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MICROBIAL DEGRADATION OF XENOBIOTIC CHEMICALS

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This thesis is submitted in partial fufilment of the requirements for the degree of Doctor of Philosophy, Council for National Academic Awards. The work in this thesis was carried out in the Department of Life Sciences, Trent Polytechnic, Nottingham, in collaboration with the Water Research Centre, Medmenham, Marlow, Bucks.

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

The candidate has completed a series of advanced studies, as outlined as registration in partial fulfilment of the requirements for the degree of Doctor of Philosophy, Council for National Academic Awards.

This is to certify that the work here submitted was carried out by the candidate himself. Due acknowledgement has been given of any assistance received.

Signed ----------

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(Director of Studies)

The conventions and nomenclature used throughout this thesis are as described in the Journal of General Microbiology Instructions to Authors, January 1988. Any abbreviations used have been defined within the thesis text.

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ABSTRACT

Microbial Degradation of Xenobiotic Chemicals
by K.O. Colquhoun.

The inherent biodegradability of eight xenobiotic chemicals which have exhibited aberrant behaviour in degradation tests were investigated. Three of the chemicals investigated; benzene-1,3-disulphonic acid (BDSA), 2-chlorobenzoic acid (2CBA) and cis, cis, cis, cis-1,2,3,4-cyclopentanetetracarboxylic acid (CPTCA) were not found to be inherently biodegradable. t-Butanol was removed in a semi-continuous activated sludge (SCAS) test, but the mechanism was found to be volatilization not biodegradation, a fact overlooked by other workers. Three of the remaining four chemicals, 3-chlorobenzoic acid (3CBA), N-methylaniline (NMA) and tetrahydrofuran-2,3,4,5-tetracarboxylic acid (THFTCA) were degraded in SCAS tests after acclimatization.
Hexamethylenetetramine (hexa) was not degraded in the SCAS test, but it was degraded during 28 day die-away tests. It has been demonstrated that hexa can be degraded by unadapted activated sludge and soil at levels as low as 10^{-8}g l^{-1} using ^{14}C labelled substrate. The ability of activated sludge to degrade these chemicals found to be inherently biodegradable was not lost during a deacclimatization period of 3 months. None of the chemicals investigated were found to be inhibitory to microbial growth.

Microorganisms have been isolated which can degrade 3CBA, hexa, NMA and THFTCA and growth characteristics investigated. Four microorganisms capable of degrading 3CBA were isolated and identifed as Pseudomonas species. The degradative pathway has been identified as the β-ketoadipate pathway. NMA and THFTCA were degraded by an Alcaligenes species and a Corynebacterium species respectively, both isolated from activated sludge. Hexa was degraded by two-separate species Methylobacterium isolated from activated sludge by enrichment. The hexa, NMA and THFTCA degrading isolates appear to be the first reported.

The results obtained are discussed in context with what has been reported by other workers, both in relation to biodegradation in general, and biodegradation testing in particular.

CONTENTS

1.0 INTRODUCTION.

1.1 Biodegradation and Xenobiotics.

As of November 1977 the American Chemical Society's Chemical Abstract Services computer registry of chemicals contained 4,039,907 distinct entities. The extent to which the number of chemicals on the register has been growing is estimated to be approximately 6000 per week (Maugh, 1978). Richardson (1985) has given the number of known chemicals to be in the order of 7×10^6. The majority of these compounds are not in every day use, with $10^4 - 10^5$ regularly in use in industry, agriculture, the home and elsewhere (Richardson, 1985).

One of the major contemporary problems has been the appearance of thousands of new chemicals released into the biosphere as products of the synthetic chemical industry, particularly the many novel compounds used in agriculture as pesticides and herbicides (Slater and Somerville, 1979). Many of these compounds have not occurred in the biosphere prior to their synthesis by man and may be considered as environmentally foreign or *xenobiotic* compounds (Slater and Somerville, 1979 and Slater, 1981).

The term *xenobiotic* (stranger to life) is derived from the Greek words *xenos : a stranger, foreign, strange*; and *bios* : life, or course of life : For the

environmental microbiologist or chemist xenobiotic usually implies "foreign to the biosphere" (Hutzinger and Veerkamp, 1981). Another term which is often used synonymously with *xenobiotic* is *anthropogenic* since it appears to be a scientific term for man-made. In Greek, *anthropos* means man, human, and *gignesthai* to become, be born. However, strictly speaking "anthropogenic" does not mean man-made but man-making - the origin of man - as is evident in anthropogenesis (the study of man's origin and development). On the other hand the term *biogenic* (of biological origin, produced by the biosphere), which is of similar linguistic construction as "anthropogenic" is commonly accepted by the scientific community, (Hutzinger and Veerkamp, 1981).

Adjectives which are used more or less interchangeably with "*xenobiotic*" or "*anthropogenic*" are : "*man-made*", "*synthetic*", "*environmental*", "*alien*" and "*pollutant*". Most often the full term includes "*chemicals*" or "*compounds*" e.g. "*man-made compounds*". The word "*chemical*" itself somehow seems to imply unnaturalness since organic substances referred to in their context as natural compounds (e.g. vitamins, proteins and carbohydrates) are seldom spoken of as "*chemicals*". (Hutzinger and Veerkamp, 1981).

Some xenobiotic compounds are sufficiently similar to existing natural or biogenic compounds to be degraded by existing mechanisms, but others are not susceptible to

biological transformation. Consequently, compounds in this latter category may persist in the environment for considerable periods of time and may become serious environmental hazards. These compounds are said to be "*recalcitrant*". The concept of recalcitrant compounds was discussed by Alexander in 1965. Referring to the introduction of structurally novel compounds, notably pesticides, detergents and synthetic chemicals that may not degrade in the environment, Alexander stressed the requirement for research in order to understand how these materials are degraded. He urged caution of Gale's microbial infallibility principle (Gale, 1952) which proposes that microorganisms capable of oxidizing any compound that can be theoretically oxidized exist or soon arise whenever and wherever these compounds are found. Microorganisms can metabolize not only naturally occurring but a whole range of man-made compounds such as phenols and amines (anilines) hence Gale's principle. Over the past 30 years mankind has witnessed a dramatic rise in the production of a diverse variety of synthetic organic chemicals that are released into the environment often in only small quantities but sometimes in larger amounts. A significant number of these materials are destroyed by biodegradative activities of microorganisms in soils, waters and sewage works, biodegradation here being considered as the conversion by biological agencies of a complex organic material into one or more

simpler substances (primary biodegradation). When biodegradation is complete, the elements of which the original molecule was composed are released in an inorganic form (ultimate biodegradation or mineraliz ation), (Alexander, 1973).

