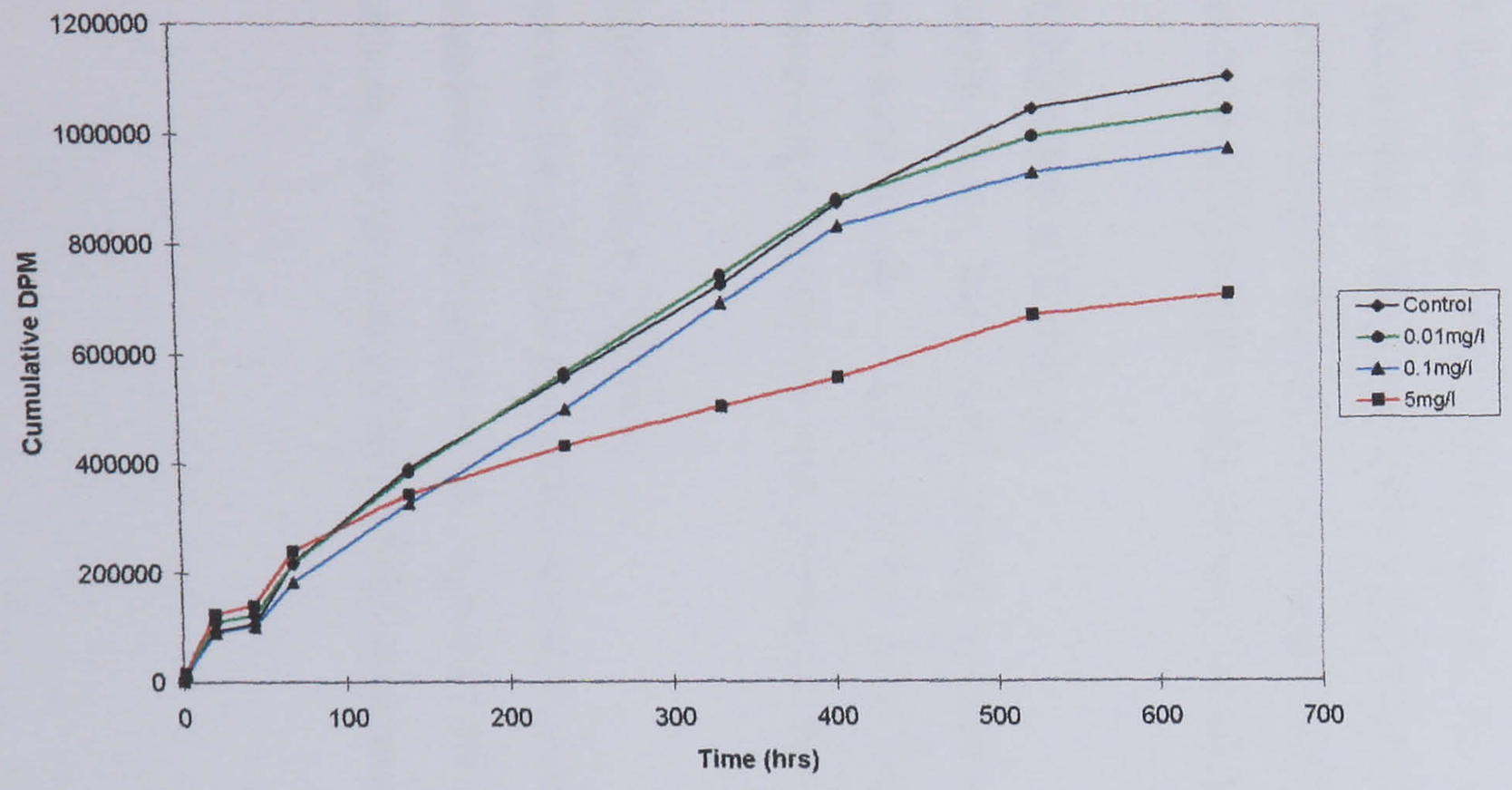


Figure 4b: Carbon Uptake by Bacteria (Filte counts)

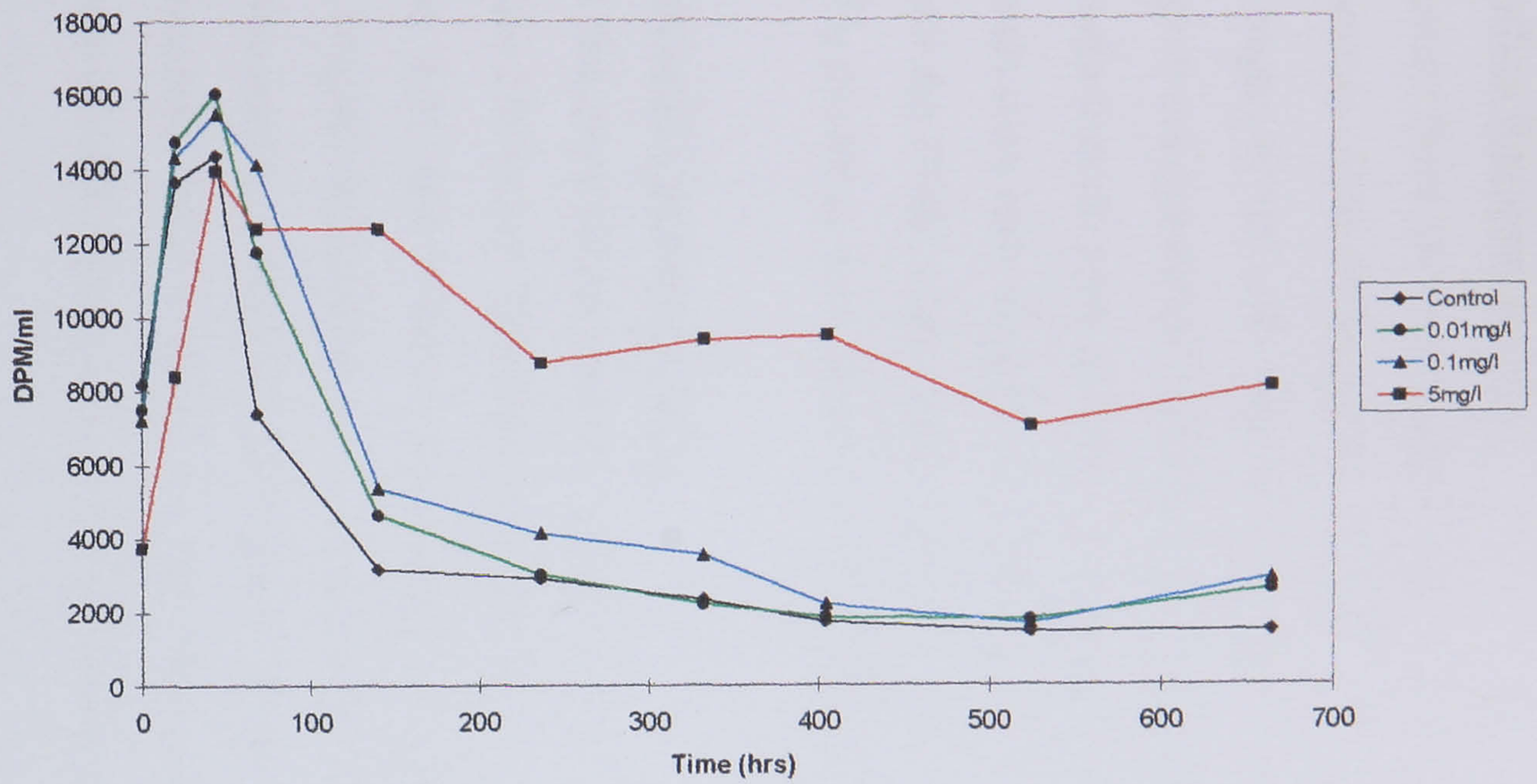


Figure 4c: Free Carbon in Solution (Wash Counts)

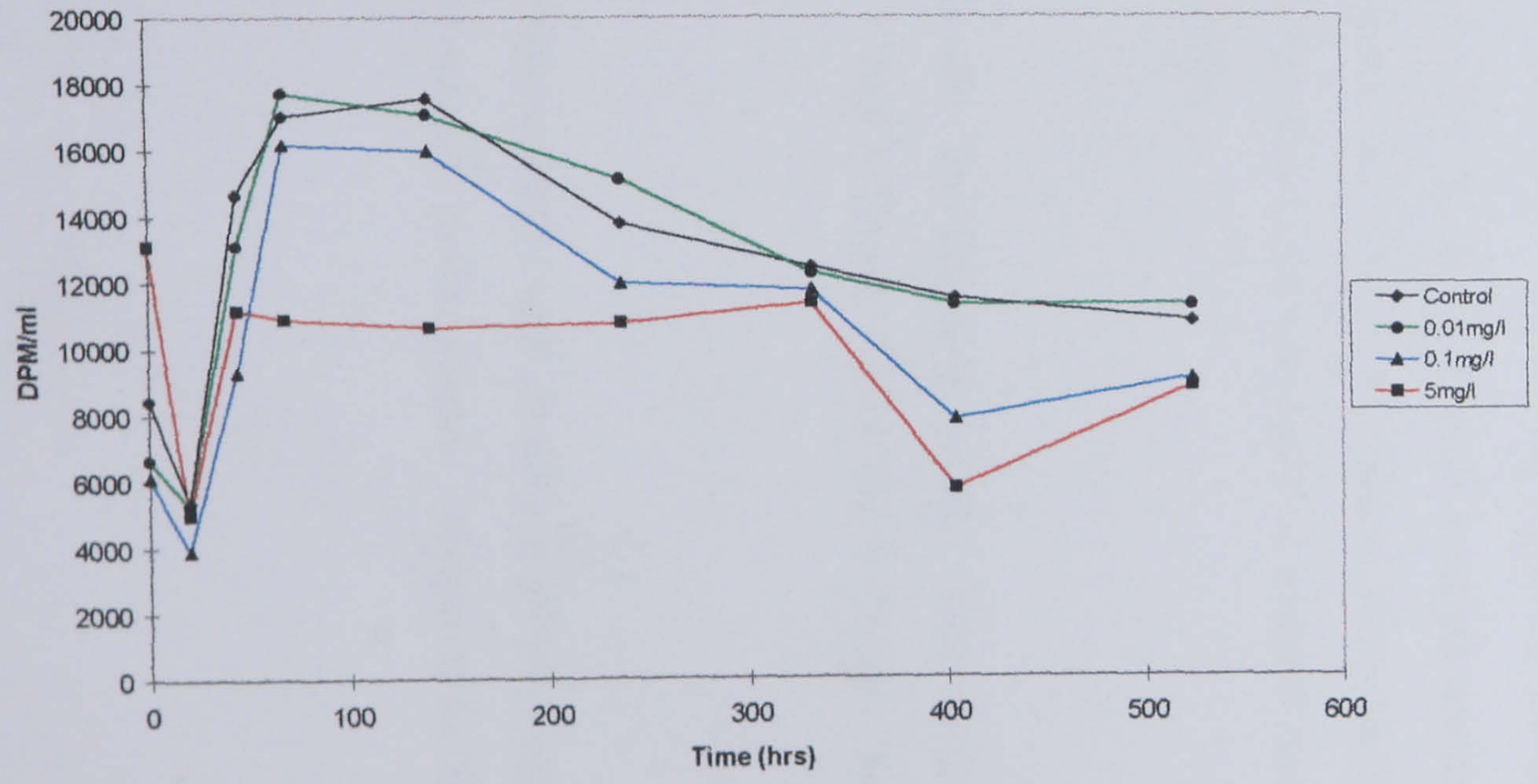
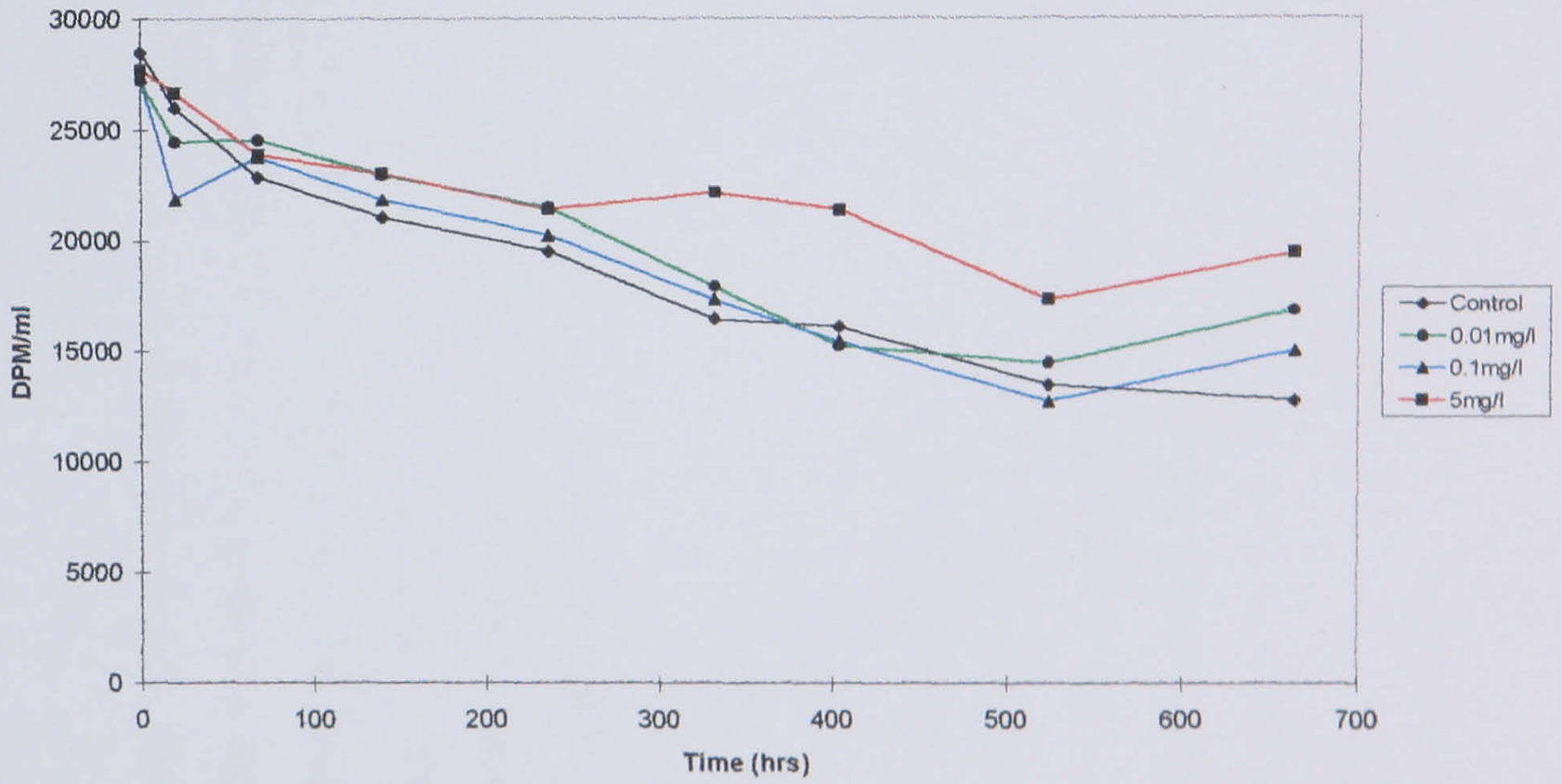


Figure 4d: Total Carbon in Solution (Total counts)



Carbon Respired as CO_2

Results from these measurements showed there was an initial high rate of CO_2 production for the controls. After 48 hours the rate became steady, and remained so over the next 350 hours, when it began to tail off. The 0.01mg/l jars showed very similar patterns to the controls. Both the higher concentrations showed an initial increased production of CO_2, but after 48 hours rates of production dropped and both remained lower than the control jars. In the case of the 0.1mg/l jars levels were only slightly reduced, however in the 5mg/l jars rate of CO_2 elution was much lower until the 524hr stage when rates of production were very similar for all samples. Total levels of CO_2 produces were around 65% of the controls.

Bacterial Uptake of Carbon

In this experiment controls again showed the initial peak in uptake, which quickly levelled out after 200 hours. This low level remained, with a slow decrease throughout the experiment. Both the 0.01 and 0.1mg/l concentrations showed slightly higher peaks than the controls. The 0.1mg/ml concentration remained higher than control for the rest of the experiment. The 5mg/l concentration showed inhibition of uptake during the initial peak, however concentrations then remained close to the peak level, decreasing slowly through the rest of the experiment. They were at least 3 times higher than the controls for the rest of the experiment.

In this experiment as in the last there was a definite impact on the 5 and 0.1mg/l concentrations. In this case there also appeared to be some effect initially in the 0.01 mg/l concentrations. Effects seen in this case there appeared to be a dose related. This was not the case in Experiment 2 where 5mg/l showed an increased level of carbon in bacteria when compared to controls and the 0.1mg/l showed a decreased level compared to controls.

Free Carbon in Solution

Results from the wash counts confirmed the results for the bacterial counts. There were decreased levels of free carbon in solution for the 5 and 0.1mg/l, whilst the 0.01mg/l had concentrations that are very similar to the controls.

Total Carbon in Solution

Results for all concentrations showed little difference from controls with exception of the 5mg/l treatments. This showed an increased presence of total activity in the system, compared to the controls, in the second half of the experiment.

5.3.4 Experiment 4

Antibiotic: Amoxycillin

Carbon Source: [U^{14}C] Protein Hydrosilate (amino acid mixture).

Duration 296 hours (13 days)

Activity added: 426.5 Bq/ml (22590 DPM/ml)

A two way analysis of variance was carried out on all results (5% level)

Results from experiment 4 can be seen in Figure 5.5

This experiment was the same as Experiments 2 and 3, except for the antibiotic added being amoxycillin instead of oxytetracycline. Though differences between the treatments were seen none of them, except for total counts, proved significant when a two way analysis of variance was carried out (5% level)

Carbon Respired as CO^2

In this experiment CO^2 evolution for the highest 5mg/l jars was similar to controls for the first half of the experiment after which they were reduced compared to controls. The jars containing 0.1mg/l and 0.01mg/l of amoxycillin initially showed increased CO^2 production compared to the controls. However, half way through the experiment the controls showed a large increase in production which resulted in levels being the same as controls for the second part of the experiment.

Bacterial Uptake of Carbon

In this experiment results, though not significant, showed a difference between the controls and the 5mg/l treatment. There was reduced uptake of carbon initially, followed by a change which resulted in slightly elevated amounts of bacterial carbon. Differences were small and statistically harder to determine because the results were all in the same range, the differences were related to the shape of the line. Differences were much smaller than in the oxytetracycline experiments.

Free Carbon in Solution

In this experiment no treatment showed consistently higher or lower levels of free carbon in solution. After an initial large reduction in levels of free carbon present there was a small steady increase of free carbon, this increase was greater for the controls and smallest for the 5mg/l concentrations.