The persistence of man-made chemicals may be a necessary requirement for compounds designed specifically for technical use, or of compounds, like insecticides and herbicides directed towards specific targets to increase length of effectiveness (Alexander, 1973; Neilson et al., 1985). In general, however, persistence of man-made chemicals in the natural environment is an undesirable property. For example, components of industrial waste effluents introduced into waterways by whatever means, if not acted upon by the aquatic microflora, may detrimentally affect wild life, reduce the quality of drinking water, and be responsible for unsightly accumulation of waste products. A classic example of this phenomenon was the introduction of the first synthetic detergents in the 1950's. These detergents now described as "hard", were only partially removed in sewage treatment and passed through treatment works, largely unchanged, into rivers. It was common, at least in Britain, to see large banks of foam created by aeration in activated sludge plants and also at weirs and sites of turbulence on polluted rivers. The problem, caused by the inability of aquatic bacteria to

degrade sufficiently rapidly the highly branched chain alkyl benzene sulphonates (based on tetra-propylene), was satisfactorily solved by replacement of the "hard" type with a biodegradable "soft" product in which the degree of branching had been considerably reduced. (Painter and King, 1985).

In recent years there have been no comparable widespread spectacular effects of non-biodegradable chemicals, due to the fact that few other synthetic chemicals are used so widely or in such large quantities as synthetic detergents. There is, however, concern over chemicals which although not used in very large quantities are known to be persistent and to accumulate in aquatic organisms. Some of these chemicals have been detected far from the initial sites of use. The pesticide dichlorodiphenyl-trichoroethane (DDT) for example has been detected at concentrations of up to $2\mu g\ l^{-1}$, in the Sargasso Sea.

Chemicals if discharged into the aquatic environment have the potential to cause damage in a number of ways. They can inhibit microorganisms directly in sewage treatment processes and in rivers, thus possibly seriously interfering in aerobic treatment of waste waters and in the self purification processes in bodies of water and may be toxic to aquatic organisms e.g. fish. Absorbed and insoluble chemicals may interfere in the anaerobic treatment of sewage sludge, which if it is

subsequently applied to land as fertilizer, may inhibit essential microbial action in soil and/or become assimilated into plants. It is possible that animals and man could have thus become affected by drinking polluted water and eating contaminated food. Hence, some form of scrutiny of chemicals is necessary before they are put into widespread use and in many countries this examination has been embodied in appropriate legislation.

In Britain and the EEC chemicals in use for stated purposes before 18/9/81 are termed "existing chemicals", under the Health and Safety Executive (HSE)/Department of the Environment (DOE) Notification of New Substances Scheme (HSE, 1982) and can be manufactured, marketed and used without restriction or submission to the EEC Commission, provided that they were registered as "existing" chemicals before 31/12/82. The water authorities have the right under UK legislation (Control of Pollution Acts COPA 1 and 2, 1974) to insist on removal or reduction in concentration of a given chemical in industrial discharges to sewers or rivers, providing that it can be reasonably shown that a specific chemical is responsible for an environmental problem e.g. inhibition of nitrification or toxicity to fish.

New chemicals, on the other hand, are subject to the EEC regulations set out in "Council Directive 67/548",

6th Amendment Official Journal of the European Communities, No L259/10, 15/10/79 (the so-called Dangerous Chemicals Directive). The purpose of this Directive is to approximate the laws, regulations and administrative provisions of the Member States on : a) the notification of substances, and b) the classification, packaging and labelling of substances dangerous to man and the environment, which are placed on the market in the Member States. This directive requires the production of a technial dossier supplying the information necessary for evaluating the foreseeable risks, whether immediate or delayed, which the substance may entail for man and the environment. The dossier requires information on the uses, toxicity, biotic and abiotic degradation of a new chemical and the possibility of rendering the chemical harmless. Provision is allowed in the Directive for Competent Authorities (DOE and HSE in the UK) to request further details of toxicity and ecotoxity studies from the notifier of the chemical.

It should be stressed that the Directive does not set out to ban a chemical per se, but to acquire sufficient information to enable the Competent Authority (DOE/HSE) to make a hazard assessment. If a chemical appears to be a hazard, DOE would warn the UK Water Authorities to look out for it so that it would be dealt with under COPA. However, most if not all chemical producers co-

operate with the Competent Authority so that few if any new "hazardous" chemicals should get onto the market.

1.2 Entry and fate of xenobiotic compounds in the biosphere.

Before man started large scale industrial activities, the concentrations of the organic chemicals on the surface of this planet remained more or less constant with biosynthesis and biodegradation being held in equilibrium by the integrated activities of plants, animals and microbes (Leisinger, 1983). We are now faced with certain industrial chemicals (xenobiotics) which do not readily participate in the global cycles of carbon, nitrogen or sulphur (Dagley, 1978); this was restated by Slater (1982). Such compounds cause problems of disposal and may, if they escape containment, lead to adverse effects on the environment. Chemicals exhibiting transitory or permanent accumulation have been termed "pollutants" or "environmental pollutants", expressions which stress their undesirable effects on the environment. In relation to the total volume of organic compounds involved in the carbon cycle the formation of chemical pollutants by human activities is modest. The turnover of organic matter in the carbon cycle by photosynthesis and biodegradation amounts to approximately 2×10^{11} tonnes per year as compared to world oil production,

$3x10^{8}$ tonnes per year and to the synthesis of organic chemicals $2x10^{8}$ tonnes per year (Leisinger, 1983).

The producers of chemicals are often made solely responsible for the entry of chemicals into the environment. This is not justified since the responsibility for the proper storage, use and disposal of chemicals is handed over to a wide variety of consumers. The routes of entry into the biosphere of chemicals have been described by Leisinger (1983) and to a lesser extent by King (1984); they are summarized in Figure 1. Major entry points of pollutants into the environment are aqueous effluents from domestic or industrial waste treatment plants (pathways 3 and 4, Figure 1). Quantitatively less important are toxic exhaust gases from waste incineration facilities (pathway 3, Figure 1). New compounds are formed during incineration of waste compounds, e.g. dioxin. The problem may be overcome by burning waste compounds with a plasma torch at 10,000°C to produce harmless exhaust gases (Wigerson, 1987). Pollution arising from biological waste treatment plants (pathway 4) is due to organic chemicals that are recalcitrant or insoluble and thus escape degradation in conventional systems. Furthermore, the toxicity of an organic compound may interfere with the proper functioning of the biological treatment and lead to discharge to rivers of pollutants. During the treatment process some organic compounds

adsorb onto activated sludge. They do not appear in the aqueous effluent but cause problems in sludge disposal and sludge application to agricultural land.

The problems of treating some organic chemicals in conventional biological systems are illustrated by a survey of 96 organic compounds, from the U.S. Environmental Protection Agency (EPA) list of priority pollutants, (Patterson and Kodukala, 1981 and Tabak et al., 1981). The groups of compounds described in the EPA priority pollutants list are shown in Table 1. (Keith and Telhard, 1979). Static-culture flasks screening biodegradability tests (Bunch and Chambers, 1967; Tabak and Hannah, 1979), were performed with these compounds at the 5mg l^{-1} level, using 3 successive subcultures with domestic sewage as the primary inoculum. Of the 96 compounds tested, 50% were completely degraded, 30% were insignificantly or not degraded at all and the remaining 20% only partially degraded. One compound exhibited toxic effects and 20% of the compounds tested were concentrated in sludge by factors of from 5 to 170.