Figure 5.5: DPM measurements for Experiment 4: River water with amoxycillin and uniformly labelled [^{14}C] Amino Acid Mixture

Figure 5a: Carbon respired as CO2 (Cumulative headspace counts)

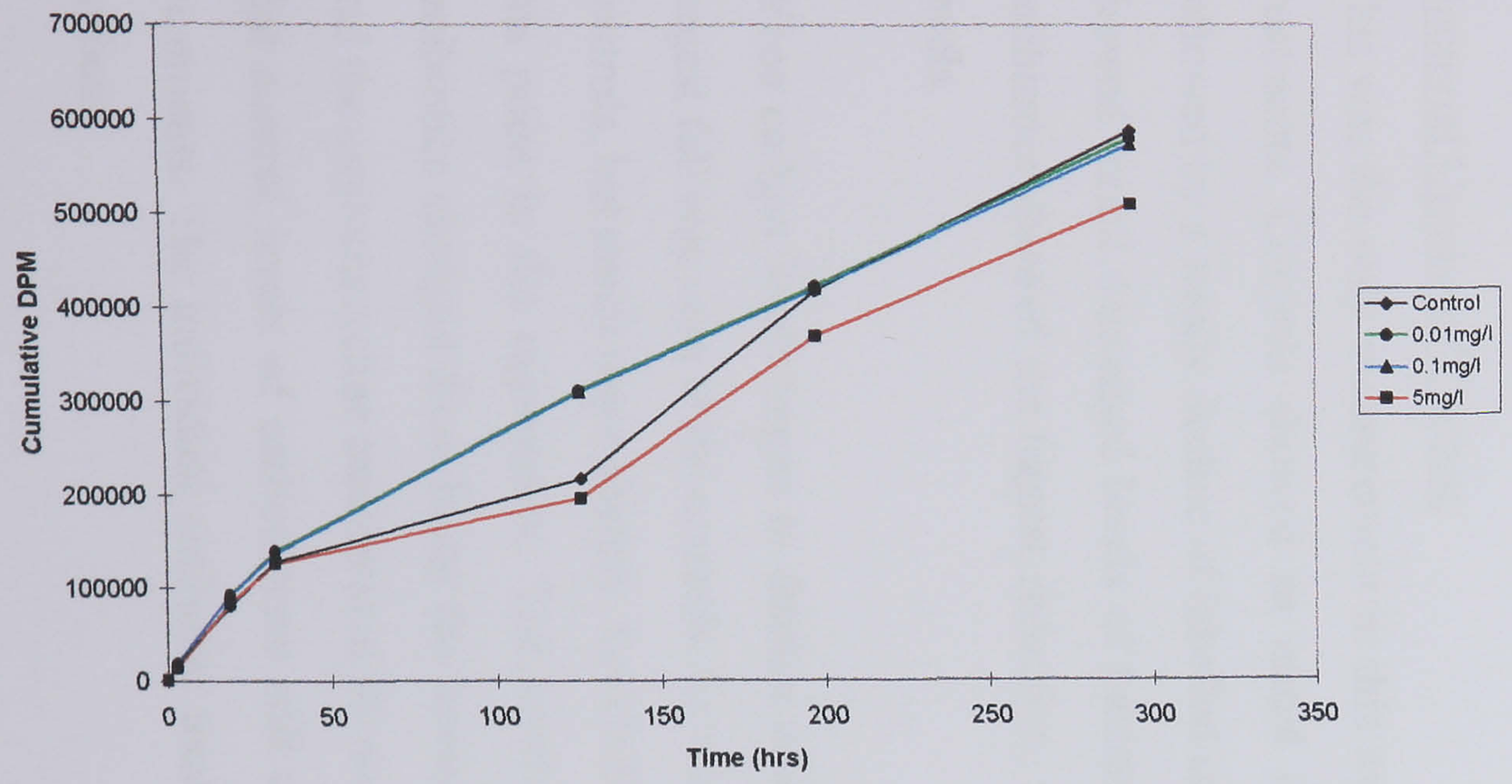


Figure 5b: Carbon Uptake by Bacteria (Filter counts)

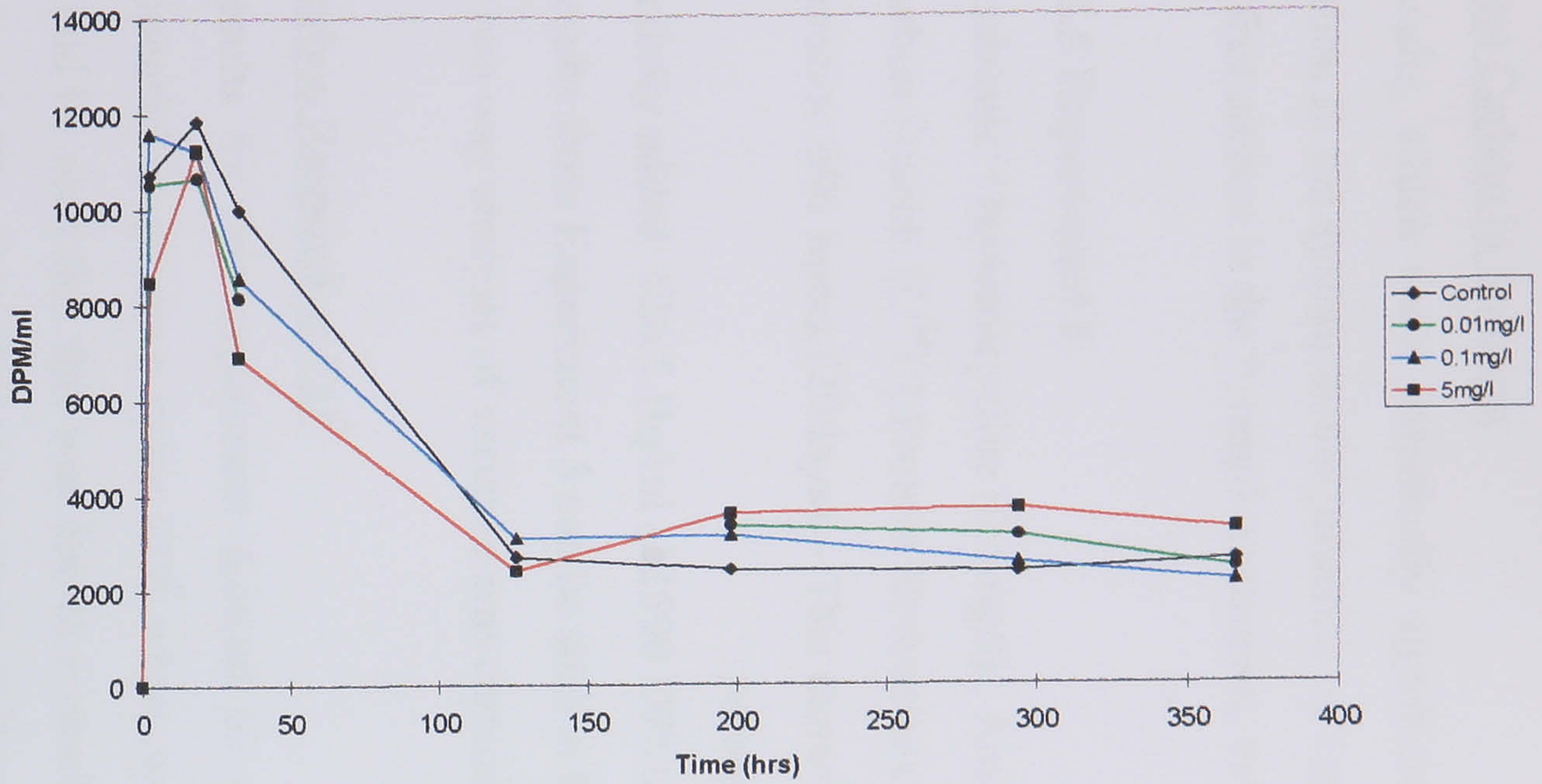


Figure 5c: Free Carbon in Solution (Wash counts)

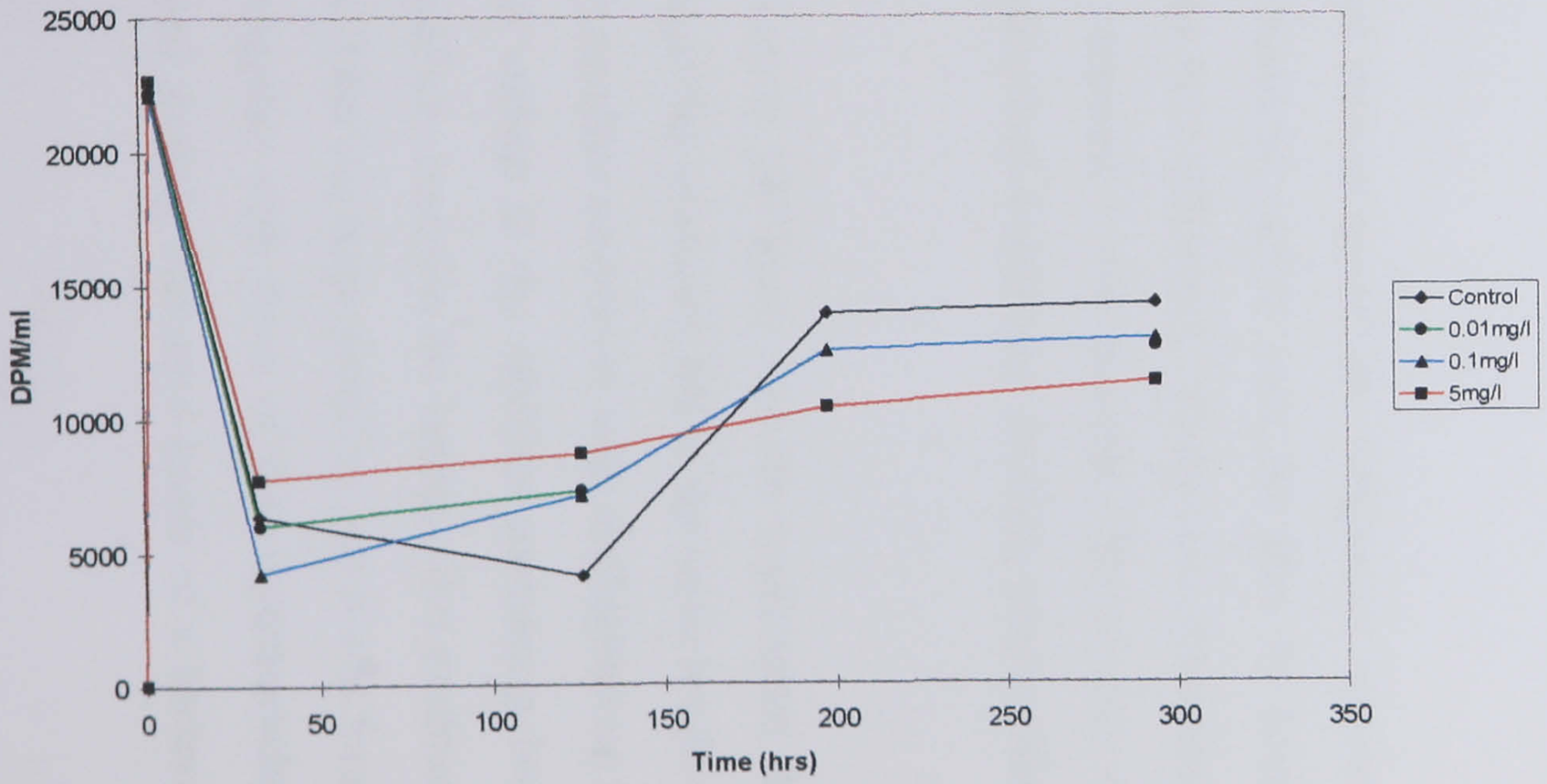
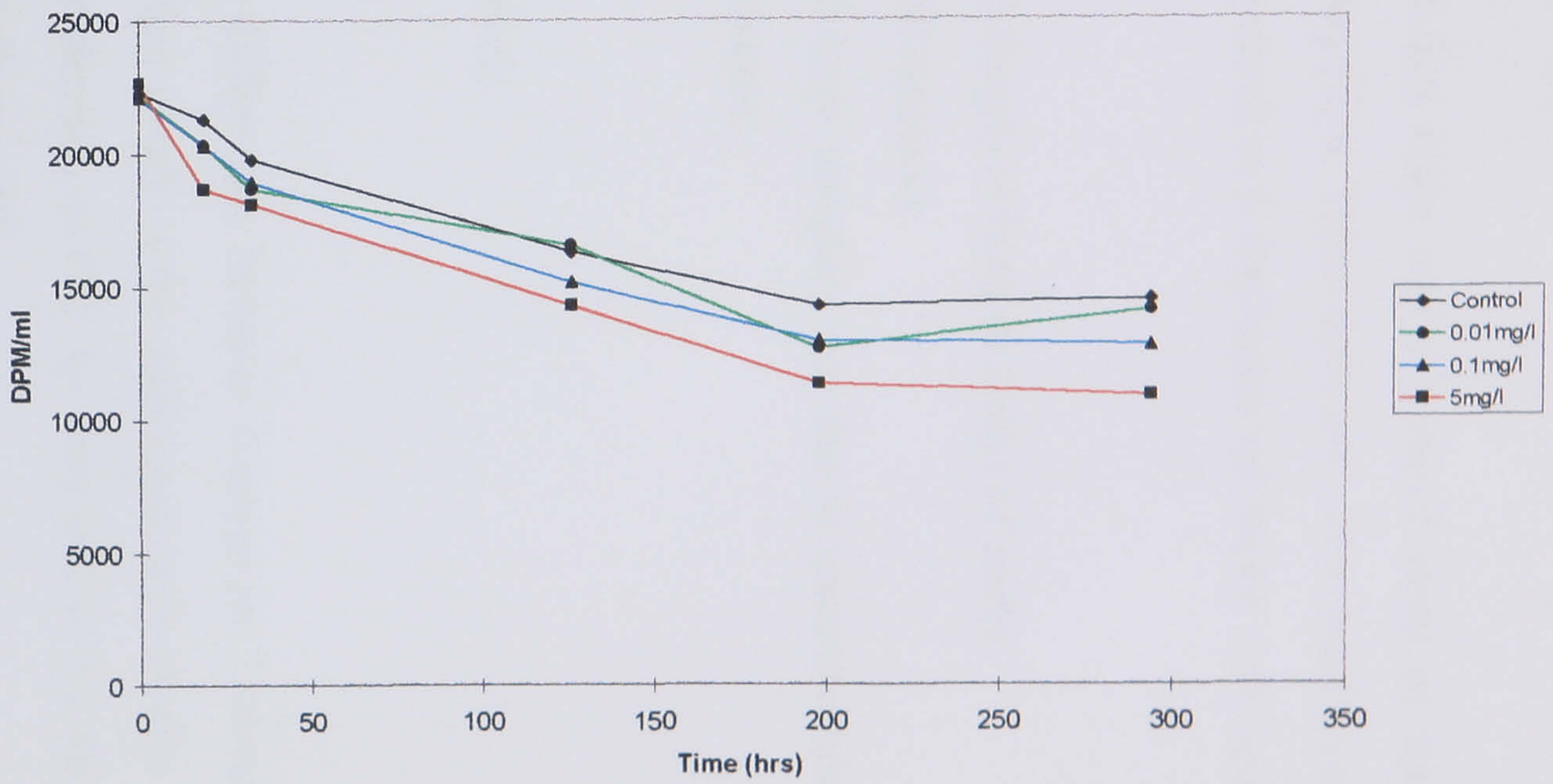


Figure 5d: Total Carbon in Solution (Total counts)



Total Carbon in Solution

Results, which were statistically significant, showed that there was a reduced level of total carbon in the system in the presence of 5mg/l of amoxycillin. There were also decreased levels of free carbon in the 0.1mg/l treatments, though not as much as in the higher antibiotic treatment.

5.3.5 Experiment 5

Antibiotic: Oxytetracycline (0.5mg/l) , Amoxycillin (0.5mg/l) or both (0.5mg/l of each).

Carbon Source: [U^{14}C] Protein Hydrosilate (amino acid mixture).

Duration 696 hours (29days) – This experiment had a large sampling gap due to uncontrollable personal circumstances.

Activity added: 426.5 Bq/ml (22590 DPM/ml)

Results from Experiment 5 can be seen in Figure 5.6

A two way analysis of variance was carried out (5% level)

Carbon Respired as CO^2

Results for this experiment showed no significant differences between treatments. Controls appeared to produce more CO^2 when averaged. When results were examined individually it could be seen that this was due to a much greater production of CO^2 by only one of the three controls The rest of the treatments all showed very similar results.

Bacterial Uptake of Carbon:

This was the only measurement in this experiment that showed significant differences between treatments. Controls showed an initial increase of bacterial carbon over the first 24 hours followed by a steady decline of labelled carbon over the next 600 hours. All the other treatments showed initial decreased levels of bacterial carbon compared to the controls. The mixture of antibiotics showed the largest reduction, with both individual treatments showing intermediate levels.

When carbon levels began to decline which occurred up to 144 hours into the experiment, the largest fall was seen in the controls. Levels in the amoxycillin treatment fell to the same levels as controls, but much more sharply. Levels for the oxytetracycline treatments were the highest up to this point in the experiment. The levels of bacterial carbon in the systems containing both antibiotics changed from being the lowest to being halfway between the amoxycillin treatment and the oxytetracycline treatment at 96 and 144 hours. After the large sampling gap it was found that control levels of carbon were still the lowest, together with those of the oxytetracycline treatments. The individual antibiotic treatments showed slightly increased levels of a bacterial carbon.

Figure 5.6: DPM measurements for Experiment 5: River water with 0.5mg/l of oxytetracycline, amoxycillin or both and uniformly labelled [^{14}C] Amino Acid Mixture

Figure 6a:Carbon Respired as CO2 (Cumulative headspace counts)

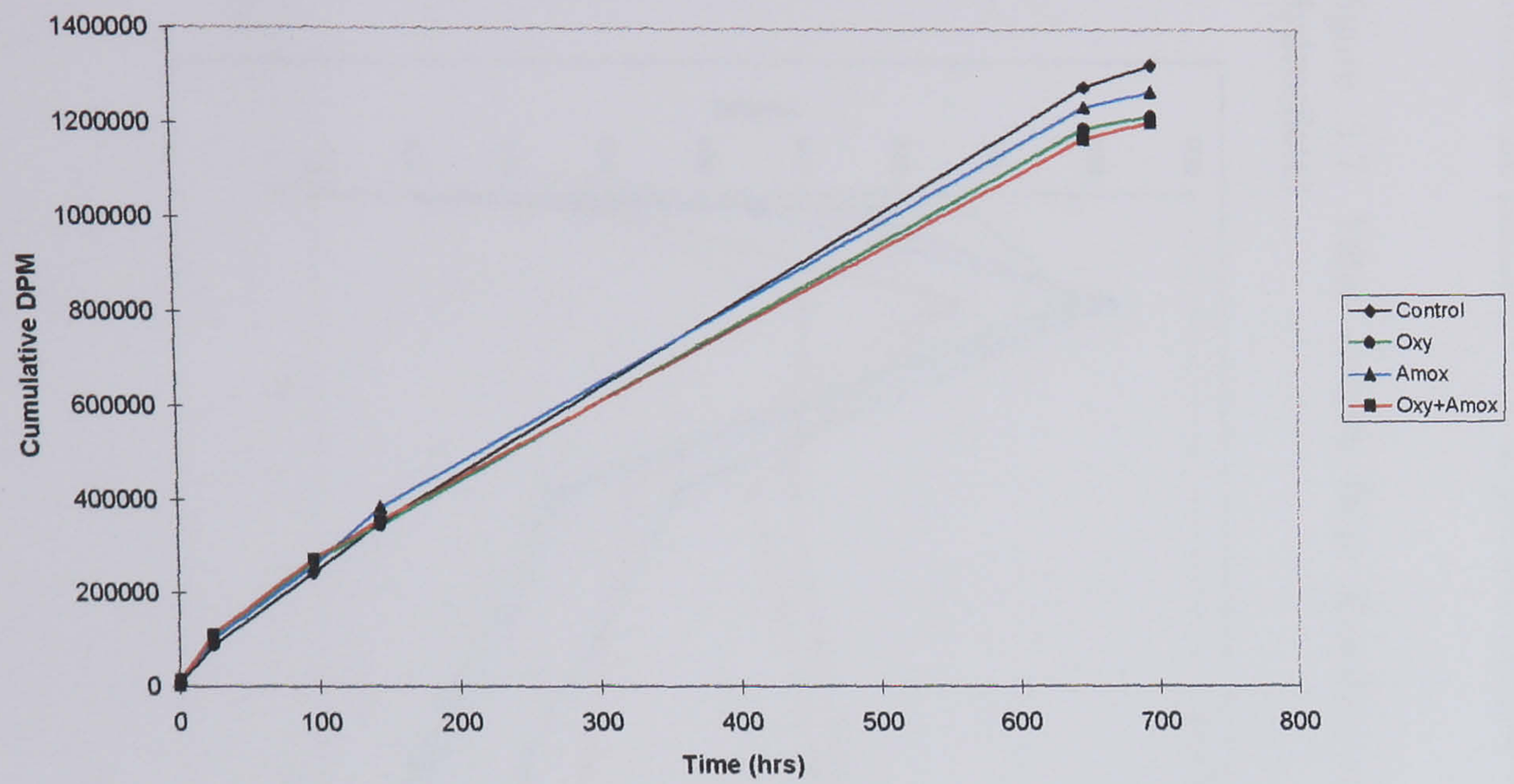


Figure 6b: Carbon Uptake by Bacteria (Filter counts)

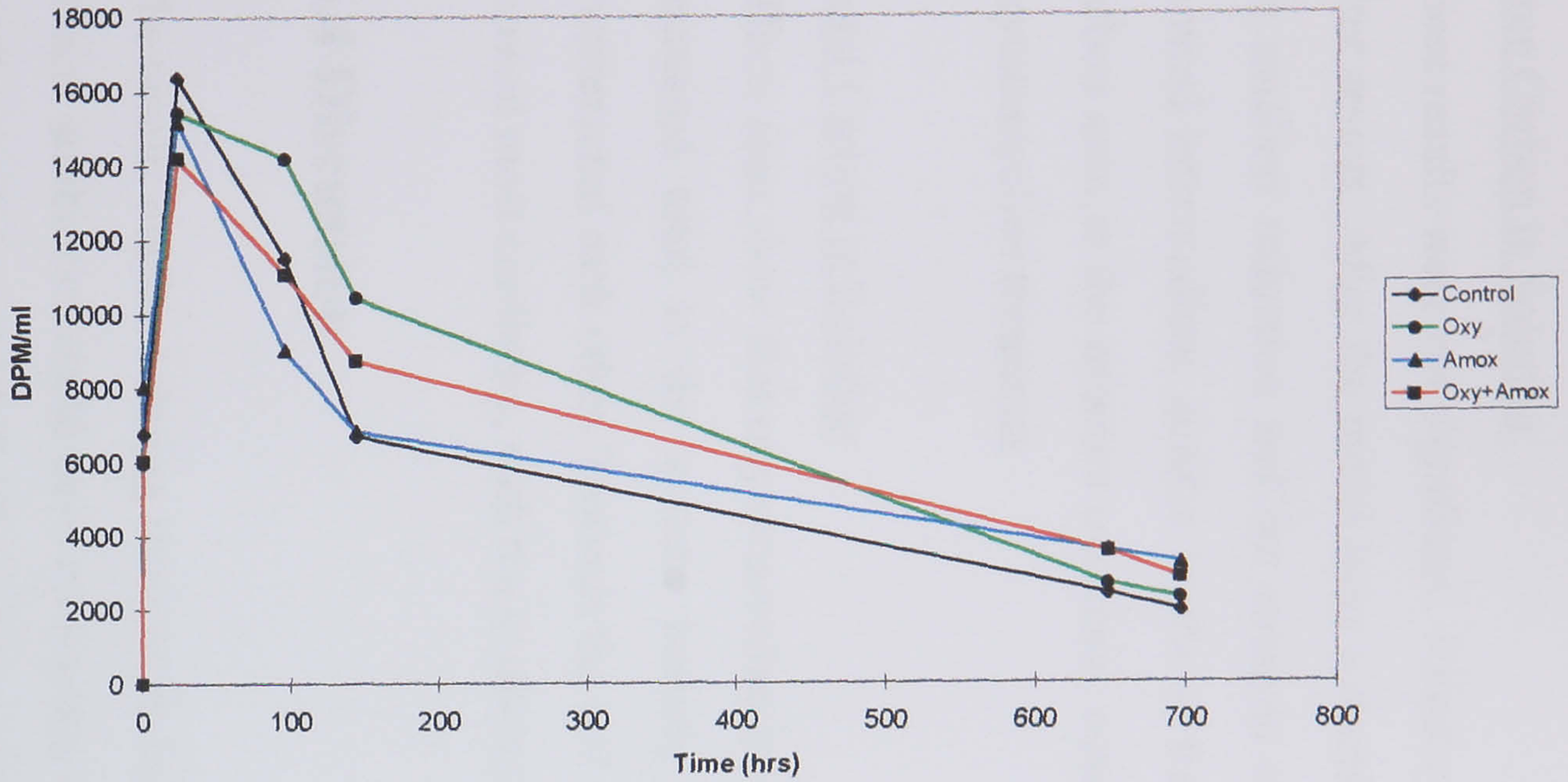


Figure 6c: Free Carbon in Solution (Wash counts)

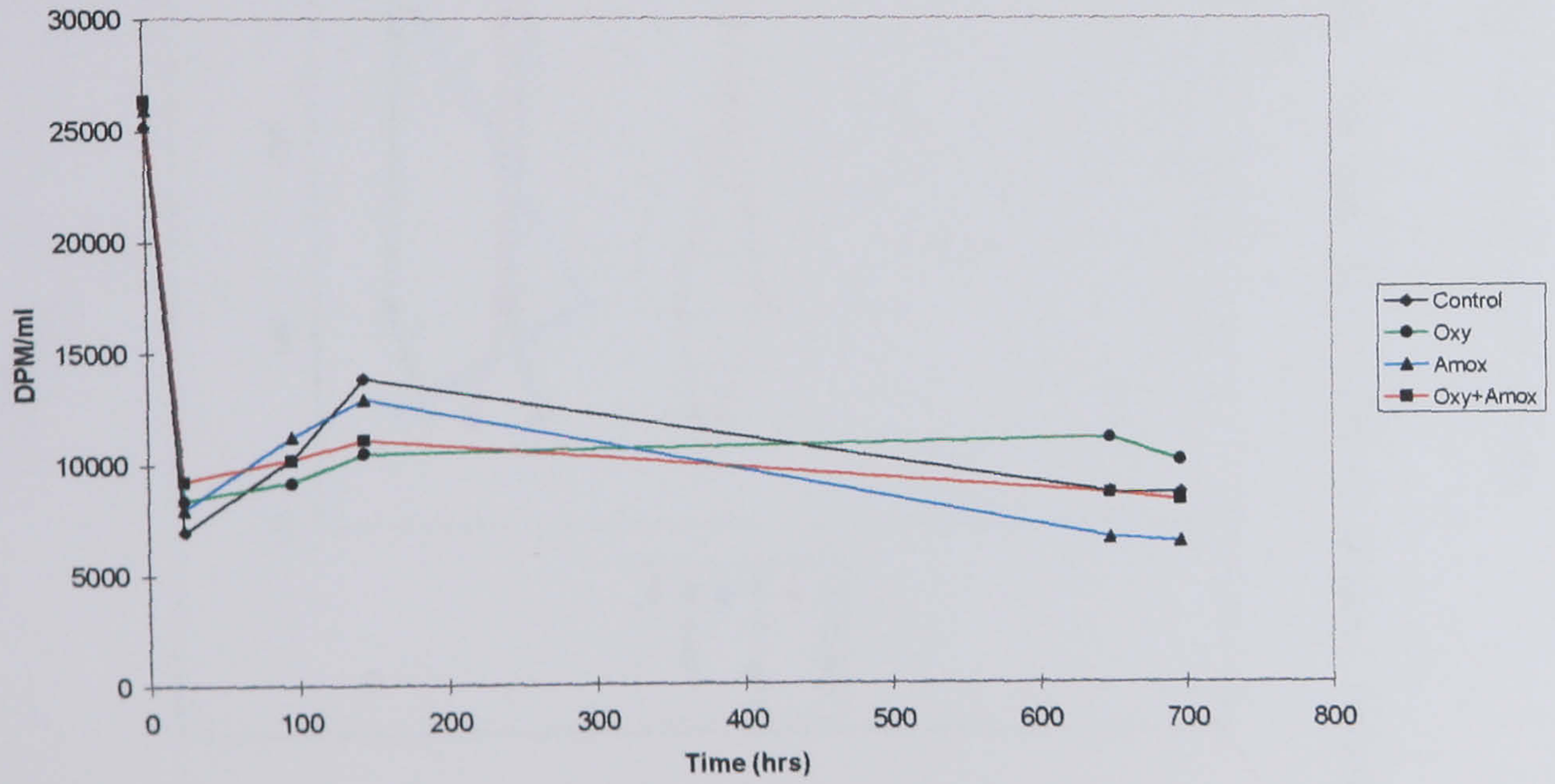
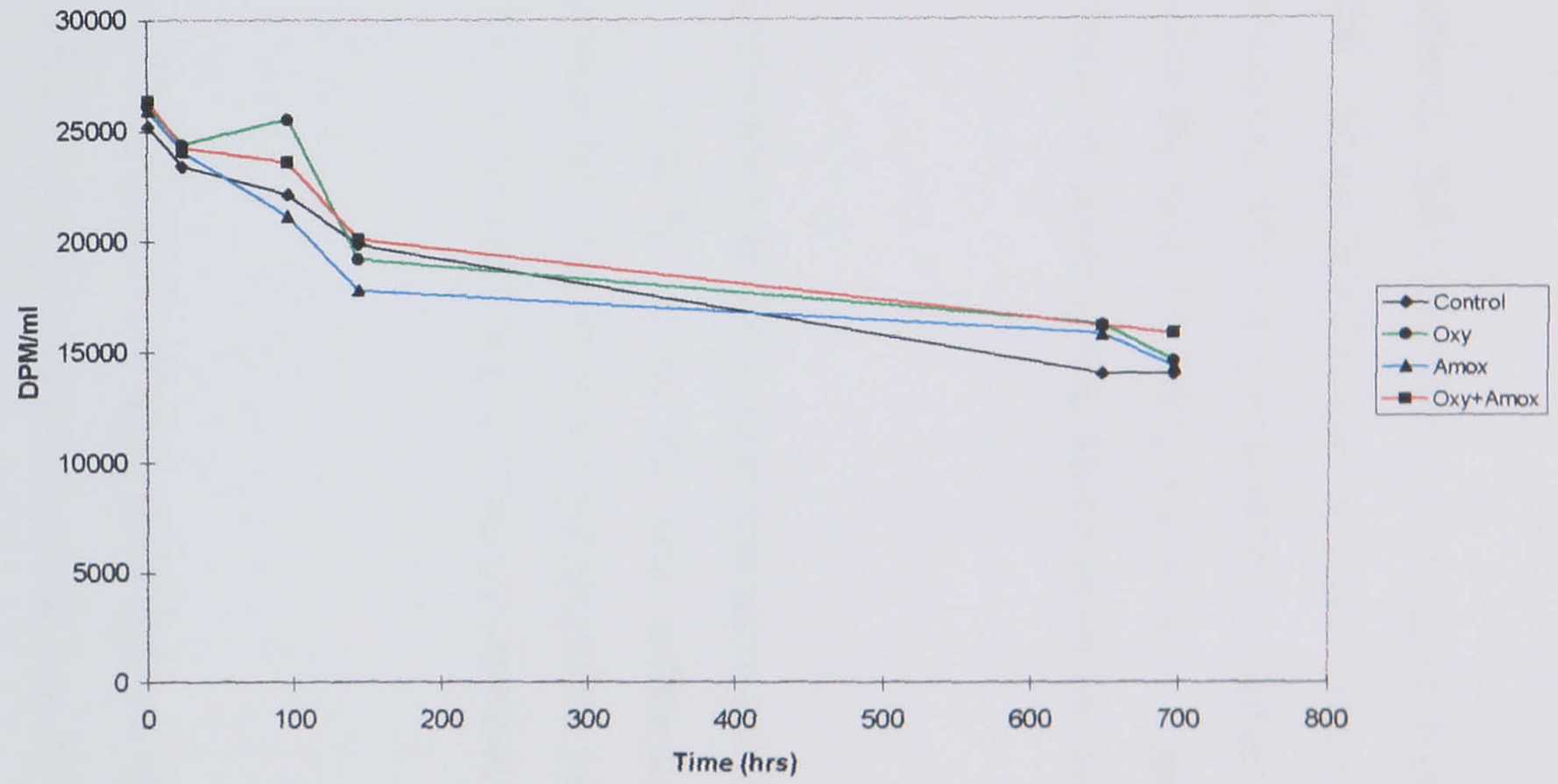


Figure 6d: Total Carbon in Solution (Total counts)



Free Carbon in Solution:

These results were not significant. Results obtained, however, did reflect those obtained with the filter counts. After the initial large reduction in available carbon, the mixture treatment showed the smallest reduction and the controls the largest reduction. The single antibiotic treatments showed intermediate, similar levels of free carbon. After the first 24 hours, the levels of free carbon seen in the mixture treatment were always in between those of the amoxycillin and the oxytetracycline treatment.

Total Carbon in Solution

Effects seen show that oxytetracycline increased the total carbon in the system and amoxycillin decreased total in the system initially, compared to controls. Again the two antibiotics counteracted each other. Towards the end of the experiment the controls and oxytetracycline jars showed most similarity, with the amoxycillin jars and the mixture jars also showing similarities.

5.4 Discussion

The results obtained during these experiments were very informative and have shed light on the effects antibiotics could have on aquatic ecosystems. Results showed duplicate jars giving very similar readings which facilitated the interpretation of results. Figure 5.7 is an example of how similar the readings for duplicate samples often were throughout all experiments.

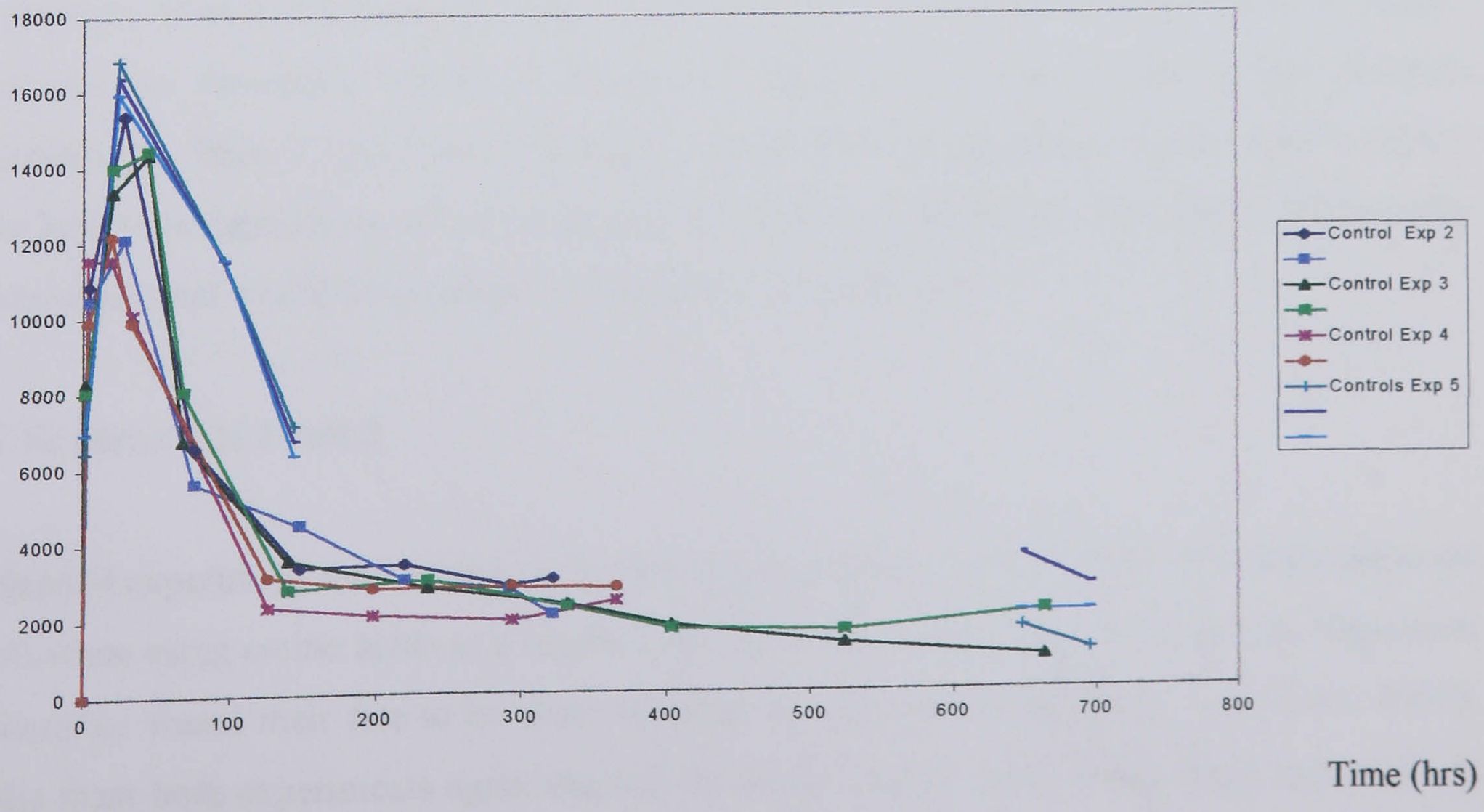
Figure 5.7: Filter counts from Experiment 3 (Antibiotic: oxytetracycline; Carbon source: Protein Hydrosilate)



In all the experiments by far the largest proportion of the labelled carbon remained free in solution and was not part of the particulate fraction. The free carbon readings were all around the 10,000 DPM/ml range whilst the bacterial counts, after the initial peaks were in the 2000 to 4000 DPM/ml range. However, the peak in filter counts and the initial reduction in wash counts indicated that over the first 24 hours of the experiment a large proportion of the amino acids and the sodium acetate (depending on the experiment) was actually taken up by the bacteria. Some of it was then broken down and respired as CO_2 but the majority was returned to solution. What cannot be determined from the data is whether the carbon was returned to solution in its original form, as a smaller fraction of the original molecule or in a completely different form. After the first few hours it cannot be assumed that the labelled carbon was still part of the compound originally added to the system.

When comparing the results for all experiments it became clear that throughout all the experiments the controls showed similar patterns of carbon utilisation. It can be seen in Figure 5.8 that filter count controls for experiments 2, 3 and 4 were nearly indistinguishable. The only differences seen were in the size of the initial peak of bacterial carbon. The controls for Experiment 5 all showed slightly elevated bacterial carbon, but levels were the same as in Experiment 3 by the end of sampling. These similarities between the controls give a strong indication that community structure of the river water bacteria remains constant over time, with similar carbon utilisation patterns. (Statistical analysis of these data was not possible due to the varying sampling times for each experiment). This fact ads weight to any conclusions reached on the effects of subinhibitory levels of antibiotics. Any large variations from controls are less likely to be due to simple variations between samples.

Figure 5.8: Filter counts for controls from Experiments 2,3,4 and 5.



5.4.1 Experiment 1

The first experiment, using oxytetracycline and sodium acetate, gave significant effects at the high concentration of antibiotic. This concentration of 5mg/l, which was used to ensure that there would be an effect detected, is not likely to be found in environmental conditions, as was determined in Chapter 3. In addition, it is higher than the MIC for some bacteria. However, it was important to know that this method was suitable for detecting the effects antibiotics have on bacteria.

Results showed that at 5mg/l there was a reduction in CO_2 respired, increased bacterial carbon and reduced free carbon in solution. Reduced respiration of CO_2 seems to indicate that there was a reduction of carbon turnover in the system. The levels of bacterial carbon remained high compared to controls. A possible explanation for this is that there were increased numbers of bacteria present in the system, which result in increased uptake of carbon. However if this were the case it would also be expect to see an increased evolution of CO_2. This did not happen. The reduction of free carbon and respired CO_2 compared to controls indicated that a much larger proportion of the labelled carbon remained in the bacterial fraction.

Another possible explanation is that there was a reduced turnover of bacteria at this concentration, resulting in the carbon being retained for longer in the bacterial fraction. The presence of the 5mg/l of tetracyclines resulted in a faster uptake of carbon followed by a reduction in the breakdown and release of carbon back into the system and its respiration as CO_2. Tetracyclines affect RNA transcription. It is possible that they reduced the ability of bacteria to break down the sodium acetate and therefore it remained much longer in the bacterial fraction.

The presence of oxytetracycline at 5mg/l had an impact on the metabolisation of sodium acetate. There was no detectable impact at all on the cycling of carbon in the system at lower concentrations, both 0.1mg/l and 0.01mg/l treatments were indistinguishable from controls. These lower concentrations where no impact was seen are still higher than any oxytetracycline concentration that would be expected in the aquatic environment.

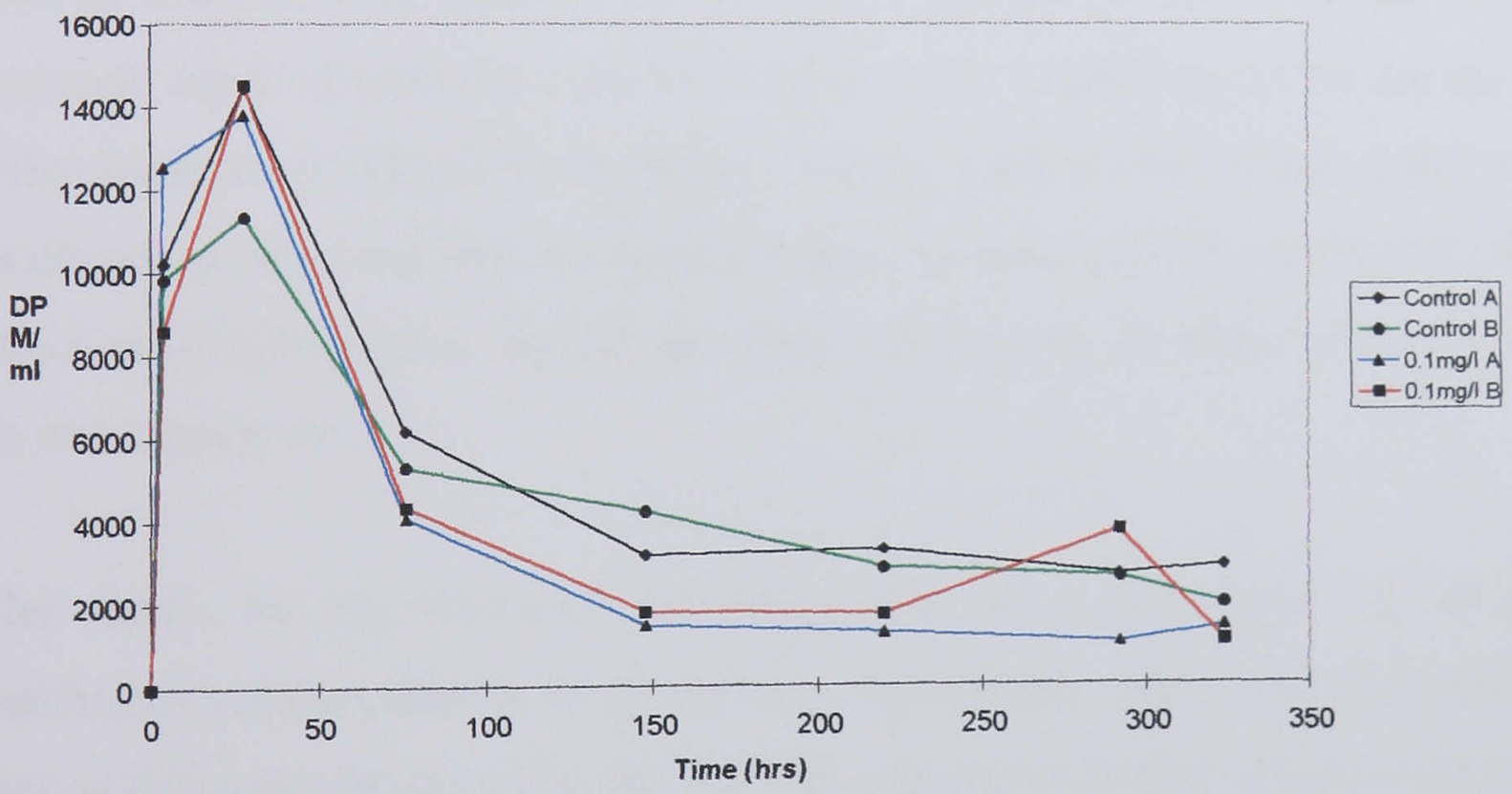
5.4.2 Experiments 2 and 3

The second experiment was carried out to determine whether it was possible to see any different effects when using amino acids as a labelled carbon source instead of sodium acetate. Reports in the literature found their fate to be more sensitive to the presence of antibiotics (Boon, 1992). Results from both experiments again showed the same kind of effect in the 5mg/l treatments as

in the case of sodium acetate. In both cases there was a clearer initial inhibition of amino acid uptake by the bacteria after which the bacterial fraction contained much more carbon than the controls. This increased carbon content was not as large as in the first experiment, which could be due to the different carbon source added. As in the first experiment, total carbon respired was also greatly reduced in both experiments, compared to controls. This again pointed to an initial inhibition of uptake followed by decreased turnover which resulted in less available carbon in solution, reduced carbon breakdown and CO_2 production.