Accidents, spills during transportation and leakage from waste disposal sites may lead to direct entry of untreated chemicals into the environment (pathway 2, Figure 1). The last route of entry (pathway 5, Figure 1) is taken by products such as pesticides and aerosol

Legend to Figure 1.

1) Recycling and reclamation of waste products.
2) Direct discharge of compounds into the environment or by accidental spillage during production.
3) Chemicals which are resistant to biological degradation in industrial waste treatment systems.
4) Chemicals entering the environment in treated effluents from domestic sewage treatment systems.
5) Chemicals entering the environment through consumer use of the product.

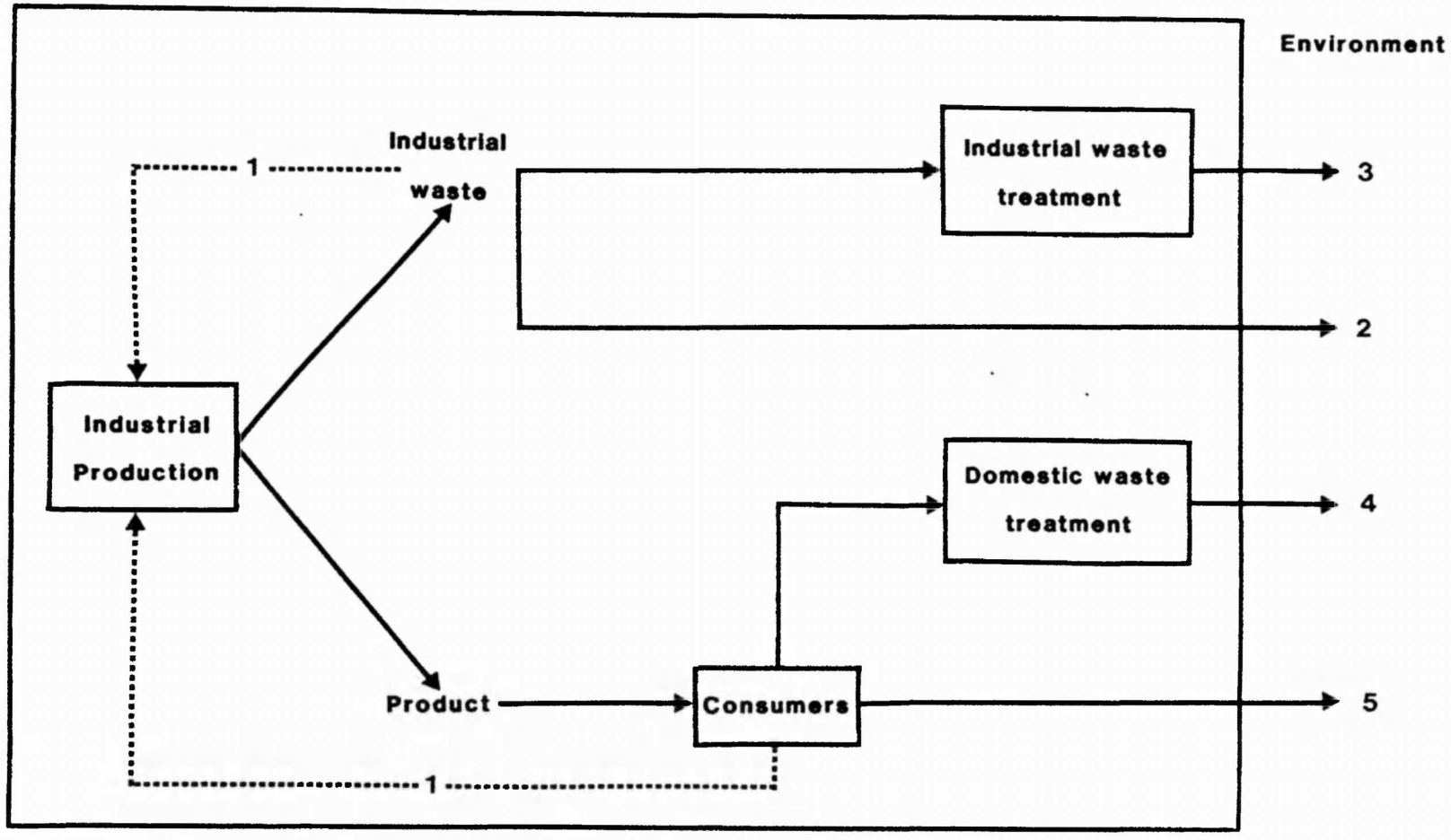


Figure 1. Entry of man-made organic compounds into the environment.

propellants whose utilization is coupled with their release into soil, water or air. Other chemicals such as certain solvents or 1,2-dichloroethane and 1,2-dibromoethane which are present in gasoline as lead scavenging agents escape containment by the consumer because of their volatility. Finally, included in this category is the great variety of pollutants deliberately released into the environment by illegal dumping.

Once xenobiotics have entered the biosphere the fate of these compounds becomes important. This is an area of great importance for assessing the environmental impact of xenobiotics. The scheme presented in Figure 2, gives an overview of the types of reactions a xenobiotic compound can undergo in the environment. Total absence of degradation of a xenobiotic compound in nature has not been demonstrated so far; 2,3,7,8-tetrachlorodi benzo-p-dioxin. (TCDD), one of the most persistent chemicals known, has been shown to be metabolized at a low rate by microbial cultures (Philipps et al., 1982).

Of the various fates described in Figure 2 for xenobiotics, complete "mineralization" of a compound is the most desirable of these processes. It generates carbon and energy for microbial growth and leads to the disappearance of the xenobiotic compound. Cometabolism (Horvath,1972) is a process by which microorganisms, in the obligate presence of a growth substrate, transform a

Table 1. Organic priority pollutants according to the U.S. Environmental Protection Agency (Keith and Teliard, 1979).