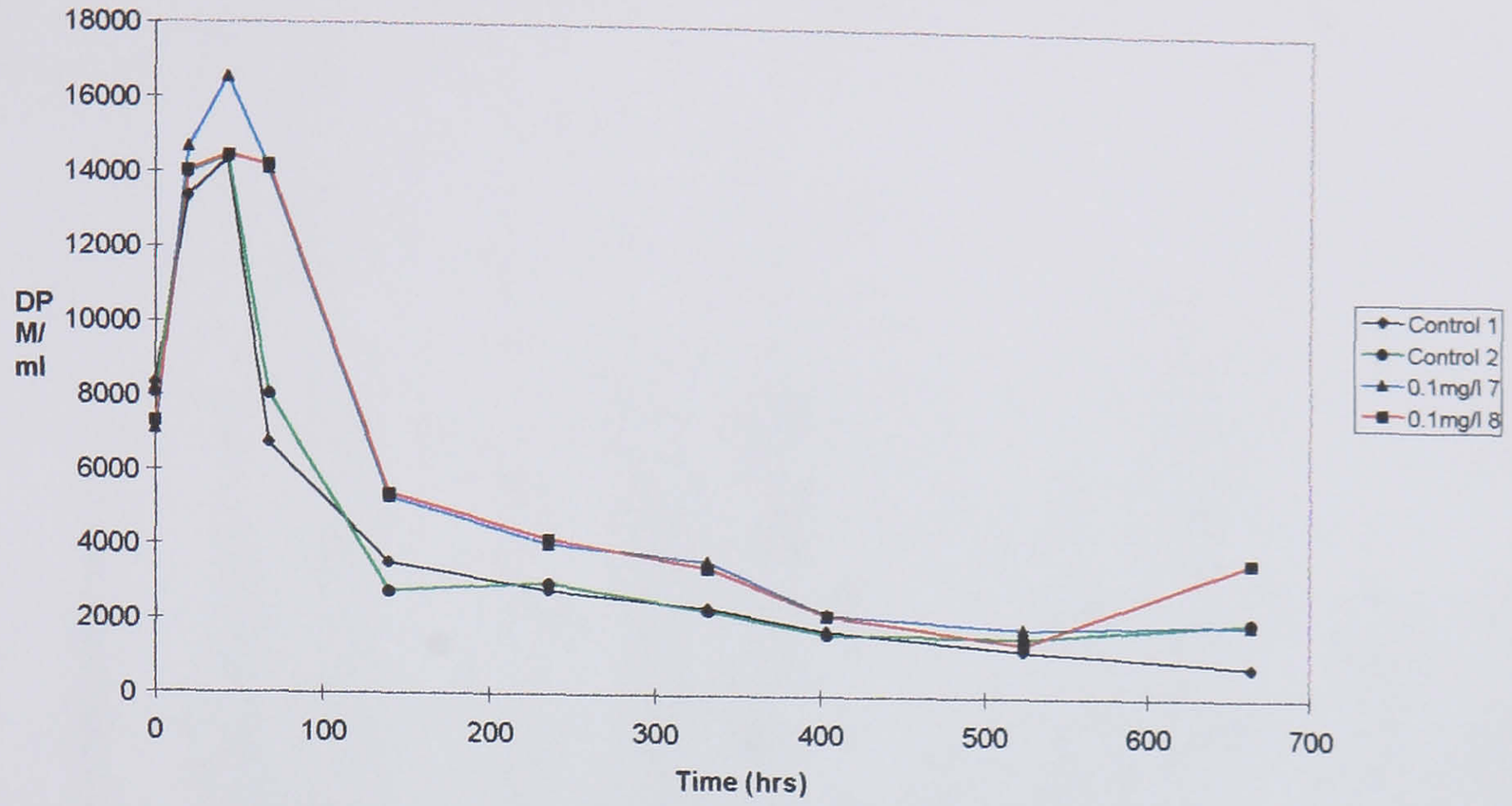
In both these oxytetracycline and amino acid experiments, unlike the first experiment, there was a an indication of an effect at lower concentrations of oxytetracycline. In the first experiment 0.1mg/l concentrations of antibiotic resulted in a decreased level of carbon present in bacteria occurring in both jars (as can be seen in Figure 5.9) which give very similar results. This was a strong indication of an effect.

Figure 5.9: Carbon utilisation by bacteria in controls and 0.1mg/l oxytetracycline jars. Experiment 2



In the second experiment, there again was a visible, though small, effect for the majority of the experiment, particularly the first 300 hours which was as long as the first experiment ran. The difference was diminished after this which suggests that any impact at this concentration is not necessarily permanent, even though the oxytetracycline should still be present. However, this time there was an increased presence of carbon in bacteria, which is the opposite of the effect seen in the previous experiment (see Figure 5.10). The differences between the controls and the treatments are in a similar range, though one is positive and the other is negative

Figure 5.10: Bacterial Uptake of Carbon by controls and 0.1 mg/l jars. Experiment 3.



The reason for one experiment showing an increase and the other a decrease could simply be as a result in differences in populations. River water samples can show variation in bacterial content, both in terms of total numbers and species composition. It is a mobile medium with a natural, constant, input of material from external sources. In addition rivers are the receiving water for many bacterial pollutants from human sources, particularly sewage and farm runoff. However, as already pointed out, the similarities in the controls, together with results detailed in Chapter 6 which will highlight how similar the overall community structure of the sample site is over time, do not support this.

The results for the 0.01mg/l oxytetracycline jars, again show no difference between the treatments and the controls. In all the three experiments carried out up to this point no effect was seen at this concentration. For this method at least, the lowest effect concentration can be said to be higher than 0.1mg/l and possibly to lie somewhere between 0.01 and 0.1mg/l.

All four measurements of labelled carbon fate can be compared by plotting a bar chart of the various DPM readings at different times and for different treatments. Figure 5.11 shows all readings taken during experiment 3 (oxytetracycline and labelled amino acids); in it all readings have been calculated to be the DPM per millilitre of river water in the jars. From the results it can be seen that only small fraction of the total carbon added to the system has been lost as CO_2. Most of the carbon remains in the system. After an initial peak of bacterial carbon, the majority of the carbon is in solution for the remainder of the experiment. Differences already highlighted between the controls and the treatments can be seen clearly.

Figure 5.11: Mean DPM per millilitre of sample counts for Experiment 3 (Antibiotic: Oxytetracycline; Labelled carbon source: Protein hydrosilate).



HC: Headspace counts; FC: Filter counts
WC: Wash counts TC: Total counts

5.4.3 Experiment 4

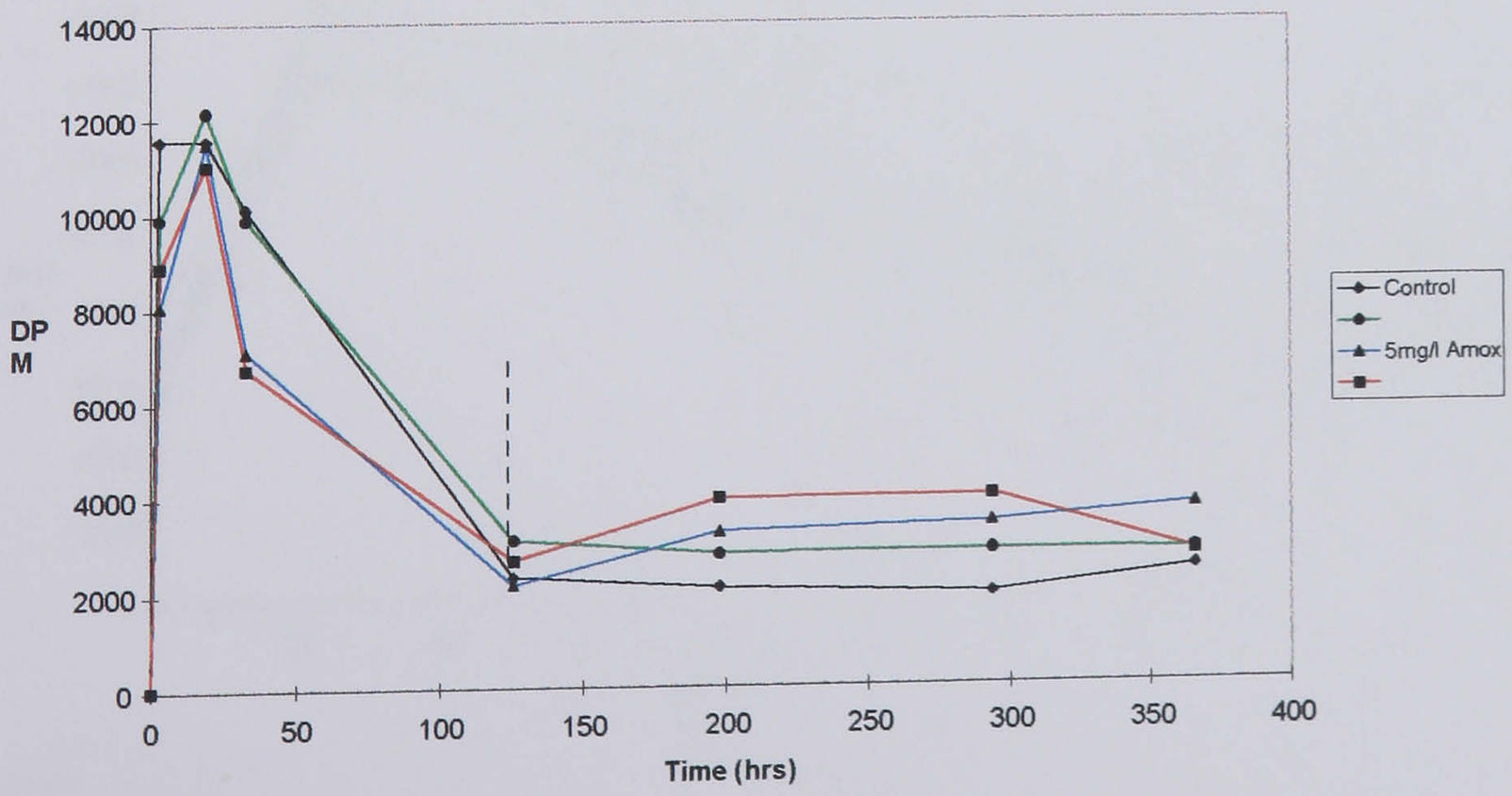
The fourth experiment carried out used the same method to look at the effects of amoxycillin on river water communities. The results from this experiment are not as clear cut as those from the previous ones. Firstly, the effect of 5mg/l, though visible was not as dramatic. Again an initial inhibition of uptake of the amino acids is seen, but in this case it was not as severe as in the case of tetracyclines. A major difference is that there was a definite peak of labelled carbon present in the bacteria, following the pattern of the controls. This is different from the effects seen in the case of oxytetracycline, where levels increase and then remain high. Interestingly, after the initial inhibition for over 100 hours, there was an increase in bacterial carbon at this concentration. This was not a large increase but it remained constant throughout the remainder of the experiment. The effect seen is initially opposite to that of the oxytetracycline. Half way through the experiment there is a change, bacterial carbon becomes greater than controls, but on a much smaller scale This effect is mirrored by the levels of free carbon in solution, which confirms the effect seen. In this experiment only the differences in total carbon levels in the river water proved to be significant. However, because of this, differences seen in the other measurements must have been real, even if they were not big enough to be statistically significant.

Overall, in the case of the 5mg/l jar, the initial reduction in CO_2 production, together with decreased uptake by bacteria of amino acids, with an increased level of free carbon in solution points to direct inhibition of bacteria in the system. Halfway through the experiment the rate of CO_2 production increases and then becomes the same as the rest of treatments, bacterial carbon increases and carbon in solution falls, compared to controls. There is a contradictory result for this concentration: whilst it produces the lowest amount of CO_2 it also has the lowest level of total carbon in solution of any of the treatments. This means that there is some fraction of carbon not accounted for, as all had the same amount of carbon added initially.

In this experiment, the results for the 0.1mg/l treatments seem to mirror the effect seen in the 5mg/l jars, including the fact that there appears to be less total carbon in the system than results suggested. Unfortunately, one of the data points is missing for the 0.01mg/l jars which makes it harder to determine if there is any indication of an effect at this concentration.

Amoxycillin is biodegradable, with the resistance arising in sensitive bacteria exposed consisting of the ability to degrade the antibiotic. In addition there is a large proportion of bacteria that are naturally less-susceptible to these antibiotics, due to their cell wall structure. Because of this it would be expected that the impact of amoxycillin would be less than that of oxytetracycline, which is not degradable, in the same situation. It would also be expected in the case of amoxycillin, for the effects seen to begin diminishing as resistance arises and the antibiotic is broken down. It can be seen in Figure 5.12 that the two treatments are very distinct until the 120 hours mark, after which the effect diminishes.

Figure 5.12: Results from duplicate bacterial uptake counts. Controls and 5mg/l of amoxycillin



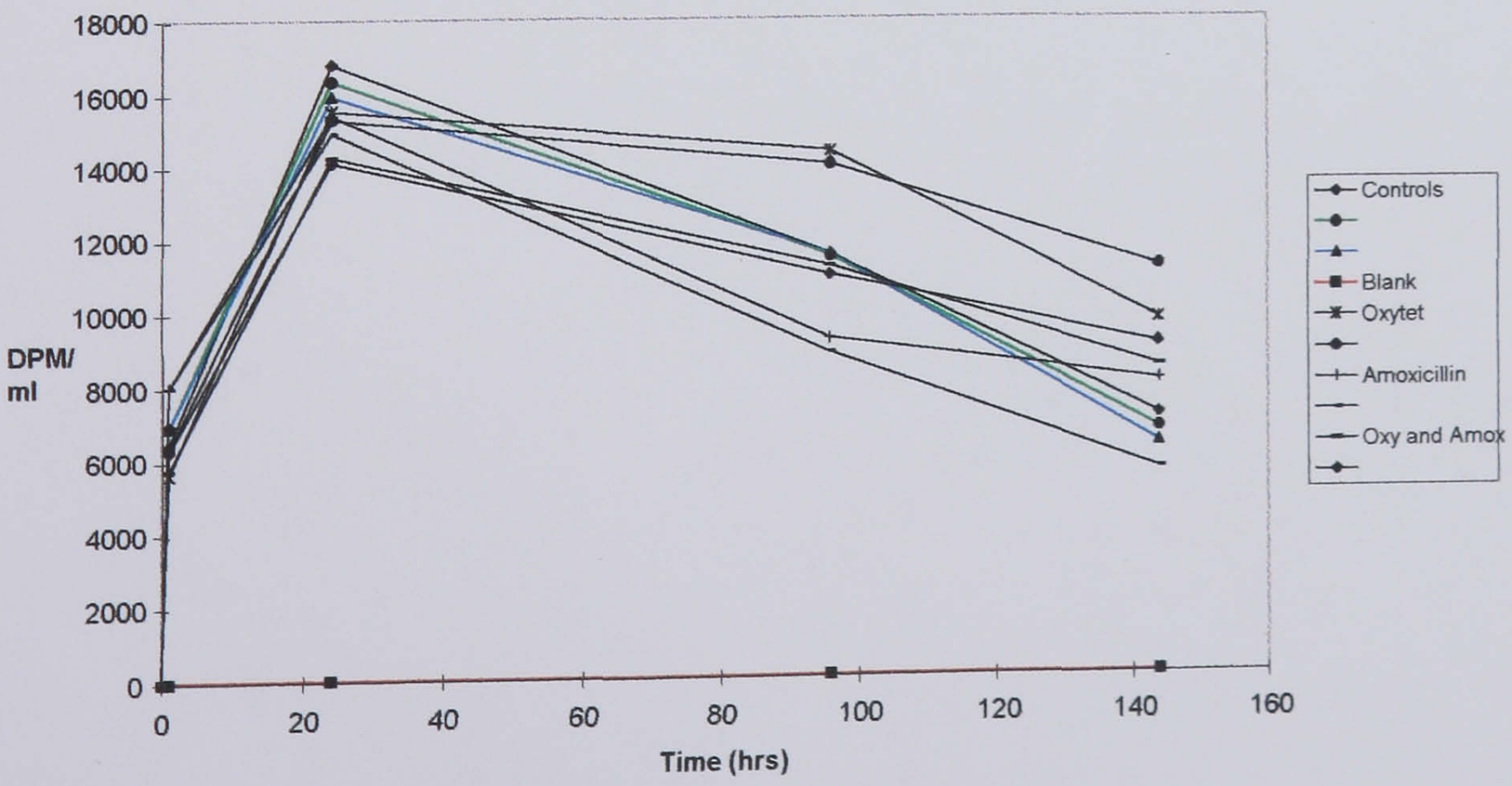
5.4.4 Experiment 5

The final experiment was carried out with the aim of determining two things: firstly, what the effect was of introducing two antibiotics into a system together. Secondly, as the previous experiments identified 0.1mg/l of both antibiotics as a concentration where there was an indication of impact, 0.5mg/l was decided on as a higher concentration that would help determine where the lowest effect concentration for both antibiotics lay.

Results from this experiment were not as comprehensive as was originally intended due to uncontrollable personal circumstances that lead to a large sampling gap. However, it was possible to see that both amoxycillin and oxytetracycline had an effect on the system at 0.5mg/l.

Initial results, after one hour, showed that there was enhanced uptake of carbon in the case of the amoxycillin treatments and reduced carbon uptake in the case of both the oxytetracycline and mixed treatment. After this and up to 96 hours after the experiment was set up, there was a change which resulted in the highest level of carbon being present in the bacterial fraction of the oxytetracycline treatment, the lowest level in the amoxycillin treatment and the mixed treatment showed intermediate levels, as did the controls. This seems to indicate that there is an antagonistic effect when both amoxycillin and oxytetracycline are present. Though the two antibiotics have very different and unconnected modes of action they appear to counteract each other's impact on the amounts of carbon present in bacteria.

Figure 5.13: Individual filter counts for first 145 hours of Experiment 5



The individual results for each jar, including the three controls, are shown in figure 5.13. and again there are strong similarities between duplicate treatments, and each treatment is distinct from each other. This supports the results obtained as it indicates that what is seen is a real effect.

Amoxycillin interferes with bacterial cell wall construction, and oxytetracycline affects protein synthesis. There are some possible ways in which the presence of amoxycillin might affect the impact of oxytetracycline. Uptake of oxytetracycline is important when it comes to its mode of action and this is why it is active against prokaryotic cells and not eukaryotic ones, as the latter are much less permeable to oxytetracycline. It is possible that the structural changes that result in the cell wall in the presence of amoxycillin may reduce uptake of oxytetracycline and therefore its impact. In addition, cells that have been treated with penicillins are not able to divide properly and as a result get larger and misformed without increasing in numbers. As a result of reduced number of bacteria it is possible that there is a reduced level of amino acids taken up as a whole by the community. Both of these possibilities would result in reduced impact of the antibiotics if they were both present. Another possibility is that the presence of amoxycillin damages the cell wall of the bacteria, which then results in leakage of, among other things , the labelled carbon molecules. If this were the case then the presence of both antibiotics would not result in an antagonistic effect, what would be occurring would be a reduced ability to detect the impact of the antibiotics present.

5.5 Conclusions

The first conclusion that can be drawn from the results obtained is that this method of monitoring ecosystems shows promise as for being a reliable method because results were reproducible. Reproducibility is often a problem when looking at environmental micro-organism samples, particularly when trying to isolate and count individual species. The reliability of this whole community experiment could be very useful and has never been demonstrated before.

These experiments showed that the presence of antibiotics in river water resulted in reduced breakdown of carbon compounds, which is a vital role bacteria play in aquatic ecosystems. This was shown by a reduction in CO_2 respired by the systems and reduced levels of free carbon in solution. This was as a direct result of the carbon input into the system being retained in bacteria for longer. There is also reduced available carbon in the system as well as increased bacterial carbon. From this it can be concluded that the bacteria could be slowing down the turnover of carbon, thus limiting its availability to other parts of the ecosystem, such as algae.

Effects were seen for both antibiotics at 5mg/l and 0.5mg/l, with indications of some effect at 0.1mg/l of oxytetracycline. Effects were not seen at lower concentrations. The effects of oxytetracycline and amoxycillin were different. Both reduced CO_2 production, though for different reasons. Amoxycillin reduced uptake of carbon from solution, whilst oxytetracycline increased uptake and then delayed its release back into solution. When both were present, they counteracted each other and resulted in a reduced impact as measurable using this method.

The work has shown that there was definite measurable effect due to the presence of antibiotics, though only at higher concentrations than would be expected in the environment. Indications of interactions between different antibiotics affecting carbon cycling were also found.

Chapter 6: Changes in carbon utilisation patterns in aquatic bacterial communities due to the presence of antibiotics

6.1 Introduction

The taxonomic classification and characterisation of microbial species and communities is a task fraught with difficulties due the similarities between different species and the large number of species usually present. As a result various other methods have been developed to try and identify species and communities. In addition, the identification of bacteria has been de-emphasised and the study of what microbes do in nature has been concentrated on (Hobbie J.E, 1988).

Community level approaches have looked at the ways of using characteristics of the community as a whole, which in themselves are a reflection of the species composition of that community. One method available involves looking at the ability of different communities to utilise various compounds as carbon sources. The determination of single carbon source utilisation by bacteria can be carried out using commercially available microtitre plates. These contain a different compound in each well, together with tetrazolium violet, which changes colour when reduced and is an indication of respiration as a result of bacteria utilising the carbon source. Biolog plates were originally developed to identify single microbial strains. The pattern of utilisation of the carbon sources is a unique fingerprint for each species. Garland and Mills (1991) used Biolog plates and the pattern of utilisation of 95 different single carbon sources to differentiate between freshwater, soil and hydroponic microbial communities and between different freshwater microbial communities. This method has also been used to distinguish between different rhizosphere communities (Garland, 1996).

"Not all organisms in the community are equally important in determining the nature and function of the entire community. A relatively few species (or species groups) generally exert a major controlling influence by virtue of their numbers, size, metabolism or other activities. Within these groups, species which largely control energy flow and strongly affect the environment of all other species are known as ecologically dominant."

Bianchi and Bianchi (1995)

It can be seen that looking at carbon utilisation patterns would therefore be particularly relevant when investigating what kind of impact antibiotics might have on bacterial communities. At a given moment in time, microbial communities from a particular environment, for example river sediment or river water, have a characteristic and reproducible pattern of carbon utilisation. This pattern is a direct reflection of the composition of the microbial community.

Changes in the community, whether it be in the proportion of each species making up the community, in species composition due to the loss of some organisms, or in the ability of particular species to utilise various carbon sources could affect the pattern of colour changes seen in the Biolog plates. Antibiotics at low concentrations may have an impact on community health and structure and therefore an impact on freshwater ecosystems. These impacts might well be reflected as changes in carbon utilisation patterns. In addition the effect of low levels of antibiotics on individual bacterial species seen in the laboratory may not be reflected in the ecosystem which may be able to compensate for any changes occurring, and this could also be determined by looking at patterns of carbon utilisation.

6.2 Materials and Methods

6.2.1 Sampling

Samples of river water and sediment were taken, using sterile collection techniques, from the River Thames at Medmenham over an 18 month period, from May 1996 to November 1997. Topsoil samples were taken twice at the Medmenham WRc site over the same 18 month period.

6.2.2 Experiment Setup

Experiment 1: Method validation

River water samples were directly inoculated into the Biolog GN microplates. 150µl of river water were added to each well. Soil and sediment samples were processed as follows: 20g of sample was weighed out and then stomached with 20ml of 0.85% sterile NaCl solution on a high setting for 4 minutes. The liquid part of the sample was then poured off and left to stand for 30 minutes after which 15ml of sample was pipetted off and diluted 1:2 with 0.85% NaCl. 150µL of sample was then pipetted into each well.

All plates were incubated at 22°C until colour began to develop, after which readings were taken approximately every two hours for up to 48 hours using a microplate autoreader (Model EL311, Biotek Instruments) set at 590nm. The control well, which contained the dye but no carbon source, was used as a blank against which to read the wells in each plate. Appendix 3 is a list of the carbon source contained in each well.

Data analysis was carried out following the method of Garland and Mills (1991). The Average Colour Well Development (ACWD) for each plate reading was determined, by adding up the values for the wells in each plate and dividing by 96, the total number of wells. Readings were normalised by dividing the values for all wells of a plate by the ACWD for that reading.

After this, Principal Component Analysis (PCA) was carried out on the normalised values of readings for each plate that had similar ACWD. In this case an ACWD of 0.7 was used. The Genstat 5, release 3,2 statistics package produced by the Lawes Agricultural Trust was used to carry out the calculations. PCA is a form of multivariate analysis which permits reduction of the 96 values obtained for each plate down to one or two values that can then be plotted on a two dimensional graph. Results from each plate can then be easily compared with each other.

Experiment 2: Determination of changes in carbon utilisation pattern in river sediment samples containing oxytetracycline.

A 200g sample of sediment was weighed out, placed in a beaker and 500 ml of river water added. To ensure full oxygenation, air was bubbled through the water using a pump connected to diffusers. Samples were covered to prevent contamination by airborne bacteria and reduce evaporation. After 48 hours when the sediment had settled and the water became clear oxytetracycline was added to the flasks so that the final concentration was 5mg/kg. Sampling was carried out at weekly intervals when 20g samples of sediment were removed and prepared as in Experiment 1. Data analysis was carried out as in Experiment 1.

Experiment 3: Determination of changes in carbon utilisation patterns in river water samples to which various mixtures of oxytetracycline and amoxycillin have been added.

Water samples were collected from the River Thames at Medmenham. 200ml was placed into a 250ml sterile conical flask and various amoxycillin and oxytetracycline concentrations added. Two flasks had 0.5mg/l of amoxycillin added; two flasks had 0.5mg/l of oxytetracycline added; two flasks had 0.5mg/l of both amoxycillin and oxytetracycline added (1mg/l of total antibiotic). Two more flasks were set up as controls with no antibiotic. Flasks were covered, protected from light and aerated using a pump connected to air diffusers. Samples were taken from flasks and used in the same way as water samples in Experiment 1.

Bacterial Counts and Antibiotics Resistance

Water and sediment samples were taken at the same as time as sampling was carried out for the Biolog plates and counts carried out to determine changes in total numbers of bacteria and changes in resistance levels in the population.

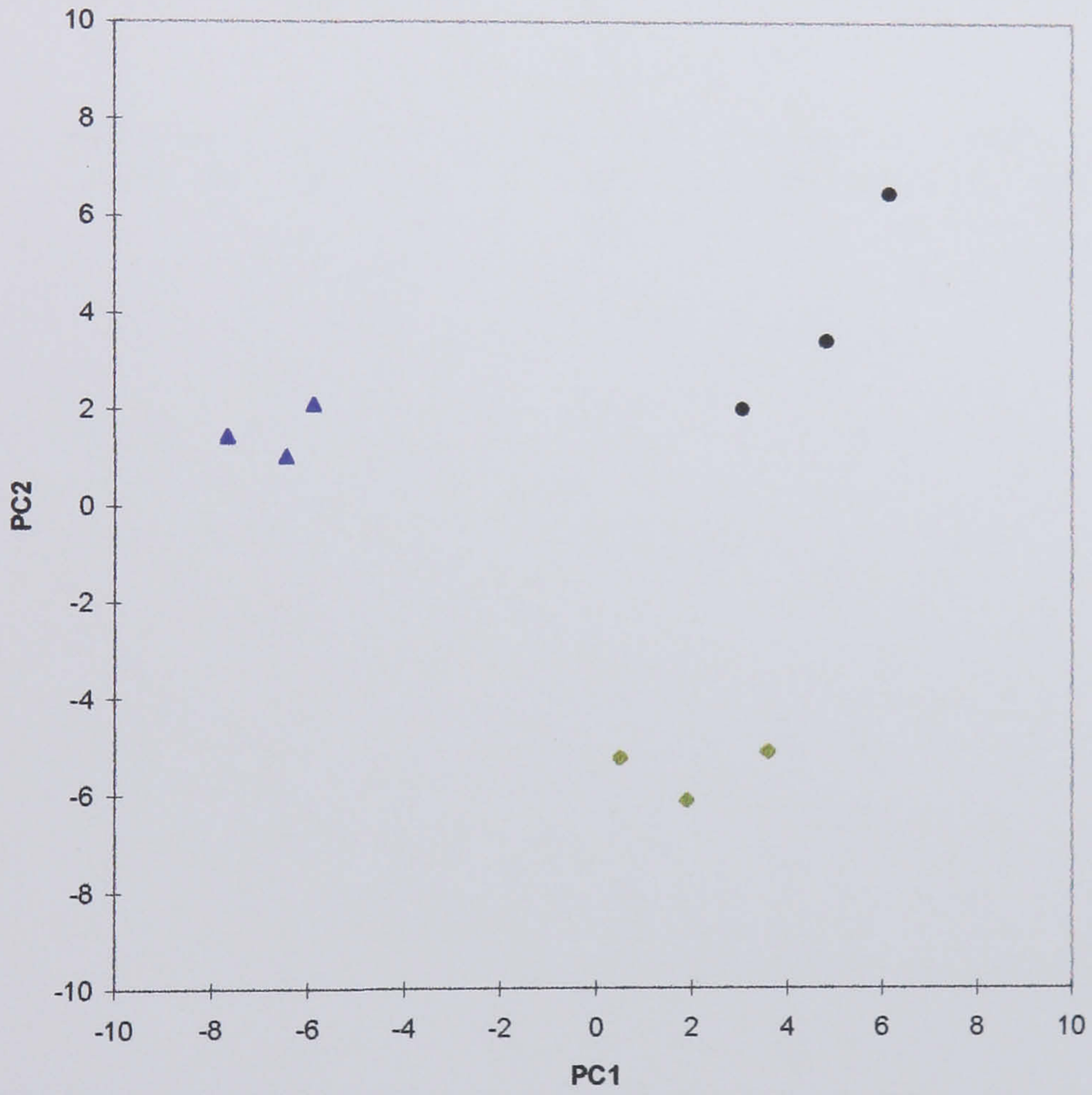
0.1 ml samples of 10-fold dilutions in a quarter strength Ringer's Solution were plated out onto Oxoid Yeast Extract Agar plates. These were incubated at 22°C for 7 days and the number of colony forming units (CFU) counted. Samples were also inoculated onto Yeast Extract Agar plates containing various concentrations of oxytetracycline, amoxycillin or both. Plates were incubated at 22°C for 7 days and then counted.

6.3 Results

6.3.1 Experiment 1

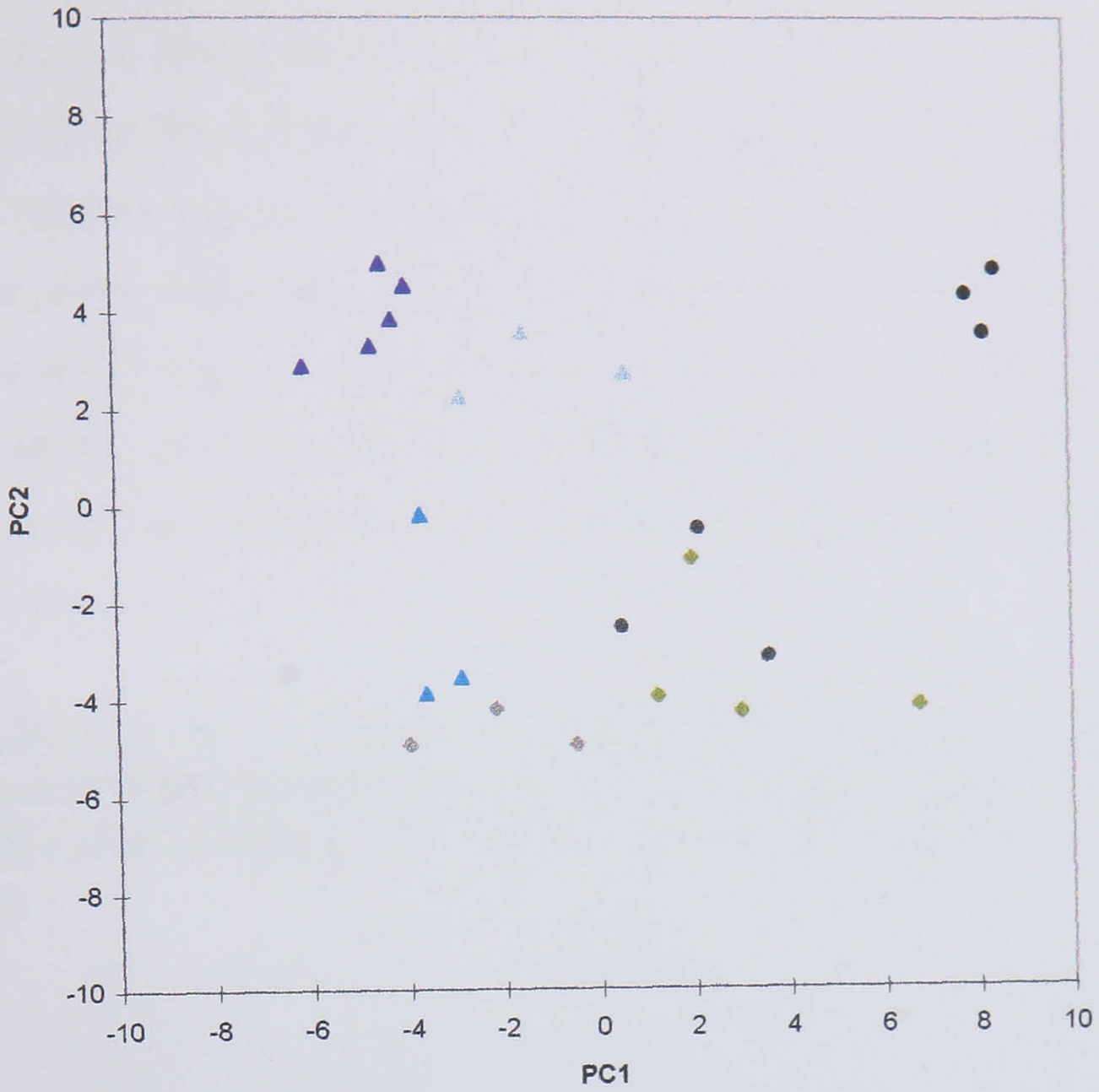
The results from the first experiment demonstrated that Biolog plates are capable of distinguishing between microbial communities from different environments. Three samples of each community were compared with each other. The first two derived principal components accounted for 50% of the variations in the samples (29 and 21%, respectively). The resulting plot can be seen in Figure 6.1. There was a clear differentiation between the communities and strong similarities between the samples. Based on these results further work was carried out.

Figure 6.1 .Results of PCA of results of sole carbon source utilisation patterns of three different communities (River water (▲) , River sediment (♦) and Soil (●).



Once all experimental work had been completed it was possible to collate the data from all of the controls and combine them to produce a larger sample set for the above three communities. The results, seen in Figure 6.2, again show strong association amongst different river water samples and different sediments. The two different soil samples are very dissimilar, possibly due to seasonal variation in the soil community. The first sample was taken in January, and the second in March, 15 months later. There is also a clear distinction between different samples of the same communities. In this case the first two principal components account for 21% and 15% of the variations between plates, respectively, with a total of 36% of variation accounted for.

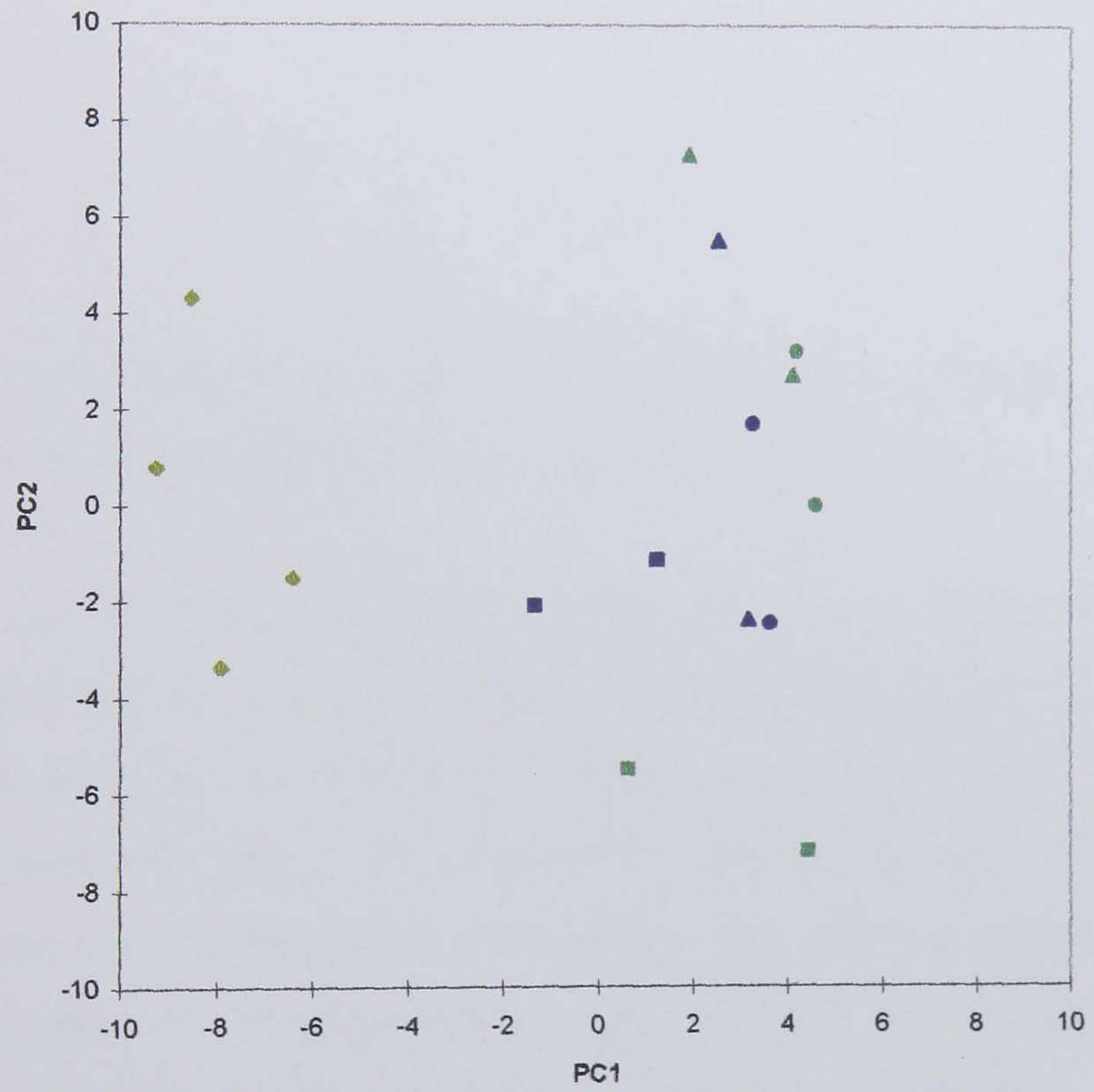
Figure 6.2: Results of PC analysis of single carbon source utilisation patterns for River water (Δ), River sediment (O) and Soil (◊) samples. Different shading represents samples taken at different times.



6.3.2 Experiment 2

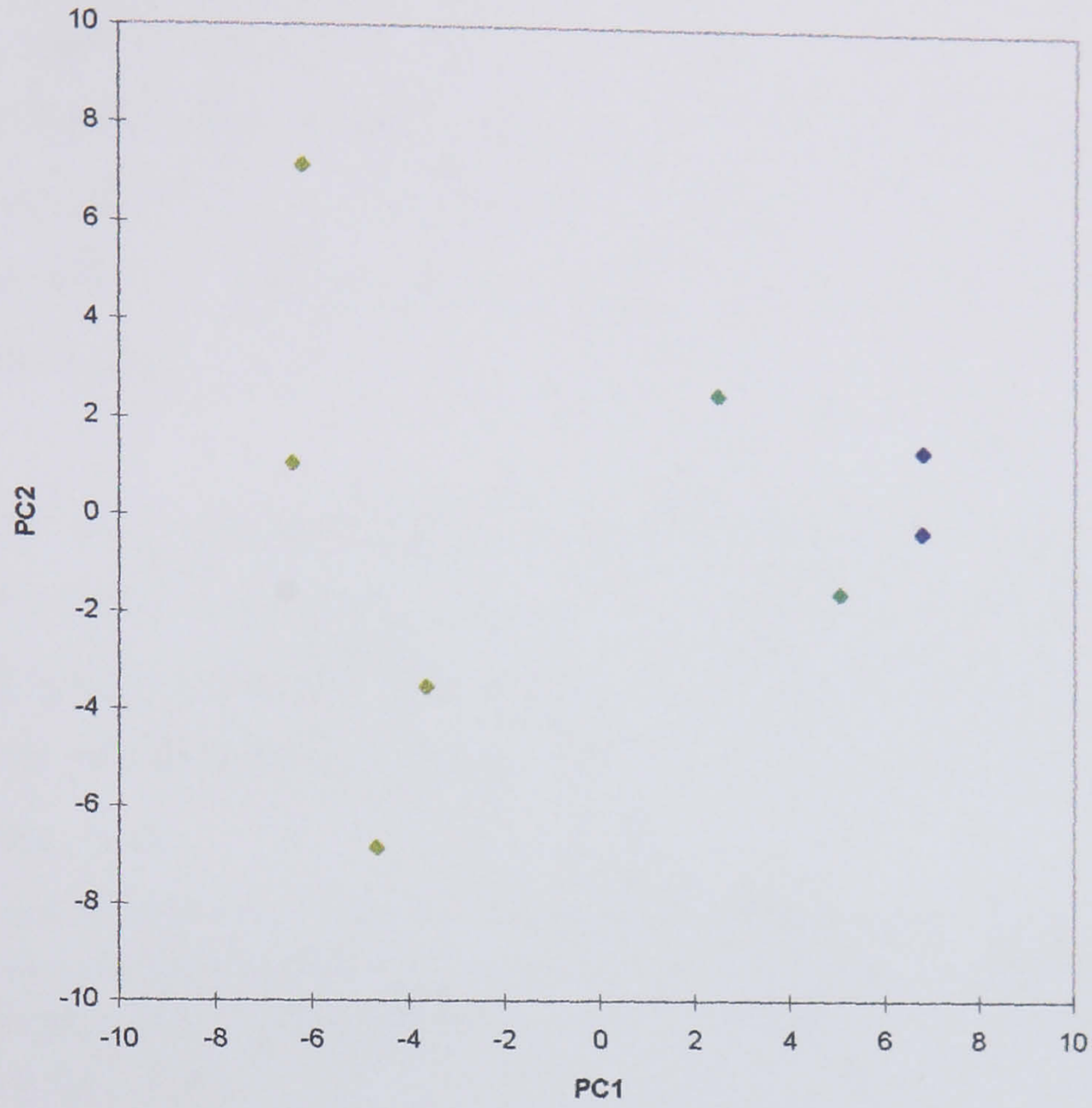
Results from the first run of this experiment in which four jars were set up, with two controls and two to which 5mg/kg of oxytetracycline were added, can be seen in Figure 6.3. Firstly, they showed that once sediments are placed in the jars the pattern of sole carbon source utilisation changes with time, even without the presence of the antibiotic. This shift is similar in both samples and is seen clearly in Figure 6.3. The jars with the added oxytetracycline are distinguishable from the controls for the first two weeks but by week three there is no separation between oxytetracyclines and controls. In this experiment the first PC accounted for 27% of the variation, the second for 17%.

Figure 6.3: Results from PC analysis of patterns of single carbon utilisation by sediment communities to which 5ug/ml of oxytetracycline had been added. . Fresh sediment samples (♦); Day 7 Control (■) and Oxytetracycline (■); Day 14 Control (●); and Oxytetracycline (●); Day 21 control (▲) and Oxytetracycline (▲)



A second run of this experiment was carried to determine if these results could be duplicated. Unfortunately due to uncontrollable personal circumstances, the experiment was interrupted after only 7 days. However, it was possible to see that there again was a difference between the controls and the samples containing antibiotics after seven days of antibiotic being present. Again, the fresh samples of sediment are clearly distinct from the control jars that contained sediment to which no antibiotic had been added. There does appear to be an indication of some separation between the controls and the oxytetracyclines along the first axis, though the difference is not large.

Figure 6.4: Results of PC analysis of sediment samples containing 5µg/ml of oxytetracycline. Day 0 Sediment Samples ( ). Day 7: Control ( ) and Oxytetracycline( );



In both sediment experiments the positions of the original sediment samples were the same and the experimental populations shifted in the same direction.

Results from plate counts of sediment samples from the jars, seen in Table 6.1, showed that the only major change in resistance seen in the jar that contained 5mg/l of oxytetracycline was a tenfold increase in resistance to 50mg/l of oxytetracycline. However, counts of resistance colonies obtained were still only 0.1% of the total culturable bacteria. No other change in relation to the presence of oxytetracycline was seen. Results obtained also show that by setting up the system a change in the populations occurred. Total numbers decreased whilst the proportion of bacteria able to grow in the presence of antibiotics increased.