Chemical Class	Number of compounds
Aliphatics	3
Halogenated aliphatics	31
Nitrosamines	3
Aromatics	14
Chloraromatics (including TCDD)[a]	16
Polychlorinated biphenyls (PCB's)	7
Nitroaromatics	7
Polynuclear aromatic hydrocarbons	16
Pesticides and metabolites (including DDT)[b]	17

[a] TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin

[b] DDT = 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane

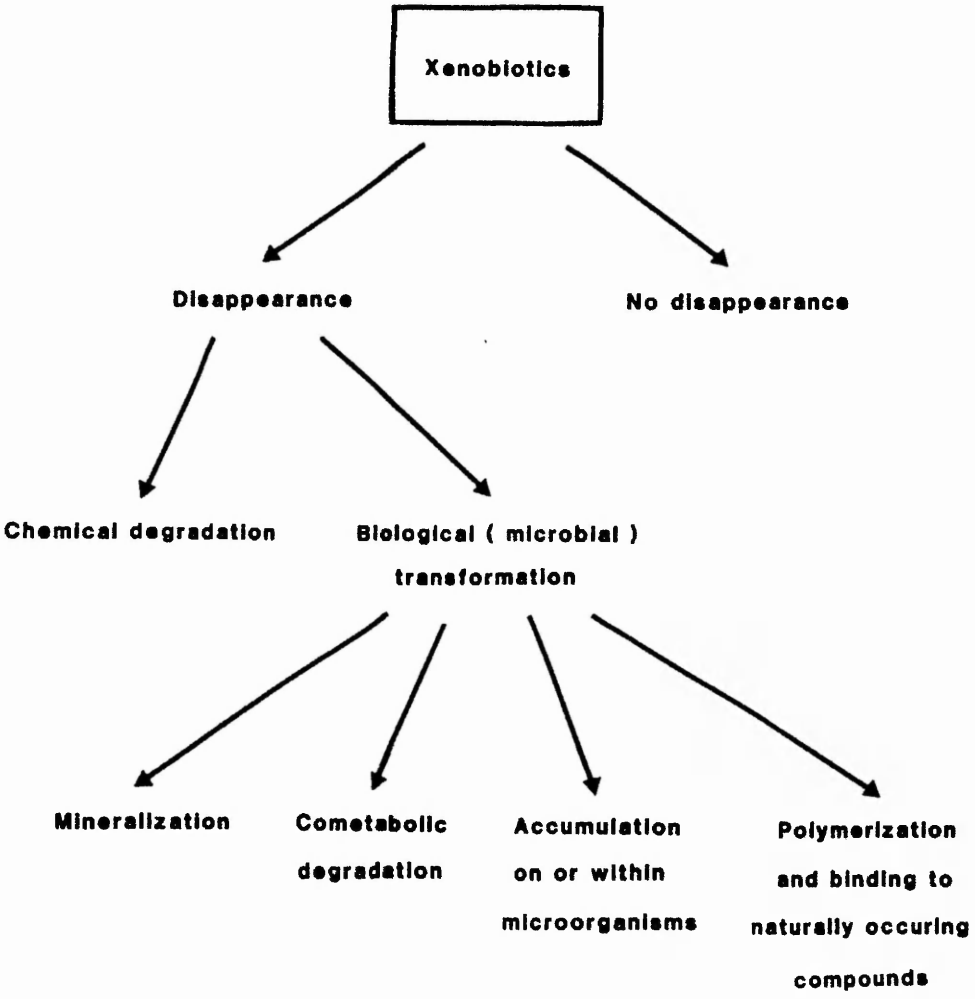
non growth susbtrate. Although cometabolizing organisms do not derive benefits from the metabolism of non-growth substrates, cometabolism is thought to play a significant role in the degradation of xenobiotics in nature, but this assumption is hard to prove (Harder, 1981). Cometabolic transformations in the environment do not necessarily result in the complete oxidation of xenobiotic compounds but may lead to the accumulation of transformation products with increased or decreased toxicity compared to the original compound (Alexander, 1981). Cellular accumulation represents a further type of interaction of microorganisms with xenobiotics. This process which may be the result of active uptake or of absorption phenomena usually has adverse effects. It may lead to bioconcentration of hazardous chemicals and their entry into the food chain (Boughman and Paris, 1981).

The final possible fate of a xenobiotic compound is to bind to organic matter such as humus in soil or flocs in activated sludge. This may be a long or short term effect and there is little evidence to suggest if this fate presents an environmental hazard or not.

1.3 Testing biodegradability.

The Organisation for Economic Co-operation and Development (OECD) and the European Economic Community (EEC) have defined three levels of testing which

Figure 2. Fate of xenobiotics in the environment (Leisinger, 1983).



correspond to three degrees of biodegradability in the OECD scheme and to three levels of annual production of the chemical in the EEC scheme (King, 1984). At the first level of biodegradability testing the "base-set", or level "O", relatively low inoculum concentrations of sewage microoroganisms in minimal media are employed in batch die-away or screening tests. These tests assess whether a compound is readily biodegradable, and are the least expensive both in terms of man-hours performing the tests and the cost of materials and apparatus. The term "readily biodegradable" is a term applied to an arbitrary classification of chemicals which have passed specified screening tests for ultimate biodegradability at this level. The tests are so stringent that such compounds biodegrade rapidly and completely in a wide variety of aerobic environments and are said to be fail safe, (Painter and King, 1985). Level I testing assesses inherent biodegradability and employs semi-continuous versions of the activated sludge process, having high sludge ages, and die-away tests in which, other degradable substrates present and/or in which much increased concentrations of bacterial inoculum are used. The term "inherently biodegradable" is a term applied to a classification of chemicals for which there is unequivocal evidence of primary or ultimate biodegradation in any test of biodegradability, (Painter and King, 1985). More complex and costly simulation

tests are used at Level II to assess whether the compound under test is removed in practice.

Table 3 shows the types of biodegradability test used at each level of testing and determinands used to observe biodegradation. The screening tests are only "fail safe", that is, a compound which does not degrade in a Level "O" cannot be assumed to be non-biodegradable. Further investigations are necessary for compounds giving negative results. Having said that, positive results do not necessarily exclude further investigation. It is known that the stringency of the tests varies, decreasing generally in the order - modified closed bottle, modified MITI 1, modified OECD, modified AFNOR and modified Sturm test (Gerike and Fischer 1979, 1981). In addition there is a further test for ready biodegradability known as the International Organisation for Standardization (ISO) die-away test method (ISO, 1984), which is not considered by Gerike and Fischer (1979, 1981).

Essentially the screening tests are batch tests which usually have the test substance as the only source of carbon and energy. The compound should be soluble at the concentration under test, non-volatile or have negligible vapour pressure under the conditions of the test, not significantly adsorbable on glass or inhibitory to microorganisms. The test compound is dissolved in minimal salts medium and inoculated with a

Table 3. Types of biodegradabilty tests.