Table 6.1: Plate counts of sediment bacteria on Yeast Extract Agar containing various Oxytetracycline concentrations.

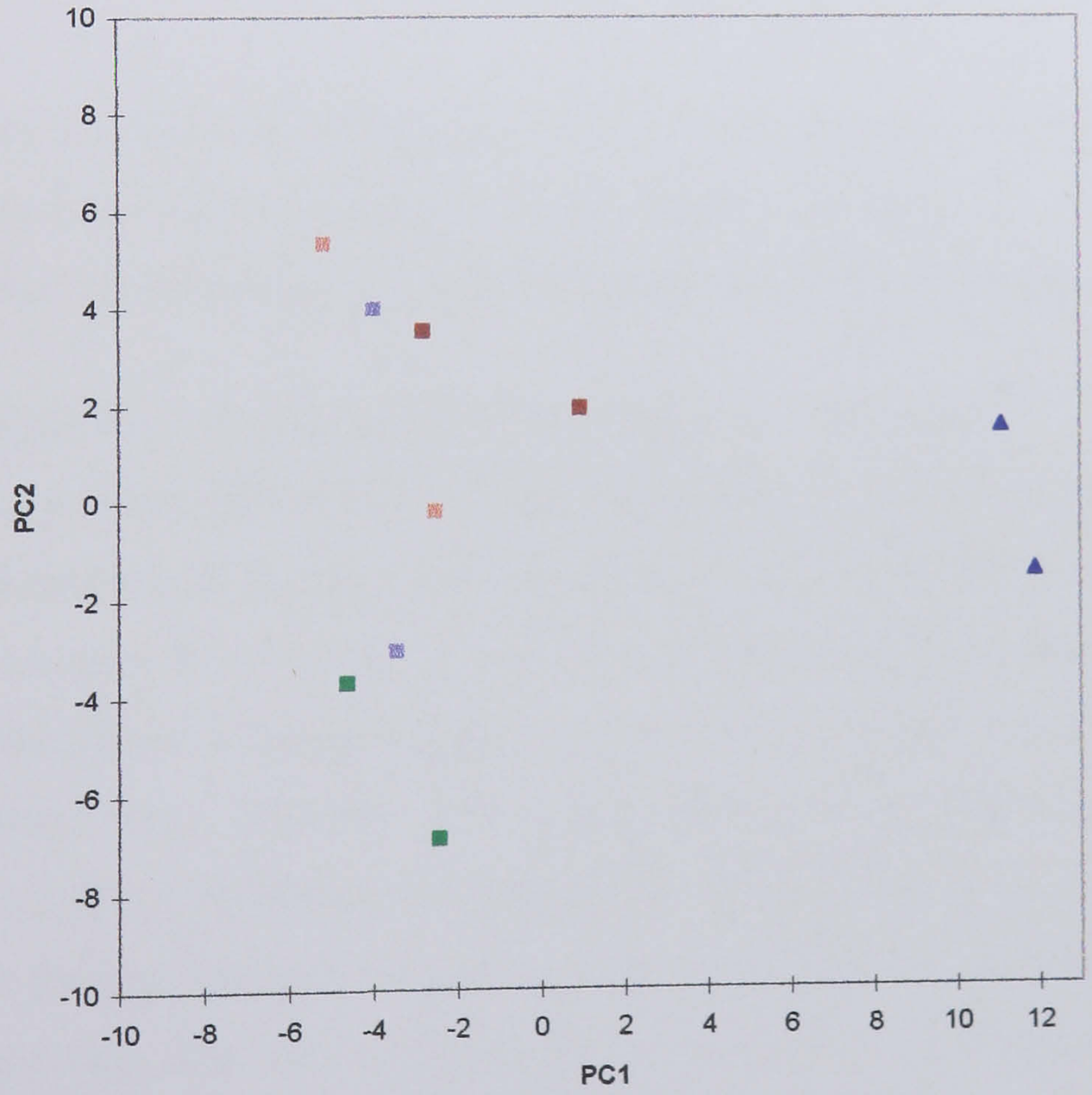
	River Sediment Day 0		Jar Sediment Samples Day 44			
	A	**B**	**Control**	**0.01mg/l**	**0.1mg/l**	**5mg/l**
Total	3.33×10^5	4.59×10^5	6.57×10^4	1.54×10^5	9.52×10^4	1.38×10^5
% Resistance						
0.1mg/l	27.27	21.57	95.65	104.94	68.00	65.74
5mg/l	1.47	1.45	9.49	5.40	8.20	9.86
50mg/l	0.01	0.01	0.03	0.02	0.03	0.11

6.3.3.Experiment 3

Two duplicate experiments were carried out in which antibiotic was added to river water samples and changes in single carbon source utilisation patterns monitored. The set up for this experiment was identical to that used to look at the fate of radiolabelled compounds in Chapter 5, except for the use of different methods to maintain aeration in the systems. Because of this, results from these different experiments are directly comparable and can be looked at in conjunction with each other.

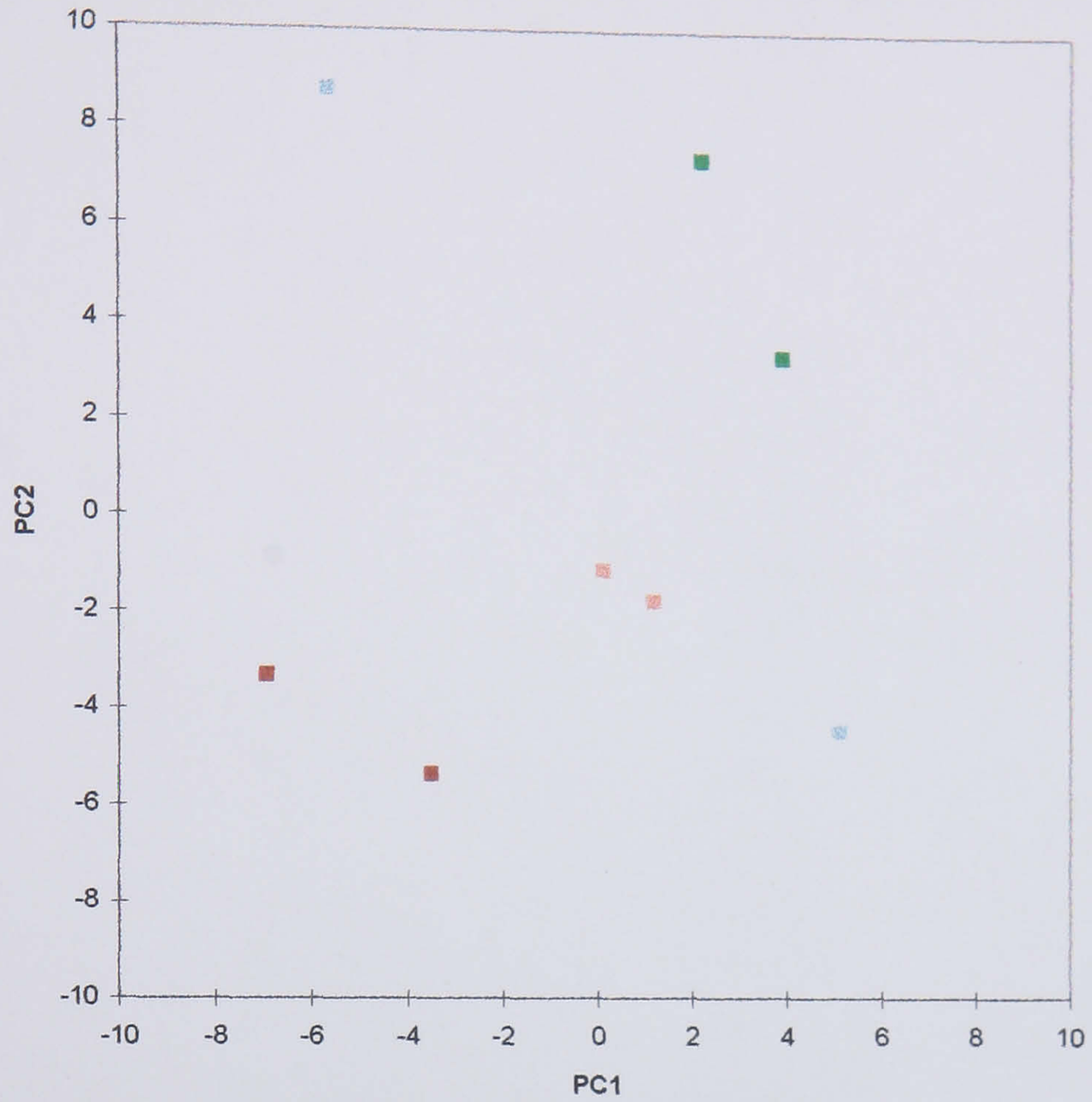
The first experiment had to be interrupted after the first week, due to uncontrollable personal circumstances. The results in Figure 6.5 showed that, as with the rivers sediment, there was a marked shift in the carbon utilisation pattern, even in the control jars. In this case, there was a greater similarity between the controls and the different treatments after 4 days than between the controls at day 0 and day 4.

Figure 6.5.: Results of PC analysis of single carbon source utilisation patterns of river water microbial communities to which no antibiotic (■), 0.5ug/ml of oxytetracycline (■), 0.5ug/ml of amoxycillin (■) or 0.5ug/ml each of amoxycillin and oxytetracycline (■), after 4 days exposure to antibiotics. Two river water samples are also included (▲).



Results from this experiment were re-analysed, excluding the two controls which were so different from the rest of the results. This forcing together of the rest of the results could be obscuring some less obvious trends that might exist in them.

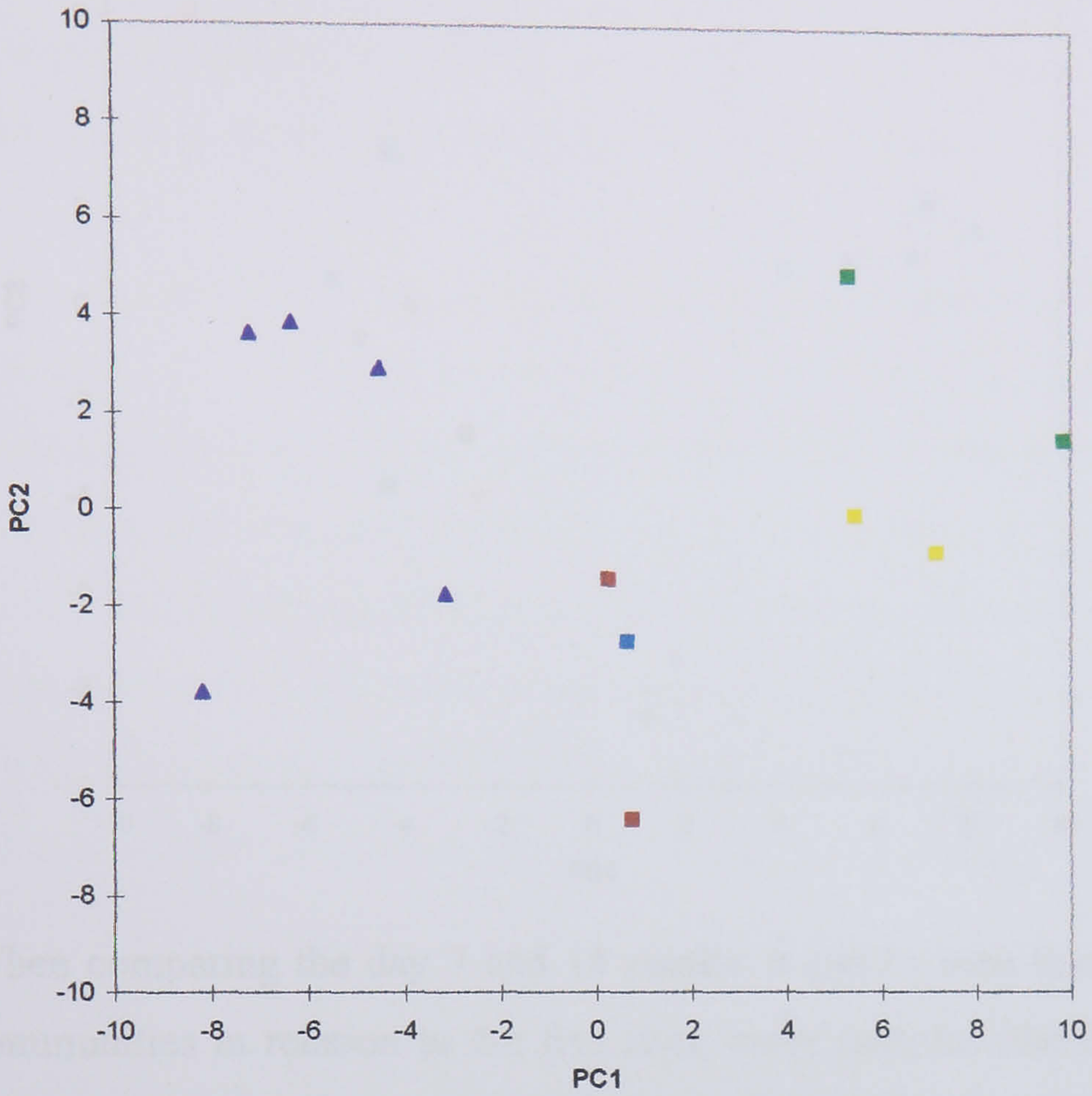
Figure 6.6: Results of PC analysis of single carbon source utilisation patterns of river water microbial communities to which no antibiotic ( ), 0.5ug/ml of oxytetracycline ( ), 0.5ug/ml of amoxycillin ( ), or 0.5ug/ml each of amoxycillin and oxytetracycline ( ), after 4 days exposure to antibiotics.



In Figure 6.6 can be seen the result of this new analysis, and it indeed becomes clear that there is a separation between the treatments, though the two controls appear to be very different. It can also be seen that all three treatments are easily distinguishable from each other.

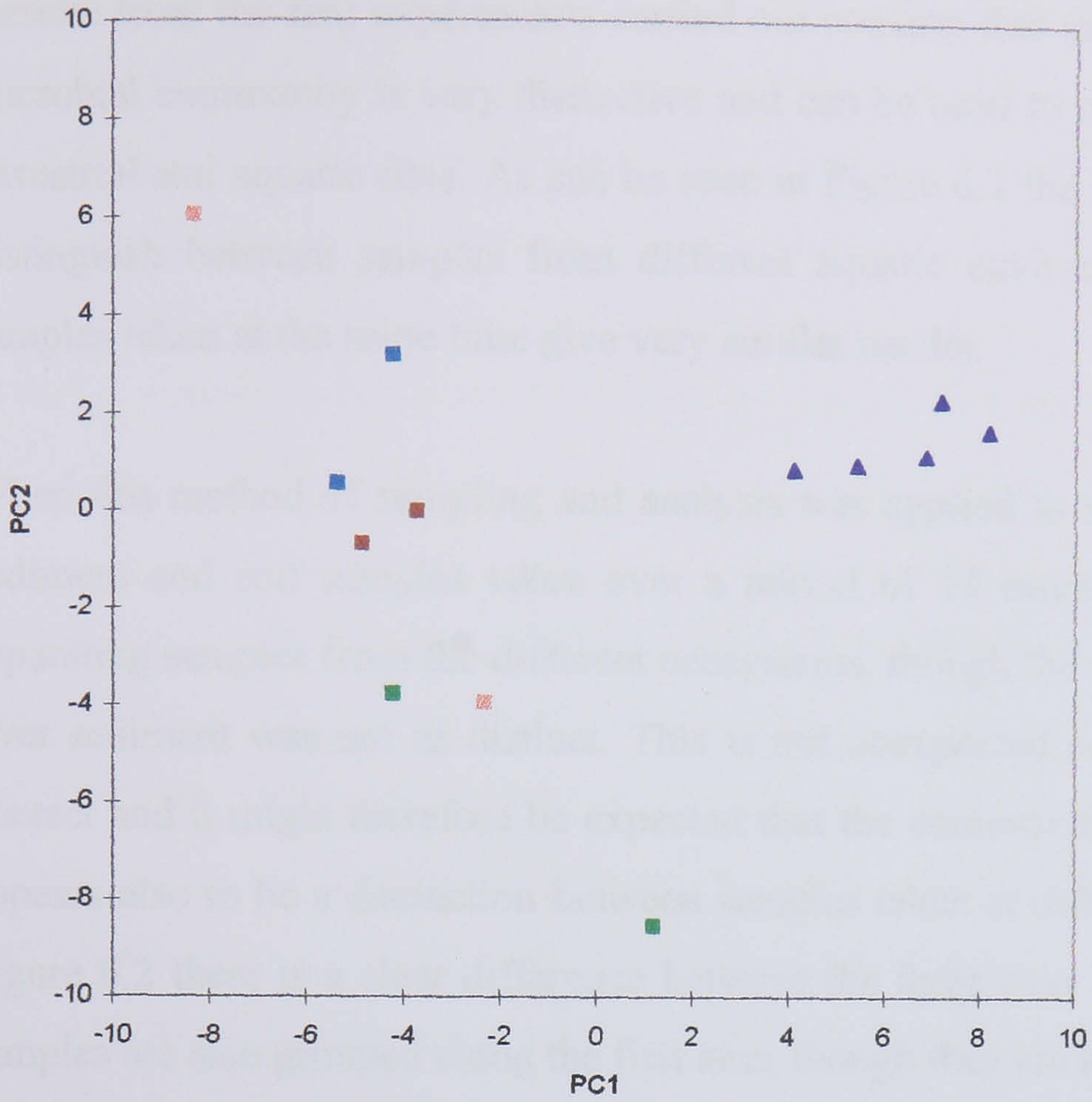
The second experiment was identical to the above, however it was possible to run it for an extra 10 days to determine whether there was a longer term impact due to the presence of antibiotics. Unfortunately, one of the control plates was spilt during transit, so results were only obtained for one control. The results can be seen in Figure 6.7 and again show that 7 days after the experiment was initiated the carbon utilisation pattern of the community changed, even when no antibiotic was present. This is a similar result to that obtained in the sediment experiments. This time there was a much clearer separation between the different antibiotic samples, and greater similarity amongst the duplicate jars. In this case, the control and the amoxycillin samples were more similar to the fresh river water samples than were the jars containing oxytetracycline. In this analysis, 38% of the variation of the samples was accounted for by the first principal component and 14% by the second.

Figure 6.7: Results of PC analysis of single carbon source utilisation patterns of river water microbial communities to which no antibiotic ( ), 0.5ug/ml of oxytetracycline ( ), 0.5ug/ml of amoxycillin ( ) or 0.5ug/ml each of amoxycillin and oxytetracycline ( ) after 7 days exposure to antibiotics. River water day 0 (▲)



After 14 days (Figure 6.8) the results from the Biolog plates again show that there is a clear separation between the fresh river water samples and the rest of the experimental samples. In this case again, the samples containing only amoxycillin appear to be very similar to the controls, whilst the samples containing oxytetracycline or both are grouped together (except for one oxytetracycline sample). In this case 34 % of the variation was accounted for by the first principal component and 15% by the second.

Fig 6.8: Results from PC analysis of river water samples to which antibiotics had been added. River water day 0 (▲), River water day 14 (■), oxytetracycline (■), amoxycillin (■), oxytetracycline and amoxycillin (■)



When comparing the day 7 and 14 results, it can be seen that the position of the experimental communities in relation to the five river water samples (the same samples were used for both analyses) had changed completely. This indicated that there had been a significant change in the communities in the intervening time.

Table 6.2: Plate counts for experiment 3.2 YEA and various antibiotics mixtures Samples taken on day 14 and correspond with results seen in figure 6.8. (A: amoxycillin, O: oxytetracycline and AO: amoxycillin and Oxytetracycline)

	Control		Oxytetracycline. (0.5mg/l)		Amoxycillin (0.5mg/l)		Oxytetracycline and amoxycillin (0.5mg/l)	
	Jar1	Jar2	Jar3	Jar4	Jar5	Jar6	Jar7	Jar8
Total Counts	$3.06x10^4$	$2.06x10^4$	$3.01x10^4$	$9.38x10^4$	$6.14x10^4$	$2.96x10^5$	$4.03x10^4$	$3.60x10^5$
(mg/ml)	% of population resistance to various antibiotic concentrations							
O 50	0.04	0.04	0.07	0.16	nd	nd	16.77	0.38
O 5	0.68	3.92	1.85	1.70	nd	nd	7.72	3.17
O 0.1	67.14	89.86	69.23	82.37	nd	nd	66.89	100+
A 50	5.65	1.77	nd	nd	29.52	2.06	6.50	7.53
A 5	29.15	32.22	nd	nd	42.38	75.15	100+	8.28
A 0.1	100+		nd	nd		77.44		
AO 50	0.02		0.03	0.10	1.05	0.05	0.05	0.36
AO 5	1.80	0.67	1.28	2.04	11.40	8.13	20.24	2.15
AO 0.1		100+	82.14	68.53	50.15	93.03		

Plate counts of samples taken on day 14 of this experiments were difficult to interpret. Duplicate jars did not show strong similarities, nor did they generally show large differences from controls.

6.4 Discussion

Results from the first experiments carried out confirm that the pattern of carbon utilisation of a microbial community is very distinctive and can be used to discriminate between samples from terrestrial and aquatic sites. As can be seen in Figure 6.1 the method is also sensitive enough to distinguish between samples from different aquatic environments. Not only this, but repeat samples taken at the same time give very similar results.

When this method of sampling and analysis was applied to a larger group of river water, river sediment and soil samples taken over a period of 18 months, it again was very efficient at separating samples from the different ecosystems, though the separation between river water and river sediment was not as distinct. This is not unexpected as water and sediment are in close contact and it might therefore be expected that the communities would show similarities. There appears also to be a distinction between samples taken at different times from the same site. In Figure 6.2 there is a clear difference between the three river water samples. The two sediment samples are also grouped along the first axis, though they are all very similar on the second axis.