Level	Type	Method	Determinand	Reference
0	Screening	Modified Closed Bottle	O_2	OECD (1981)
		Modified MITI I (Japanese Ministry of International Trade and Industry)	O_2/specific analysis /DOC	"
		Modified OECD	DOC	"
		Modified AFNOR (Association Francaise de Normalisation)	DOC	"
		Modified Sturm	CO_2	"
		International Standard Organisation (ISO) Die-away test	DOC	ISO (1984)
1	Inherent	Zahn-Wellens	DOC	OECD (1981)
		SCAS (Semi-continuous activated sludge)	DOC	", HMSO (1983)
		MITI II	O_2/specific anlaysis	OECD (1979)
2	Simulation	Activated sludge ; Husmann, Porous Pot	DOC	OECD (1981), HMSO (1983)
		Biological filters ; rotating tube	DOC	HMSO (1983)
		River ; Karlsruher	DOC	OECD (1979)

relatively small number of unacclimatized microorganisms i.e. not having "seen" the test chemical before. The test vessels are incubated in the dark at 22.5°C±2.5°C for 28 days. Samples are taken for analysis, or measurements made, at sufficiently frequent intervals to allow the calculation of the degree of removal of test chemical (or of oxygen uptake or carbon dioxide produced) in the 10 day period after degradation starts. Suitable blank controls are run with each test to allow for the activity and carbon content of the inoculum, and substances of known biodegradability such as sodium benzoate or sodium acetate are also tested to check that the inoculum is active. In some methods extra vessels can be set up containing both the test and standard chemicals in order to ascertain whether inhibition of the inoculum has occurred. Should lack of degradation of test substances be thought to be due to inhibition, the test may be repeated with a lower concentration of test compound. The amount and type of inoculum used varies from test to test (Table 4) as does the constituent make-up of the minimal media (Table 5). It has been proposed that this variation is responsible for the difference in stringency for each test and harmonization of the tests should be undertaken (Blok et al., 1985).

Table 4. Inoculum used for each test. (After Painter and King, 1985).

Level	Type	Method	Inoculum (volume or weight per litre of medium)	Concentration of bacteria (microorganisms ml^{-1})
0	Screening	Modified Closed Bottle	1 drop effluent	$0,25x10^2$
		Modified MITI I	30 mg suspended solids of "activated sludge" grown on glucose peptone medium for at least 1 month.	$2-10x10^5$
		Modified OECD	$0,5ml^{-1}$ effluent	$0,5-2,5x10^2$
		Modified AFNOR	Resuspended bacteria filtered from surface water /effluent and counted.	$2-8 \times 10^5$
		Modified Sturm	1% supernatant of homogenised activated sludge	10^4-2x10^5
		ISO Die-away	10ml suspended solids	$3x10^5$
1	Inherent	Zahn-Wellens	1000mg suspended solids of activated sludge	$0,6-3x10^7$
		MITI II	100mg suspended solids of activated sludge	$0,7-3,3x10^6$

Table 5. Composition of media. (Values expressed as mg l^{-1}, except total PO_4 which is $x10^{-3}M$). (After Painter and King, 1985).

Constituent	Modified Closed Bottle	Modified MITI I	Modified OECD	Modified AFNOR	Modified Sturm	ISO Die-away test
KH_2PO_4	8,5	22,5	8,5	300	17	8,5
K_2HPO_4	21,8	66,3	21,8	-	43,5	21,75
$Na_2HPO_4\ 2H_2O$	33,4	133,8	33,4	2000	66,8	33,4
Total PO_4	0,375	0,94	0,375	7,8	0,59	0,375
$MgSO_4 7H_2O$	22,5	67,5	22,5	50	22,5	22,5
$CaCl_2$	27,5	82,5	27,5	37,8	27,5	27,5
NH_4Cl	1,7	5,1	20	-	3,4	2,5
$(NH_4)_2SO_4$	-	-	-	300	40	-
NH_4NO_3	-	-	-	175	-	-
$FeCl_3,6H_2O$	0,25	0,25	0,25	-	1	0,25
$FeCl_3$,EDTA	-	0,1	0,1	-	-	0,1
$MnSO_4,4H_2O$	-	0,04	0,04	-	-	0,04
$(NH_4)_6Mo_7)_{24}$	-	0,035	0,035	-	-	0,035
H_3BO_3	-	0,057	0,057	-	-	0,057
$ZnSO_4,7H_2O$	-	0,043	0,043	-	-	0,042
Yeast extract	-	0,15[a]	0,15[a]	5	-	0,015
Trace elements	-	-	-	Yes[b]	-	-

[a] Or 1ml l^{-1} of a solution containing, 0,2mg biotin, 2,0mg nicotinic acid 1,0mg thiamine, 1,0mg p-aminobenzoic acid, 1,0mg pantothenic acid, 5,0mg pyridoxamine, 2,0mg cyanocobalamine and 5,0mg folic acid in 100ml distilled water.

[b] 1ml l^{-1} of a solution containing, 0,1g $FeSO_4,7H_2O$, 0,1g $MnSO_4,H_2O$, 0,025g K_2MoO_4, 0,025g $Na_2B_4O_7$, 0,025g Co $(NO_3)_2,6H_2O$, 0,025g $ZnCL_2$ and 0,01g NH_4VO_3.

There are some elaborations on these basic screening tets which make them more likely to yield positive results for those substances which are degraded only by cometabolic processes. For example, in the Bunch-Chambers method (Bunch and Chambers, 1967) yeast extract is added (55mg l^{-1}) as an alternative substrate to the test substance with 10% sewage as inoculum. At weekly intervals, 10ml of the culture is subcultured into fresh yeast extract-test substance medium. At each transfer the concentration of test substance is determined by specific analysis to assess the degree of primary biodegradation.

There are three tests included in the OECD scheme for testing compounds for inherent biodegradability (Table 3). Originally the Zahn-Wellens method was put forward as a screening method but, since experience showed that it was considerably less stringent than the other screening tests, it was reclassified as a test for inherent biodegradability. A much larger inoculum (1000mg sludge solids l^{-1}) and higher concentrations of test substance (50-400mg C l^{-1}) are used than in screening tests. To allow for any adsorption of test substance onto sludge, a sample is taken 3 hours after mixing, and further samples taken at 1 to 3 day intervals for analysis of DOC, after filtration. The higher concentration of inoculum gives a greater

opportunity for more test compounds to be degraded within the 28 day period of incubation.

With the SCAS method (HMSO, 1983) for assessing inherent biodegradability, domestic sewage and the test substance (5-20mg C l^{-1}) are added daily to activated sludge in a suitable vessel which is aerated for 23 hours. Sludge is then allowed to settle for ½ to 1 hour, the supernatant is discarded or collected for analysis, fresh sewage plus test substance are added and the cycle is repeated. Control units receiving no test substance are operated simultaneously. Tests are usually continued for up to 3 months or more, if necessary, during which time the concentration of DOC in the supernatants of test and control units are compared to determine how much if any removal has occurred. It is assumed that the sludge in the test unit would oxidise the sewage components to the same extent as the control, so that any residual DOC, after subtraction of the control effluent value from the test value, would be derived from the test substance. The pattern of removal over several weeks can usually help in distinguishing between adsorption and biodegradability, but any doubt can be dispelled by using the acclimatized sludge as an inoculum in a screening test.

The Japanese MITI II respirometric method (OECD, 1981) is less stringent than the MITI I screening test since it employs a higher concentration of inoculum (100

mg l^{-1}) and 30 mg test substance l^{-1}, but it is the most stringent of the three tests for inherent biodegradability.

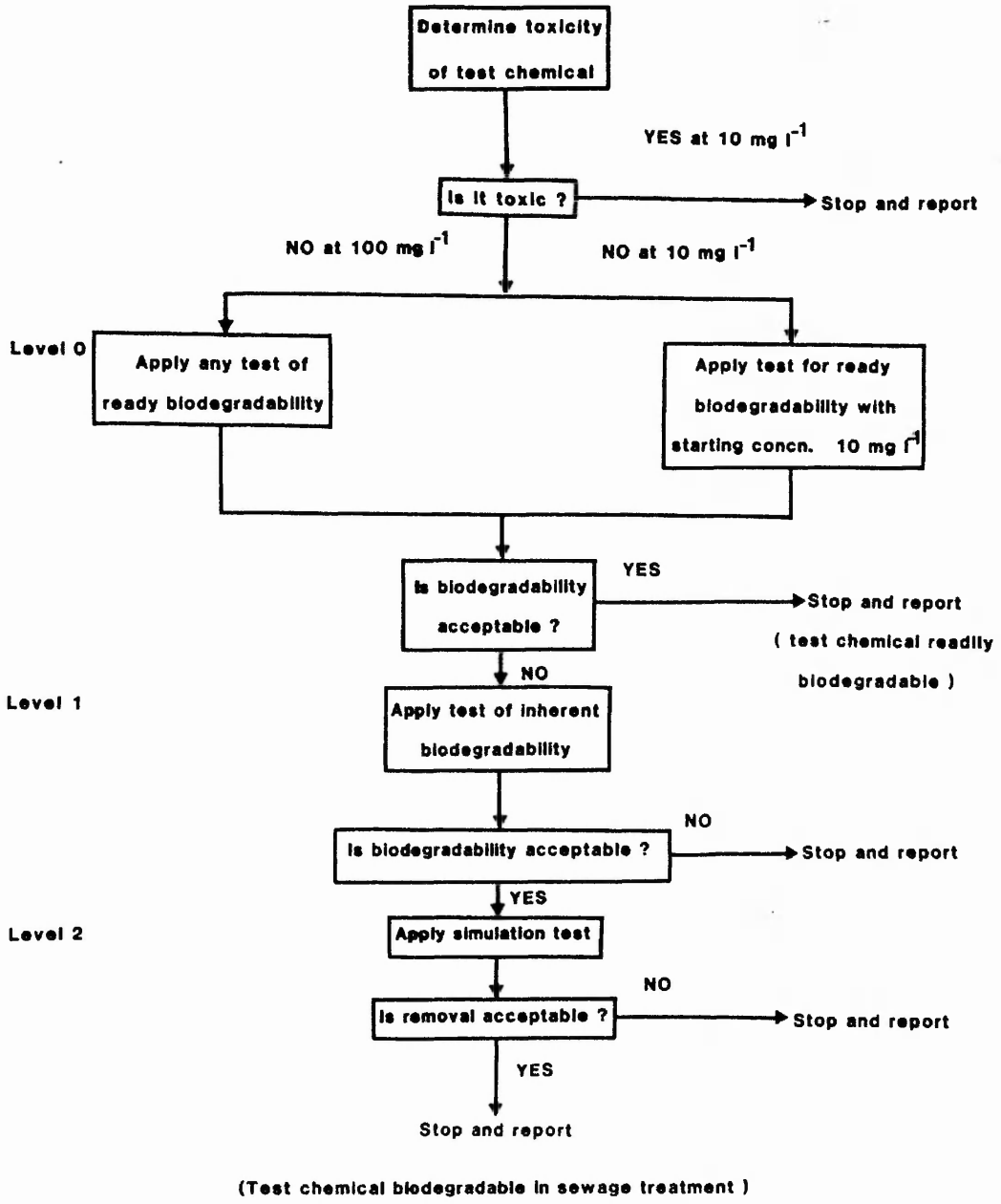
Substances which are degraded by the Zahn-Wellens or SCAS methods do not necessarily degrade readily in the environment : those substances which do not degrade in the inherent tests are considered to be non-biodegradable. To decide the fate of inherently degradable substances it is necessary to subject them to a test which simulates the environment into which they will be discharged. Tests simulating conditions in rivers such as the Karlsruher test (OECD, 1979) have been devised but little seems to have been reported on their use.

Because the majority of chemicals are discharged to sewers much attention has been given to simulations of activated sludge systems and to a lesser extent biological (percolating or trickling) filters. The latter will not be discussed here as little work has been reported using this system. The Husmann Activated Sludge Unit used in the OECD confirmatory Test as a simulation test was originally applied to the assessment of the biodegradability of surfactants (detergents). It has been adapted for general use with soluble organic chemicals by the substitution of DOC analysis for the specific analyses of surfactants. To overcome the discrepancies associated with the variable nature of

real sewage, a synthetic sewage is used; it is applied to the 3 litre aeration vessel at a rate of 1 h^{-1}, giving an average retention time of the liquid phase of 3 hours and the dissolved oxygen concentration is kept above 2mg l^{-1}. The mixed liquor passes to the settlement tank of about the same volume from which treated effluent overflows into a collection vessel, prior to analysis for DOC or a specific chemical. Settled sludge is returned to the aeration vessel by means of an air lift pump, however, the rate of return is so high that the settled sludge does not become anaerobic as it does in practice. Sludge is wasted from the system twice weekly to maintain the concentration of suspended solids in the aeration vessel at 2.5g l^{-1}.

A strategy which may be adapted when testing compounds for biodegradability is described in Figure 3. It consists of carrying out tests in a series of steps, starting with the relatively simple and inexpensive methods and progressing to the more complex and expensive ones. The steps are related to the different categories of biodegradability recognised by the OECD and to the amounts produced per year. Any of the screening tests are applied after testing for toxicity of the test compound. A positive result in any of the screening tests indicates the substance is readily biodegradable and need not be further examined.

Figure 3. Strategy for testing biodegradability of soluble chemicals (HMSO, 1983).



However, a negative result cannot be taken as evidence that the compound is not biodegradable, further tests should be made such as a test with a higher concentration of inoculum or with inoculum which has previously been acclimatized with the test compound. Another variant is to apply the Bunch-Chambers method to test for cometabolism. If these fail the Zahn-Wellens or SCAS methods should be applied.

For compounds found to be inherently but not readily biodegradable which may be discharged into sewers the next step is a simulation step such as the OECD confirmatory test. In cases of little or no biodegradation or a negative result, the simulation test could for example be repeated with a lower concentration of test compound over a longer time period.

1.4 Aims of the present study.

The project, to investigate the acclimatization of mixed cultures (activated sludge) to named test compounds, was initially proposed and funded by the Water Research Centre (WRc), Medmenham. The time taken for acclimatization to take place, the increase in sludge activity towards test compounds and whether de-acclimatization of the sludges takes place after exposure to the test compounds has been discontinued were specifically of interest to the WRc. Intermittent production and discharge to sewers of industrial

chemicals is a problem in that microbial populations are exposed to such chemicals at infrequent and irregular intervals, and populations may not get the opportunity to adapt. Part of the study was to determine whether activated sludge having been exposed to a test compound which it adapts to and degrades (acclimatization) retains the ability to degrade that compound after subsequently not being exposed to it for several months (deacclimatization).