In the case of the soil samples, which were taken from exactly the same spot 18 months apart, the results show that they are clearly different. There are various possible explanations for this. There may have been some drastic event that has resulted in a shift in the microbial community, though the site appeared undisturbed. Another, more likely, explanation is that the difference seen is due to naturally occurring seasonal variations in the community. Soil ecosystems experience considerable changes in temperature and humidity throughout the year. These seasonal variations may also partly account for the variations in the water and sediment samples. A possible cause of variations between samples from the sediment, and particularly the water, is the presence of non-native bacteria that have been washed into the water and are only present for a short time as they are not capable of surviving in this environment. The type of these bacteria present will vary according to conditions and will have an effect on the ability of the sample to utilise the various carbon sources.

The second set of experiments involved adding oxytetracycline to a final concentration of 5mg/kg of total mass (water and sediment). The sediment was then sampled to determine if there were any detectable effects on the carbon utilisation patterns. The reason for carrying out this experiment using just oxytetracycline was due to the fact that tetracyclines bind to various kinds of soils in a reversible way, as shown by Sithole and Guy (1987a, 1987b). Amoxycillin was not used in these experiments as it is quickly degraded in soils and sediments. It has already been shown in Chapter 3 that it would only be present for a short time and that sediment would not be acting as a reservoir in the environment.

Oxytetracycline is not biodegradable, so it is possible that it remains in the system at high concentrations or it may be bound and inactive and with no detectable effect. The high concentration of oxytetracycline used (much higher than would be expected in the environment) was to ensure that any effect would be clearly visible, even if the antibiotic were partly inactivated. In this experiment river water was placed on top of the sediment, to keep the system functioning as normally as possible for as long as possible. The water from the system was not sampled. Being in such close proximity to the sediment, which would be disturbed every time it was sampled, it would have contained a much larger and varied population than river water in the environment would do and therefore results from this analysis would have had no bearing on what might happen *in situ*.

The first observation from these experiments was that there was a major impact on the microbial community which resulted in a clear change of carbon utilisation patterns occurring when samples were taken from the environment and placed in the microcosm system. In both experiments there is a clear separation between the fresh sediment samples and the control samples to which no antibiotics were added. This indicates that by simply taking sediment from a river bed and placing it in an experimental setup there is an impact on the population. This may be due to the fact some bacterial species do not survive or do not thrive while others may find it a more hospitable environment, possibly due to the removal of competing species. This finding was backed up by the plate count results obtained. Again they showed a shift in the population in all systems, with or without antibiotic, as a result of setting up the experiments. A greater proportion of the population in all jars was resistant after 44 days. This suggests either that simply being in the jars induced resistance, or that resistant bacteria survived better in the jars. If resistant bacteria survive better, this suggests that resistance to oxytetracycline could confer other advantages to bacteria other than ability to survive in the presence of the antibiotic. The possibility that resistance carries unknown advantages has been suggested in the past (Bennet and Linton, 1986).

This initial shift continues throughout the first oxytetracycline experiment with each set of controls being dissimilar to each other, indicating that after the initial change due to setting up the microcosms, the population continues to change. This is not a naturally occurring shift in population as from the results of Experiment 1 (Figure 6.2) it can be seen that samples taken at different times from the same site are similar to each other and do not vary significantly with time. These results highlight the difficulties in carrying out experiments that attempt to mimic in some way the natural environment, and whilst this does not make them any less valid it is important to remember this when interpreting the results obtained.

Because of the change in population due to setting up the experiment it is only possible to compare the results from the microcosms containing the antibiotics with the results from the controls sampled at the same time. The results from the first experiment show that there was clear separation between the controls and the oxytetracycline samples after one week. This difference became smaller after two weeks, and after three weeks there was no distinction between tetracyclines and controls. It is interesting also to note that the shift seen in the controls was reflected in the oxytetracycline samples, the changes in all the populations seem to be moving in a similar direction. The second experiment, which had to be interrupted, again showed a separation between the initial sediment and the controls, and there was also a separation along the first axes between controls and oxytetracycline jars. Both sediment experiments showed similar trends when it came to changes in carbon utilisation. The similarities between the two experiments confirm that real effects were occurring in the sediment microbial community, as it existed in the experimental system.

It was clear that there was an impact on the population from the presence of the tetracyclines, at least for the first week. After the first week this difference between the populations decreases. It was known from previous work mentioned in Chapter 1 that oxytetracycline is not biodegradable. The system used in this experiment was a closed one and therefore the antibiotic at an overall concentrationof 5mg/kg must still have been mostly present. If all should be removed from the water and present in the sediment, concentrations there could have been as high as 17.5mg/kg which is well above the MIC for sensitive bacteria. There are possible explanations for the lack of visible effect after three weeks. Firstly, there may have been an initial impact when the antibiotics killed sensitive bacteria, after which those that survived became resistant and recovered and the population converged with that of the control system. If this were the case it would be expected that there would be a large increase in the resistance of the bacterial population, in particular that a large proportion or all of the population would be able to grow in the presence of 5mg/l of oxytetracycline. In this case, it would be unlikely that the population would then return to being similar to that of the controls.

Another possibility is that the antibiotic is no longer present in a free and active form in the system after the first week. It could have bound to the sediment and been inactivated, so that the source of impact was removed from the system and the population then returned to normal. In this case it would be expected to see an increase in resistance in the population, but not as large because there would be a proportion of the population that was only inhibited, not killed, by the presence of the antibiotic and this would recover after the antibiotic became inactivated by binding. Resistance in the population would then be slowly lost due to the removal of any selection pressure favouring it.

From the population counts carried out on sediments to which 5mg/l of oxytetracycline had been added it can be seen, in Table 6.1, that even after 44 days there were no major changes in resistance pattern that were attributable to the presence of tetracyclines. Therefore, the most likely explanation is that the antibiotic is not present in its active form throughout the whole experiment. Sithole and Guy's experiment (1987b) showed that binding of oxytetracycline to soil was not instant, but reached equilibrium after around 18 hours. This might explain the initial difference from the controls, which then disappears. This time scale would be sufficient for the oxytetracycline to kill and damage bacteria sufficiently for the change in population to be detected. Once the majority of the antibiotic is removed by binding, then the bacteria begin to recover and the community is sufficiently unchanged to converge eventually with the control samples.

The third set of experiments looked at the impact of 0.5mg/l of antibiotics on the water fraction of a river ecosystem. Lower concentrations of antibiotics were used, as there was no sediment present and they had been shown to have an impact in the radiolabelling experiments.. A system which contained both antibiotics was also set up to determine the effects of a combination of antibiotics on the system. From the results in Chapter 5 it is known that the presence of both amoxycillin and oxytetracycline counteract each other, with carbon cycling patterns similar to controls, whereas amoxycillin alone decreases the carbon fraction in bacteria and oxytetracycline alone increases the carbon fraction in bacteria.

Results from experiments using the Biolog plates again demonstrated that in river water there was a great change in the microbial community's ability to utilise the carbon sources as a result of taking the samples and setting up the microcosms. In the first experiment, which was interrupted, it was again clear that this was the case. In this case it was harder to distinguish amongst the treatments and the controls, with the possible exception of the oxytetracycline jars which were grouped in the lower left hand part of Figure 6.5. When the analysis was repeated without including the original controls, it was then possible to see a clear distinction between all three different treatments.

The results showed that the treatment with amoxycillin and oxytetracycline differed from both of the single antibiotics ones. This is an important factor and is highly relevant to the question of what are the possible impacts of mixtures of antibiotics on the aquatic ecosystem. It is not possible from these results to say what kind effect there is, only that the overall utilisation pattern of the 95 carbon sources is different in a system that contains both antibiotics. It cannot be said what kind of interaction there is, only that there is a different effect on the community when both antibiotics are present.

When this experiment was repeated and sampled 7 days after adding the antibiotics to the system it was possible to see a clear distinction between the samples, even when the original controls were included in the analysis. Again, there was a clear distinction between the original river water samples and the rest of the results. The treatment containing the amoxycillin was very similar to the control, and these were more similar to the original samples than were the treatments containing the mixture or just the oxytetracycline. These effects were again visible after 14 days, with the controls and amoxycillin samples being very similar and the samples containing oxytetracycline distinct. However, the distinction is not as clear as at the 7-day stage.The presence of the antibiotics has a clear impact on the microflora of the river water even after 14 days. The effect of amoxycillin on its own appears to be minimal, though when present with oxytetracycline it results in a clear distinction between it and the oxytetracycline samples.

The effect of the oxytetracycline on the system appears to be longer lasting than in the case of the sediment samples. This would be expected as the oxytetracycline would not be removed or inactivated to a great extent (due to the absence of sediment in the experimental setup). The populations do not seem to be converging as in the case of sediment experiments, probably for the same reason. Further sampling might have given more information on how long the effect lasted, but this was impossible due time constraints. In the first experiment, the impacts of oxytetracycline, amoxycillin and the mixture of both are distinct from each other. In the second experiment the amoxycillin had less of an impact, being very similar to the controls. Interestingly amoxycillin and oxytetracycline resistance levels were higher not only in the two jars containing both antibiotics, but also in the jar that contained only amoxycillin. This would suggest that though the amoxycillin appeared not to have a major impact on the system's ability to utilise carbon source, plate count results however, do indicate that it does have an effect. As amoxycillin affects cell wall structure, maybe it is reducing the ability of bacteria to take up oxytetracycline and therefore protecting them from its adverse effects. It is also possible that cells walls become leaky, as suggested in Chapter 5 and as a result oxytetracycline itself does not stay in cells long enough to affect them.

6.5 Conclusions

Results obtained from the first set of experiments show that that the analysis of carbon utilisation patterns of microbial communities is a valid method for distinguishing soil and aquatic ecosystems. The method appears to be sensitive enough to also be able to distinguish between the small variations that occur when sampling the same site at different times. Unfortunately previous published work did not distinguish between samples taken at different times from the same site (Garland and Mills,1991). Results from PC analysis only distinguished between the types of communities. It is therefore not possible to determine whether the same effect was found. Further work would be required before this could be confirmed. If this method is so sensitive, it could be a useful tool for monitoring impacts on the aquatic environment *in situ*. A database could be established that contained baseline data for a particular community. If this community were regularly monitored, any major change in the community could be used as an early warning signal of a disturbance that could be caused by pollution.

From the soil experiment using oxytetracycline, it can be concluded that even at the high concentration used, which is highly unlikely to be found in the environment, there was no detectable long-term impact. Indications were that oxytetracycline bound quickly to sediment and became inactivated. After exposure, the bacterial communities converged, giving indistinguishable results. This was important from an environmental point of view, as it could be an indication that in the case of the ecosystem being exposed to tetracyclines it would recover again quite quickly after the antibiotic was bound and inactivated. However, it has to be remembered that a significant difference was shown between the communities in the experiments and the naturally occurring community. It is possible that those bacteria that were most sensitive to changes in their environment, as seen when setting up the experiment, were also those that are most affected by antibiotic. If this were the case, there could be a much more severe and longer term impact on the ecosystem than seen in the microcosm experiments.

The first conclusion from the river water experiments is that the more sparse microbial community present shows a long term, possibly permanent change as a result of setting up the microcosms. It can also be concluded that there is an impact occurring in the case of amoxycillin, though the level of this impact is difficult to determine from the work carried out as the experiments gave quite dissimilar results. It is known that gram-negative bacteria are often less sensitive to β-lactams and a large proportion of river water bacteria are gram negative. β-lactam antibiotics are also likely to be degraded quickly in the environment, within weeks, as pointed out in Chapter 1. However as amoxycillin is by far the most commonly used antibiotic in the UK it was still important to use it during this work.

Oxytetracycline alone had an impact on the system at 0.5mg/l and the effect was still visible after 14 days. This effect would not necessarily be seen in the environment as the oxytetracycline is likely to be slowly removed by binding to particulates and sediment, which were not present in this system. However, should there be a steady input of antibiotic at these levels, over a period of time, then there is the possibility of a permanent impact.

In conclusion, there appears to be little permanent change in river water and sediment bacterial communities after exposure oxytetracycline and amoxycillin, which belong to two groups of important antibiotics. At this stage it cannot be definitely stated that this is the case under ambient conditions, but this is a fairly strong indication that this may be the case. However should the input of antibiotics be a continuous one over a long period of time it is likely that the impact on the bacterial communities would be severe. This may well be the case for many sewage treatment works that discharge effluent into rivers. The effect would be most important on the bacteria in the river water, rather than the sediment and the presence of mixtures of antibiotics would not only make an impact on the ecosystem more likely, but it would also increase the likelihood of resistance developing in the bacterial community.

Chapter 7: Final Discussion and Conclusions

This project was initiated because there was so little information on the impact of antibiotics on the environment. The data available were very limited and it was impossible to produce even the vaguest estimate of what quantities of antibiotics were reaching the environment in the UK, or what impact they might be having. The work presented here, on this very new area of research, must be regarded as only the first step in determining the possibilities of environmental impacts of antibiotics and in developing methods that might be of use in detecting these impacts.

Research carried out to obtain information on the quantities of antibiotics prescribed in the UK highlighted the lack of accessible data available. It soon became obvious that this was due to a complete lack of concern of the possible impacts antibiotics might be having. When this project began in 1994, no controls or guidelines existed within the farming industry. There was no central record kept of antibiotics prescribed to humans. It was common practise for GP's to prescribe antibiotics for all kinds of flu or cold infections, even though they are completely ineffective against viral infections. Since then, there have been considerable changes in the attitudes towards antibiotic consumption and in prescription habits. The main reason for this has been increased concerns over microbial resistance. Numerous articles in the general press have increased public awareness of the problems of bacterial resistance to antibiotics. There is increasing resistance found in the causative agents of diseases, such as tuberculosis, that has resulted in increased worldwide incidence. Methicillin Resistant *Staphalococcus aureus* (MRSA) has been on the increase. The emergence of bovine spongiform encephalitis (BSE) has resulted in increased concerns over farming practices and how they might affect human health. Among other issues that have become of concern is the use of antibiotics as growth promoters in agriculture. Because of all these factors, antibiotic use has become a very topical issue over the last three years.

Work detailed in previous chapters has provided, for the first time, estimates of the amounts of antibiotics currently used in human medicine in the UK, together with amounts of these that could be reaching the environment. Methods for analysing and concentrating tetracyclines and β-lactams in sewage and river water were developed. Results indicated that any antibiotics present in sewage were below the limits of detection of these methods. In river water, however, tetracyclines were detected at very low concentrations, confirming the findings of the only other report on the presence of tetracycline in the River Thames. This study found two additional tetracyclines, oxytetracycline and doxycycline. All were found in two different analyses, which suggests that they are ubiquitous in river water at µg/l concentrations. Various compounds were found, in river water and sewage, which appeared to be the breakdown products of amoxycillin and cephalexin. Though inactivated, these compounds could still present a threat to the aquatic environment.

Various assays and microcosm systems were used in novel ways with the aim of identifying any sublethal impacts that antibiotics could have, at concentrations estimated and found in the aquatic environment during this project. A growth assay was successful at detecting effects at low concentrations, and determined that even though bacteria may be resistant to antibiotics. there was still a visible reduction in growth. It was also shown, in the case of amoxycillin, that although individual species in the community may be sensitive, the community as a whole will compensate for this by increased growth of resistant bacteria. In the case of tetracyclines, it was found that both sediment and water communities as a whole were sensitive to some concentrations and this was reflected in the plate count results. A further finding was that there was no increase of toxicity when the three antibiotic groups, cephalosporins, penicillins and tetracyclines, used in the experiments were combined. This was confirmed by bacterial counts that showed that resistance to tetracyclines was the same in the presence or absence of amoxycillin.

Microcosm experiments showed that there was an impact on the way that dissolved organic carbon was taken up and broken down by river water bacteria in a closed system. Both tetracycline and amoxycillin had impacts over a period of weeks, in different ways. A mixture of both antibiotics had different effects from either one individually. This difference was also confirmed by work looking at the utilisation patterns of various carbon compounds by aquatic and sediment bacteria exposed to antibiotics. Systems containing both antibiotics showed differences from those containing only one, and from the controls. In addition, it was shown that tetracycline in river would be removed and inactivated by binding to sediment, suggesting that a large reservoir of tetracyclines in river sediments is a possibility.

Results from the isotope experiments showed the control systems set up over a period to be very similar indeed. However, the carbon utilisation experiments showed that setting up the experiments resulted in a shift in the population from that in the environment. This suggests that though the population does change, the changes are not random and the system always responded in the same way. To confirm these results experiment using larger number of replicates would need to be carried out. This was not done in the case of the isotope experiments due to time limitations and in the Biolog experiments due to the cost limitations, as the plates where expensive .

The growth assays and both microcosm systems showed effects at similar concentrations, from 0.5 to 0.1mg/l of antibiotics. The growth assays showed that a mixture of antibiotics had no different effect from the single antibiotics the samples were most sensitive to. However, both the microcosm experiments showed a different effect of combining antibiotics. This indicates that growth is not the best measurement of antibiotic impact on bacterial communities, though it does benefit from being a quick and easy method. It also suggests that the cocktail of antibiotics, and other cytotoxic compounds reaching the aquatic environment through sewage, have potentially harmful effects alone and in combination.

Effects on carbon cycling have been demonstrated in two different ways. Antibiotics both inhibit the level of carbon compounds broken down and change the range of carbon compounds that the aquatic communities can utilise. In the specific case of β-lactams and tetracyclines, the threat may only be a temporary one. The first do not bind to sediments and are probably quickly degraded during sewage treatment and when reaching rivers. The second are likely to be bound to sediments and particulates, and inactivated. This will not be the case for all compounds. In addition, tetracyclines are not biodegraded and degraded very slowly in any other way. As a result of this there is likely to be a large reservoir of inactive antibiotic bound to sediments. They are not permanently bound and could be released back into the environment under changing conditions. Indeed, it is possible that the levels detected in river water are a direct result of the slow leaching of tetracyclines from sediments into river water.

In retrospect, results could have been more comprehensive if the various unsuccessful single species bioassays had not been attempted. The work with microcosms proved that these were a more reliable and informative method for determining the impact of antibiotics. Time saved could have been used to carry out more work on microcosms, and to analyse the changes in the levels of antibiotics in the microcosm systems using HPLC. This would have given a more complete picture of the fate and impact of antibiotics.

Overall, it is unlikely that there are individual antibiotics in river water in the UK at high enough concentrations to be considered a threat to aquatic ecosystems or human health. The combination of antibiotics present may have a stronger impact, particularly on resistance levels, but no strong indication of this was seen during this project. It can also be concluded that other compounds used medically could also be reaching the aquatic environment with unknown effects. A possible threat could exist if any of the antibiotics entering the environment had effects other than antibacterial. It has been suggested that this may be the case with tetracyclines, which could have endocrine disrupting characteristics.

One final important point is that though in the UK antibiotics may not be a major environmental problem this may not be the case in other parts of the world. In many countries antibiotics are sold over the counter without prescription. This combined with inadequate or non-existent treatment of sewage before effluent enters river systems may result in much larger quantities of antibiotic entering aquatic ecosystems. In addition, if there were reduced rainfall, compared to the UK, this could result in reduced dilution of sewage and therefore increased impacts. This could be a possible threat to the environment and, through increased antibiotic resistance. to human health.

7.1 Further Work

Many of the methods used in this project were developed specifically to address the issues of antibiotics in the environment. Because of this, there was little opportunity for validation work to be carried out and this is an area that would need to be further addressed.

In the case of antibiotic usage in the UK further work would be needed to determine the accuracy of the very rough estimates for hospital inputs, by obtaining data from as many more hospitals as possible. In addition, it might be feasible to obtain data from the Department of Health on a regional level or local level, thus increasing the accuracy of estimates of antibiotics reaching sewage works and river systems from GP prescriptions.

No standard methods for the analysis of antibiotics in environmental samples exist. Because of these all the methods used were novel. It would be important to carry out further work to validate the methods used. This would require the introduction of internal standards and the repeated analysis of spiked samples to determine accurate and reproducible limits of detection and extraction efficiencies. In addition, successful analysis in more than one laboratory would confirm without a doubt the presence of antibiotics and their breakdown products in rivers.

Analysis of river sediments for tetracycline would confirm whether there is a large bound tetracycline reservoir in riverbeds. Should this be the case, laboratory experiments to determine whether it could leach into clean water would establish if it has the potential for harming the aquatic environment. Work should also be carried out to determine if the breakdown products of β-lactam compounds could be confirmed in sewage and the aquatic environment using HPLC. Their fate and toxicity could be of interest. Repeating the microcosm systems with larger experimental set-ups would be beneficial for various reasons. Using bigger tanks (at least ten litres) would allow performance of experiments in which both the sediment and the water were sampled so that interactions between sediment and the overlying water could be investigated.

Use of a flow-through system in which the water entering the system has a constant level of antibiotic added to it would also be valuable.

This would be the closest possible imitation of a river water ecosystem and would provide further information on whether the effects seen in the above experiments are likely to be seen in the environment. It is also possible that working on a larger scale, particularly with a flow-through system, would reduce the changes seen in the communities due to the setting up of the experiment. This would produce results that could be interpreted more reliably. This could be combined with HPLC to determine accurately antibiotic fate, and concentration changes with time.

In addition, results from the Biolog carbon utilisation patterns could be used to identify the carbon compounds whose utilisation was most sensitive to the presence of antibiotics and other toxic compounds. A radiolabelled form of these compounds could then be used in closed system experiments to obtain more information on exactly how their breakdown is affected by the presence of toxicants. It might also be possible to develop a method to determine the fate of compounds in the larger tanks containing both sediments and water (though still a closed system). A further possibility would be the use of these compounds individually to look at the detail of the differences between samples. Utilisation rates curves could be drawn up for each compound and comparisons made, to see if they are the same for those communities that PCA shows to be similar. They could then be used to look for changes when the ecosystems are exposed to antibiotics.

For all the above suggestions, more combinations of different antibiotics, and in larger numbers (and more replicate experiments), would also be of interest, as impacts changed when antibiotics were combined. These experiments would also more representative of ambient conditions, where many different types of antibiotics are likely to be present simultaneously. The possibility of combining antibiotics with other pollutants such as organic compounds or heavy metals could also be considered.

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APPENDIX 1: MUVS analysis of variance results

Two way analysis of variance, without replication

MUVS of E.coli exposed to oxytetracycline and amoxycillin

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Antibiotic	11.82061	4	2.955151	3.856563	0.022243	3.006917
Wavelength	52.10825	4	13.02706	17.00071	1.3E-05	3.006917
Error	12.26025	16	0.766266			
Total	76.1891	24				

Two way analysis of variance, with replication

MUVS of duplicate samples of *E.coli* 10650 grown in various concentrations of oxytetracycline

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	32.02	5	6.40	34.49	7.98E-18	2.34
Wavelength	39.86	11	3.62	19.52	1.89E-17	1.92
Interaction	21.48	55	0.39	2.10	0.001582	1.51
Within	13.37	72	0.19			
Total	106.73	143				

MUVS of duplicate samples of *E.coli* 10650 grown in various concentrations of amoxycillin

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	36.07	6	6.01	13.09	3.77E-10	2.22
Wavelength	24.00	10	2.40	5.22	8.03E-06	1.96
Interaction	9.70	60	0.16	0.35	0.999979	1.49
Within	35.37	77	0.46			
Total	105.13	153				

MUVS of duplicate samples of *E. coli* grown in various concentrations of oxytetracycline and amoxycillin

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	29.14	4	7.29	28.79	2.28E-13	2.53
Wavelength	43.19	11	3.93	15.51	9.7E-14	1.95
Interaction	3.95	44	0.09	0.35	0.999766	1.58
Within	15.18	60	0.25			
Total	91.46	119				

MUVS of duplicate samples of *E.coli* grown in various concentrations of cephalexin.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	55.55	4	13.89	69.03	9.1E-22	2.53
Wavelength	34.60	11	3.15	15.64	8.25E-14	1.95
Interaction	5.25	44	0.12	0.59	0.964581	1.58
Within	12.07	60	0.20			
Total	107.47	119				

Effects of two concentrations of oxytetracycline on the MUVS of three different river water bacteria.

RWB1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	668.90	2	334.45	185.66	1.09E-25	3.16
Wavelength	110.02	18	6.11	3.39	0.000224	1.79
Interaction	162.83	36	4.52	2.51	0.000912	1.62
Within	102.68	57	1.80			
Total	1044.43	113				

RWB2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	133.04	2	66.52	86.25	5.75E-18	3.16
Wavelength	43.67	18	2.43	3.15	0.000508	1.79
Interaction	22.55	36	0.63	0.81	0.74503	1.62
Within	43.96	57	0.77			
Total	243.21	113				

RWB3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Antibiotic	46.21	2	23.11	219.14	1.74E-27	3.16
Wavelength	6.76	18	0.38	3.56	0.00013	1.79
Interaction	9.26	36	0.26	2.44	0.001266	1.62
Within	6.01	57	0.11			
Total	68.24	113				

Appendix 2: Counts of DPM obtained for radiolabelling experiments (Chapter 5)

Counts of DPM obtained for Experiment 1: Radiolabelled sodium acetate and oxytetracycline added to river water

HEADSPACE COUNTS (DPM/ml)										
Time (h)	Control		Blank		0.01 mg/l		0.1mg/l		5mg/l	
	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
3	31.3	22.1	0.6	0.5	61.3	33.1	45.0	34.2	21.7	30.4
20	729.3	400.6	0.0	24.6	892.1	676.8	741.5	747.2	574.8	604.4
44	836.2	954.0	0.9	199.3	792.2	812.2	845.4	812.0	788.6	770.0
116	2395.7	2545.1	0.7	1.4	2339.1	2325.1	2248.4	2277.2	1795.5	1736.7
164	1452.9	1723.7	0.9	0.0	300.9	1232.8	1369.6	1308.7	870.8	888.3
212	1371.0	1712.8	29.4	2.8	429.5	1617.3	1290.3	1288.5	791.5	779.2
284	2665.2	2034.9	1.7	1.2	744.0	2842.1	3168.3	2348.4	1027.4	1269.6
356	2103.7	1534.7	3.1	1.0	986.2	1603.6	1959.4	1825.6	2274.9	1064.0

FILTER COUNTS (DPM/ml)										
Time (h)	Control		Blank		0.01 mg/l		0.1mg/l		5mg/l	
	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
3	9949.1	7227.8	109.1	50.3	18630	11685	17723	15212	9147.5	12993
20	16126	12808	64.7	28.6	15835	16374	14913	16301	17728	21474
44	17178	12933	43.4	95.6	14099	16502	13544	16549	16392	20079
116	7700.2	8034.5	50.9	22.7	6474.4	7456	6260	9595.3	23539	23908
164	1317.9	4525.4	31.8	41.7	5892.6	6095.5	5079.7	5258.4	21913	16656
212	4004.7	3831.8	23.1	19.8	3419.1	3792.5	1251.4	3197.1	19958	22926
284	3211.4	3588.1	31.1	33.2	3067	2711.7	2216.6	2723.7	11674	22245
356	3451.3	2818.8	98.9	38.4	2748.3	2054.8	2248.9	2923.8	6349.7	17040

WASH COUNTS (DPM/ml)										
Time (h)	Control		Blank		0.01 mg/l		0.1mg/l		5mg/l	
	1	2	3	4	5	6	7	8	9	10
0	56900	54279	56161	61983	56126	56864	56619	52723	61217	50316
3	41833	47981	59045	61896	31998	40424	32971	39124	53774	42757
20	30414	36223	59080		30508	31948	30632	29677	28931	27309
44	30671	35443	59069	61599	34136	33734	33654	31915	33681	29434
116	45019	45672	59877	62962	42607	44980	43138	42675	32650	30608
164	44617	38570	57789	60635	21643	41787	41748	42123	30805	32173
212	33127	35741	56505	57873	21788	33150	40327	41261	31863	28338
284	29506	32772	60761	61612	23373	27209	28370	32478	38875	23690
356	22392	30598	61166	62568	23187	22811	22435	28749	31103	26559

TOTAL COUNTS (DPM/ml)										
Time (h)	Control		Blank		0.01 mg/l		0.1mg/l		5mg/l	
	1	2	3	4	5	6	7	8	9	10
0	56900	54279	56161	61983	56126	56864	56619	52723	61217	50316
3	53624	58849	58703	61605	56936	56906	57594	56618	60265	61205
20	58611	64899	62259	65857	60755	67476	65804	60473	60736	56381
44	57254	67229	57861	59055	61705	57847	59024	51920	62286	64569
116	51614	55228	58691	65932	50352	54660	49773	53777	54298	56507
164	47869	46445	60945	61996	43322	52070	48491	51899	54131	61394
212	40401	46379	54133	62734	33354	44258	44323	45531	57442	57018
284	33320	40368	51274	59449	30824	42281	33697	37348	50621	49450
356	32108	35372	59740	73304	32904	31638	28696	37695	47636	50548

Counts of DPM obtained for Experiment 2: Radiolabelled amino acid mixture and oxytetracycline added to river water

HEADSPACE COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
4	69.5	74.8	1.5	3.4	81.1	78.3	79.6	82.7	84.2	95.3
28	277.7	292.7	3.3		299.5	333.6	283.7	303.4	338.5	375.0
76	1445.6	1489.1	10.9	0.5	1450.6	1542.3	1451.1	1463.5	1350.0	1407.2
148	1053.2	5.8	6.2	1.0	878.0	759.3	914.8	943.6	684.3	699.3
220	867.1	2.5	8.3	1.2	781.0	812.9	761.6	745.7	569.8	561.1
292	1070.4	7.6	9.4	3.4	768.5	874.5	732.5	734.2	540.9	549.2
322	447.6		2.5	1.7	307.0	634.7	545.8	642.5	198.6	
FILTER COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
4	10863	10462	120.5	43.9	12561	13691	13398	9133.2	5725.8	6009.3
28	15380	12094	88.6	54.9	15852	14151	14666	15403	9434.2	12310
76	6550.2	5607	177.3	135.5	4908.9	6564.9	4295.2	4566.7	8623.2	7897.7
148	3348.1	4462.8	107.3	53.5	3309.6	4040.7	1541.7	1883.1	8834	8458.5
220	3422.1	3010.6	125.1	114.7	3137.5	3702.7	1344.5	1804.8	6820.1	6547.5
292	2824.2	2754.6	91.6	71.4	3464	1897.3	1073.1	3968.2	5374.8	6570.8
322	3029.9	2061.5	79.4	62.2	2153.2	3567.2	1476.8	1096.3	5165.8	5531.4
WASH COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	26508	26693	21526	25446	25307	25770	25222	26664	26786	24960
4	13531	15455	21652	27175	12264	11694	11801	14092	10548	19362
28	7993	8619	21304	23757	8350	9358	8916	9155	14201	10723
76	15976	15591	21498	27257	15945	16267	16842	16021	12873	12835
148	17637	8815	21573	26946	18257	17403	18818	18483	14176	13702
220	17833	9019	21943	28002	18272	17584	19203	18885	14842	14514
292	17407	9083	22322	26719	17079	13245	18194	8812	14796	14875
322	20520	13409	22286	27494	19217	19170	17482	15892	23310	22990
TOTAL COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	26508	26693	21526	25446	25307	25770	25222	26664	26786	24960
4	311111	24441	20680	27556	25010	27108	26575	32765	29391	23806
28	27322	26633	21874	26421	25759	25860	25035	26712	25591	24287
76	24538	23898	21069	26602	22376	23990	22437	22691	23283	23760
148	22947	17040	23324	28286	20234	19736	20740	22259	23815	22995
220	20531	15947	22577	23994	20926	21507	20015	20610	21311	19585
292	20520	13409	22286	27494	19217	19170	17482	15892	23310	22990
322	21046	15313	21660	27496	21862	16876	19341	21382	21325	22938

Counts of DPM obtained for Experiment 3: Radiolabelled amino acid mixture and oxytetracycline added to river water

HEADSPACE COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
1.5	51.9	60.4	2.1	2.1	49.2	311.6	71.9	57.7	78.8	250.7
20	414.5	459.6	2.9	2.9	386.7	382.8	470.8	347.0	524.0	431.5
44	40.8	102.3	0.8	0.7	47.9	99.5	44.9	84.5	57.2	133.5
68	648.3	686.5	3.6	3.9	658.6	484.6	538.9	437.8	640.4	551.5
140	1065.3	1160.6	4.7	5.4	1141.5	995.5	1033.0	815.6	692.3	644.8
236	1084.3	1199.6	8.6	8.7	1247.1	1254.5	1314.3	1071.5	650.9	560.5
332	1421.4	1107.4	7.1	5.5	1007.5	1649.6	1252.2	1630.7	520.4	562.1
404	962.7	1460.0	4.1	4.2	1243.9	1021.7	1240.3	1027.9	381.0	471.3
524	1080.3	2007.9	4.9	4.9	1314.6	728.6	875.3	21.6	814.8	1220.8
644	367.2	805.7	2.2	2.6	540.0	442.1	442.5	10.7	346.0	426.0
FILTER COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	
1.5	8314.7	8061.8	40.9	24.2	7131.9	7863.4	7101.8	7292.7	3879.5	3570.2
20	13346	13976			15409	14088	14669	14005	8667.5	8076.7
44	14341	14424			16236	15901	16552	14447	13284	14587
68	6737.7	8050.4			8890.6	14638	14082	14182	11990	12785
140	3548.3	2766.3	54	51.3	4687.4	4561.2	5301.8	5388.9	12486	12239
236	2801.8	2989.5			3063.5	2976.2	4051.1	4178.2	9607.5	7846.8
332	2347	2286.2			2765.4	1609.4	3611.9	3435	11408	7259.6
404	1755.8	1668.9			1924.9	1749.4	2204.1	2167.7	8329.3	10518
524	1254.2	1593.8			2051.2	1461.8	1852.7	1427.2	7625.4	6296
664	855.5	2076.6			2824.7	2294.4	2015.5	3707.4	8001	8030.2
WASH COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
Time(h)	1	2	3	4	5	6	7	8	9	10
20	8205	8675			7205	6059	7281	4931	9670	16511
44	2780	7903			5292	5235	4510	3283	3353	6603
68	14904	14421			16415	9804	10358	8252	11774	10558
140	17528	16546	29001	28922	17700	17729	17200	15139	11340	10403
236	17998	17128			17745	16397	17052	14875	11119	10122
332	13109	14481			17674	12557	12802	11140	10204	11310
404	12779	12108			12364	12148	12087	11406	11143	11556
525	12435	10518			12137	10429	10435	5200	4183	7233
664	11966	9573			11843	10694	11402	6692	8593	8990

Counts of DPM obtained for Experiment 3: Radiolabelled amino acid mixture and oxytetracycline added to river water

TOTAL COUNTS (DPM/ml)										
Time(h)	Control		Blank		0.01mg/l		0.1mg/l		5mg/l	
	1	2	3	4	5	6	7	8	9	10
0	27981	28995	28570	26061	27347	27122	29274	25463	28021	27392
20	25421	26586			26254	22649	22532	21209	26828	26459
44	24893	25147			7210	15315	15985	16938	11577	11824
68	22345	23393			40	24544	24846	22669	24295	23437
140	20706	21436	28204	28124	24149	21827	22376	21325	23702	22362
236	20319	18760			21137	21857	21967	18550	21710	21136
332	15156	17781			19360	16501	19554	15138	24081	20274
404	18038	14129			15233	15244	16588	14280	22606	20115
524	13234	13674			15425	13432	15790	9598	17586	16998
664	12836	12717			17230	16455	15184	14851	19277	19624

Counts of DPM obtained for Experiment 4: Radiolabelled amino acid mixture and amoxycillin added to river water

HEADSPACE COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l Amox		0.1mg/l Amox		5mg/l Amox	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
3	77.8	83.9	0.9	0.6	86.4	92.0	88.1	93.2	76.7	60.9
19	319.0	359.7	1.4	0.9	406.0	377.9	362.6	415.8	389.7	339.2
33	287.2	233.0	10.6	18.6	262.5	266.9	242.7	261.1	245.5	236.8
126	1073.1	1.8	2.5	1.8	1001.4	1052.8	953.0	1112.5	836.6	0.6
198	758.3	1815.3	6.6	1.2	744.3	678.7	729.6	673.7	628.1	1582.3
294	1277.3	1070.4	4.2	20.1	1340.2	830.5	1200.6	921.8	862.1	1082.7

FITLER COUNTS (DPM/ml)										
Time(h)	Control		Blank		0.01mg/l Amox		0.1mg/l Amox		5mg/l Amox	
	1	2	3	4	5	6	7	8	9	10
0	0	0			0	0	0	0	0	0
3	11551	9897.1			10693	10347	11697	11506	8067.3	8891.4
19	11550	12158	35.4	35.4	9457.8	11864	11728	10712	11492	11012
33	10098	9888			9027.5	7264.2	9199.7	7921	7085.5	6721
126	2307.2	3091.1			0	0	2559.7	3635.6	2152.6	2649.8
198	2070.3	2769.8	34.8	24	3253	3415	3373.8	2902.1	3232.9	3929.4
294	1914.4	2810.1			3091.9	3140.9	2411.4	2693.6	3389.4	3958.1
366	2416.4	2785.4	20.5	15	2095.6	2750.7	1070	3218.2	3723.5	2734.4

WASH COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l Amox		0.1mg/l Amox		5mg/l Amox	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	22503	22117	21934	20741	21994	22376	22106	22123	23382	21952
19	6512	6208	17188	21686	5852	6124	3874	4533	7921	7499
33	7804	386			7173	7383	6758	7459	8646	8719
126	13944	13966					12883	12166	10115	10707
198	14505	14033	22970	21534	13411	11915	12985	12911	11127	11528
294	11052	10692			10123	10494	9611	11483	10320	9060

TOTAL COUNTS (DPM/ml)										
	Control		Blank		0.01mg/l Amox		0.1mg/l Amox		5mg/l Amox	
Time(h)	1	2	3	4	5	6	7	8	9	10
0	22503	22117	21934	20741	21999	22376	22106	22123	23382	21952
19	21477	21145	21559	20336	20548	20187	20287	20367	17943	19420
33	19991	19608			19250	18117	19254	18624	18304	17948
126	15981	16694			17015	16141	15042	15332	14404	14226
198	14505	14033	22970	21534	13411	11915	12985	12911	11127	11528
294	13823	15193			14310	13852	14245	11313	11052	10692

Counts of DPM obtained for Experiment 5: Radiolabelled amino acid mixture and antibiotic added to river water (0.5mg/l of each antibiotic).

HEADSPACE COUNTS (DPM/ml)										
	Controls			Blank	Oxytetracycline		Amoxycillin		Oxy and Amox	
Time (h)	1	2	3	4	5	6	7	8	9	10
1	35.765	46.815	32.66	3.745	41.81	64.61	71.97	59.335	57.74	
24	458.16	434.5	386.99	68.64	532.45	504.32	453.21	474.89	517.63	
96	893.44	998.54	703.69	1.2472	883.74	906	1010.9	751.81	909.75	1513.8
144	608.66	659.47	668.02	1.3114	447.6	458.29	622.41	870.3	491.08	507.81
648	5309.2	5392.2	7153.8	40.949	5047.5	5799	5382.2	5552.3	5280.4	5132.9
696	287.18	252	408.3	23.076	198.59	166.3	256.49	193.12	270.27	232.07
FILTER COUNTS (DPM/ml)										
	Controls			Blank	Oxytetracycline		Amoxycillin		Oxy and Amox	
Time (h)	1	2	3	4	5	6	7	8	9	10
1	6449.8	6961.7	6942.6	17.3	5640.5	6347.8	8046.9	8026	5786.1	6287.8
24	16828	16372	15949	24.6	15555	15308	15433	14937	14272	14123
96	11525	11479	11536	35.4	14359	14010	9217.1	8852.7	11206	10959
144	7102.8	6710.4	6316.9	26.7	9696	11168	8041.7	5605.3	8409.8	9043.5
648	1620.9	3566.8	2025.9	21.7	2872.5	2441.7	3716.5	3440.8	4075.2	3003.7
696	1011	2741.9	2052.7	11.9	2378.3	2172.4	3105.8	3345.1	3628.6	1995.7
WASH COUNTS (DPM/ml)										
	Controls			Blank	Oxytetracycline		Amoxycillin		Oxy and Amox	
Time (h)	1	2	3	4	5	6	7	8	9	10
0	24865	25636	25066	22827	26131	26130	26017	25809	26788	25894
24	6918	7149	6812	24015	8126	8589	8184	7742	9193	9251
96	10802	10236	9598	23531	9014	9290	10550	11873	9997	10349
144	14102	13645	13730	23447	10392	10439	12205	13538	10685	11400
648	10570	6987	8397	23207	11076	11142	6590	6594	6744	10469
696	10575	6885	8339	23516	10950	9211	6331	6411	6475	10018
TOTAL COUNTS (DPM/ml)										
	Controls			Blank	Oxytetracycline		Amoxycillin		Oxy and Amox	
Time (h)	1	2	3	4	5	6	7	8	9	10
0	24864	25635	25066	22827	26130	26129	26016	25809	26788	25894
24	24276	23003	22948	22053	24374	24391	24396	23787	23936	24535
96	21762	21755	22902	24975	25937	25088	21576	20745	22332	24834
144	21788	20892	16909	22168	15952	22394	19469	16113	18041	22113
648	14040	15166	12760	21228	15786	16593	15748	15780	16408	15870
696	13536	15875	12527	24607	14232	14870	13895	14659	16873	14714

Appendix 3: 95 carbon sources in Biolog plates (Chapter 6)

Amino Acids
D- Alalnine
L-Alanine
L-Alanyl-l-glycine
L-Aspargine
L-Aspartic acid
L-Glutamic acid
Glycyl-L-aspartic acid
Glycyl L-glutamic acid
L-Histidine
Hydroxy-L-proline
L-Leucine
L-Ornithine
L-Phenylalanine
L-Proline
L-Pyroglutamic acid
D-Serine
L-Serine
L-Threonine
D,L-Carnatine
γ-Aminobutyric acid

Carbohydrates

N-Acetyl-D-galactosamine
N-Acetyl-D-glucosamine
Adonitol
L-Arabinose
D-Arabitol
Cellobiose
i-Erythritol
D-Fructose
L-Fructosee
D-Galactose
Genitobiose
α-D-Glucose
m-Inositol
α -Lactose
Lactulose
Maltose

D-Mannitol
D-Manose
D-Melibiose
β-Methylglucoside
Psicose
D-Raffinose
L-Rhamnose
D-Sorbitol
Sucrose
D-Trehalose
Turanose
Xylitol

Carboxylic acids
Acetic acid
Cis-Aconitic acid
Citric acid
Formic acid
D-Galactonic acid lactone
D-Galacutaronic acid
D-Glauconic acid
D-Glucosamine acid
D-Glucoronic acid
α-Hydroxybutiric acid
β-Hydroxybutiric acid
γ-Hydroxybutiric acid
p-Hydroxyphenylacetic acid
Itaconic acid
α-Ketobutric acid
α-Ketoglutaric acid
α-Ketovaleric acid
D,L-Lactic acid
Malonic acid
Prponoic acid
Quinic acid
D-Saccharic acid
Sebacic acid
Succinic acid

Alcohols

2.3-Butanediol
Glycerol

Amides

Succinamic acid
Glucuronamide
Alaninamide

Aromatic chemicals
Inosine
Urocanic acid
Uridine

Brominated chemicals
Bromosuccinic acid

Amines
Phenylethylamine
2-Aminoethanol
Putrescine

Esters
Mono-methylsuccinate
Methylpyruvate

Polymers

Glycogeo
α -Cyclodextrin
Dextrin
Tween 80
Tween 40

Phosphorylated chemicals

D,L-α-Glycerol phosphate
Glucose -1phosphate
Glucose 6-phosphate

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