Eight compounds which have given inconsistent results in individual biodegradability tests and/or have given conflicting data about their degradability in different tests were chosen for investigation, (see sections 1.5.1 to 1.5.8). The initial aim of the investigation was to examine the inherent biodegradability of these compounds using laboratory scale semi-continuous activated sludge units. The patterns of "acclimatization" and "deacclimatization" of the activated sludge initially over an extended period then increasingly intermittent periods of exposure to the test compound was to be studied. Acclimatization includes those processes, such as selection and adaptation by which a mixed population of microorganisms develops the ability to degrade a compound. It also covers the situation in which the populations develop tolerances to inhibitory substances (Painter and King, 1985).

It was intended to investigate the mechanism of degradation of those compounds found to be biodegradable e.g. single species utilization of a compound as the sole carbon source, cometabolism or mutualism. Its aim was also intended to isolate organisms capable of utilizing the test compounds as their sole carbon source.

It was hoped to investigate the degradative pathways of any microorganisms isolated with respect to specific compounds. In addition it was proposed to investigate removal of environmental levels of test chemicals by the use of ^{14}C radioisotopes with activated sludge, soil and river water providing the previously unexposed microbial populations. The reason for using ^{14}C is that the standard biodegradation tests are made with levels of test chemical many times higher than would normally be found in sewage or the aquatic environment. Thus they do not test the degradability of chemicals at environmentally realistic concentrations ($\mu g\ l^{-1}$) but at artificially high concentrations ($mg\ l^{-1}$). It has been proposed by Alexander (1985) that threshold limits may exist below which degradation of chemicals will not occur. The data obtained from the standard biodegradability tests may not be relevant to the environmental fate of chemicals. Using ^{14}C labelled chemicals the inherent biodegradability of test

chemicals likely to occur in the environment will be determined.

1.5 Test compounds to be investigated.

The following chemicals have been shown in degradation tests to exhibit aberrant behaviour and/or have given conflicting data about their degradability. It is this inconsistent behaviour which has made these chemicals of particular interest and therefore the subject of this study.

1.5.1 Benzene-1,3-disulphonic acid (BDSA)

BDSA has a molecular formula of $C_6H_4(SO_3Na)_2$ and a molecular weight of 282.20 and is easily soluble in water, BDSA (Figure 4) contains 25.51% carbon.

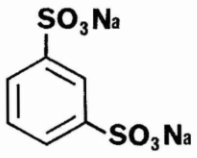


Figure 4. Structure of BDSA.

This compound was found not to be degraded in any screening test (Gerike and Fischer, 1979 and Painter et al., 1983) neither could it be degraded in the SCAS test after 12 weeks acclimatization (Painter et al., 1983). It has previously been reported to degrade at a very low rate (3.4 mg COD mg h^{-1}) with acclimatized inoculum (Pitter, 1976), while Gerike and Fischer (1981) observed it to degrade with acclimatized sludge, taken from a Husmann unit, in the Closed Bottle and Modified OECD tests. BDSA was not degraded in the 42 day AFNOR test using unacclimatized sludge, but after 4 weeks of "working in" (lag) in the Husmann test, 84-87% removal occurred (Gerike and Fischer, 1981).

1.5.2. t-Butanol.

t-Butanol, also called 2-methyl-2-propanol and trimethyl carbinol is a highly flammable compound with a melting point of 25°C, and is harmful by inhalation. It has a molecular formula of $(CH_3)_3COH$ and a molecular weight of 74.12; the molecular structure (Figure 5) has a carbon content of 64.76%.

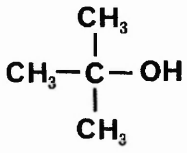


Figure 5. Structure of t-butanol.

t-Butanol is used as a solvent in paint removers, in flavours and perfumes and a denaturant for ethanol. The majority of workers reported it not be be readily biodegradable although in a ring test using a respirometric method it could be noted that 2 of the 11 participants reported a greater than 60% theoretical oxygen demand (King and Painter, 1985). t-Butanol was found not to degrade very much in activated sludge simulation tests (Gerike and Fischer, 1981 and King et al., 1984), but was found to be removed in the more lenient "square wave feed" version of the test (Gerike and Fischer, 1981). In the latter study it was found that acclimatized sludge failed to degrade t-butanol but Painter et al., (1983), found SCAS-acclimatized sludge did degrade t-butanol after 13 days.

Horn et al., (1970) reported that, after 8 weeks acclimatization in activated sludge units, 99% of the t-butanol available was removed and after making a mass balance only 1% was found to be lost by evaporation. King et al., (1985), in conditions similar to those in the Hussman method, reported that a solution containing 20mg C l^{-1} of t-butanol lost 8% of its DOC during a 3 hour period directly due to its volatilization. Romadina et al., (1984) isolated two Pseudomonas species which could degrade t-butanol and which continued to do so when added to activated sludge units.

1.5.3 2-Chlorobenzoic acid (2CBA).

2CBA is easily soluble in water after neutralization with sodium hydroxide and has a molecular formula of $C_6H_4ClCOOH$ and a molecular weight of 156.57. It is used as a preservative in glues and paints, and as an intermediate in the manufacture of fungicides and dyes. The molecular structure of 2CBA is shown in figure 6; the chemical contains 53.65% carbon.

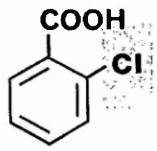


Figure 6. Structure of 2-CBA.

It is agreed that this compound is not readily biodegradable. Even with inocula acclimatized by the Sturm method, Gerike and Fischer (1981) found little or no degradation in the Closed Bottle and Modified OECD tests, but in one out of three occasions the Sturm CO_2 test was positive. It was observed by Haller (1978) that degradation of 16mg C l^{-1} occurred after a lag of 25 days in waste water at 30°C, but no degradation was found in the SCAS test after 7 weeks (Painter et al., 1983). Lund and Rodriguez (1984) also reported lack of

adaptation in a modified SCAS procedure over more than 30 days.

In a simulation test, 93% DOC was removed, surprisingly with no lag, using OECD synthetic sewage (Gerike and Fischer, 1979) but under the same conditions only 30% DOC was removed (King et al., 1984) although when domestic sewage was treated the value rose to 108% after a lag of 4 weeks.

It has been demonstrated by Shamat and Maier (1980) that 2CBA can be degraded by an enriched activated sludge in a continous flow reactor. The population of the enriched activated sludge was identified as predominantly Pseudomonas species. Cometabolism of 2CBA was demonstrated in continuous culture by Veerkamp et al., (1983). The organism responsible was found to be a Pseudomonas species. Cometabolism is the process or processes by which a normally non-biodegradable compound is biodegraded only in the presence of an additional source of carbon (Painter and King, 1985).

1.5.4 3-Chlorobenzoic acid (3CBA).

3CBA is easily soluble in water after neutralization with sodium hydroxide. It has the same molecular formula, molecular weight and carbon content as 2CBA, Figure 7.

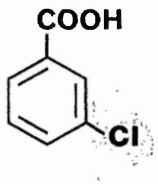


Figure 7. Structure of 3CBA.

Zahn and Wellens (1980) found this compound to be readily biodegradable, using river water as the source of inoculum. It was also found to be readily biodegradable by Painter et al., (1983) using the International Organisation for Standardization (ISO) die-away test. Gerike and Fischer (1979,1981) however, reported that it was not degraded unless an acclimatized inoculum (Sturm) was used. Lund and Rodriguez (1984) did not find 3CBA to be degraded unless the inoculum had previously been acclimatized for 30 days.

In a study by King et al., (1984), 3CBA was removed extensively in the simulation test both with domestic (lag 14 to 28 days) and synthetic (lag 7 days) sewages. Gerike and Fischer (1981) found only 30% DOC was removed with synthetic sewage although with the less stringent "square wave feed" mode 95% DOC was removed after a lag of 16 days. Square wave feeding is when the sludge is exposed to the test compared for a set period of time

after which it was withdrawn for a set amount of time. This cycle is repeated throughout the test.

Hughes (1965) found that washed cell suspensions of Pseudomonas fluorescens could utilize 3CBA as the sole carbon source after initially being grown on benzoate. Another 3CBA degrading organism, a Pseudomonas species, was isolated by Johnstone et al., (1972). In 1974, Dorn et al., isolated a 3CBA-degrading organism (by use of a chemostat) which was designated Pseudomonas B13. This organism has been studied by many workers since its original isolation particularly in genetic studies of degradation. Hartman et al., (1979) isolated a further organism capable of utilizing 3CBA, by use of a chemostat. The organism was identified as a Pseudomonas species and designated WR 912. Haller and Finn (1979) reported isolating 4 organisms capable of utilizing 3CBA. All the organisms mentioned above can utilize 3CBA as their sole carbon source.

Cometabolism of 3CBA has been demonstrated with benzoate as the co-substrate by an Arthrobacter species (Horvath and Alexander, 1970). Cometabolism by a Pseudomonas species was demonstrated by Veerkamp et al., (1983) both in batch and continuous culture with either glucose or benzoate as the co-substrate. In addition to metabolism of 3CBA by either a pure culture or cometabolism, degradation has been demonstrated by a mutualistic relationship between Alcaligenes eutrophus

and Pseudomonas B13 growing on benzoate plus 3CBA (Knackmuss, 1983).

1.5.5 cis, cis, cis, cis-1,2,3,4-
Cyclopentanetetracarboxylic acid (CPTCA).

CPTCA has a molecular formula of $C_5H_6(CO_2H)_4$ and a molecular weight of 246.17. The structure of CPTCA is given in Figure 8 and it contains 56.1% carbon.

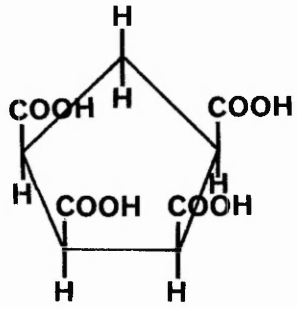


Figure 8. Structure of CPTCA.

CPTCA has been proposed as a phosphate substitute in detergent mixtures (ie a chelator of Ca^{++} to soften water). It was not degraded in any of the seven tests applied by Gerike and Fischer (1979) and neither was it removed in the SCAS or simulation tests performed by Painter (1986). CPTCA in an isomeric form (cis, trans, cis, trans) was reported by Gilbert and Lee (1980) to be degraded by about 85% after a lag of 8 weeks in the SCAS test. They also reported 0-30% removal in a ready test

depending on the stereoisomer used, but it was not stated which one was used. Gerike (1978) did not find any evidence of biodegradation during tests for both ready and inherent biodegradability.

1.5.6 Hexamethylenetetramine (Hexa).

Hexmethylenetetramine ($C_6H_{12}N_4$) has a symmetrical adamantane-like structure (Figure 9) and contains 51.4% carbon. Thermal decomposition occurs at 270°C but below this temperature the compound is fairly stable. Hexa decomposes readily under acid conditions to formaldehyde and ammonia but is stable in neutral aqueous solution (Tada 1960).

Hexa is used extensively in a variety of industries; it is used in adhesives, coating and sealing compounds, chemical detection of metals and in the preservation of hides. In addition it is also used as a cross-linking agent in the hardening of phenol-formaldehyde resin, vulcanizing rubber, as a filler in plastics, as a dye fixative, a stabilizer for lubricating and insulating oils and as a corrosion inhibitor for steel. Hexa is also used as solid fuel tablets for camping stoves and as the parent compound for high explosives such as cyclonite.

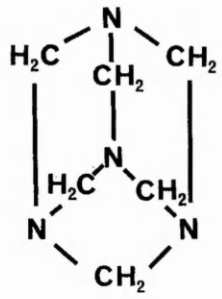


Figure 9. Structure of Hexa.

Hexa has been given an "E" number (E239) because of its use in the food industry for the marination of mackerel and herrings as a preservative and it is also used in Provolone cheese (Hannsen and Marsden, 1984). Hexa was first described by Butlerav in 1860 and used as a urinary antiseptic by Nicolour as early as 1894. The antimicrobial activity of hexa is derived from formaldehyde produced upon hydrolysis of hexa in the bladder. The degree of hydrolysis of hexa and therefore the effectiveness against infection is a direct function of the acidity of the urine (Strom and Won Jun, 1980).

Hexa is known not to be degraded readily in full-scale sewage treatment (Borne, 1976). Hexa was found, in a "ring" test to be degraded by greater than 60% ThOD by only 8 out of the 24 participating laboratories (King et al., 1985). However, in the Husmann simulation test, using either synthetic or domestic sewage, not more than

20-25% hexa (as DOC) was removed during the whole 9 weeks of the test (Painter and King, 1986).

1.5.7 N-Methylaniline (NMA).

NMA has a molecular formula of $C_6H_5NHCH_3$ and a molecular weight of 107.16 and we found it to be not easily soluble in water. The structure of NMA (Figure 10) is a secondary amine with a methyl group on the nitrogen atom and is a weak base, it is 78.58% carbon.

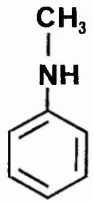


Figure 10. Structure of NMA.

NMA is an important starting material for the synthesis of certain dyes.

On five out of six occasions King and Painter (1985), found NMA was not degraded in ISO die-away tests, but on the sixth attempt greater than 90% DOC was removed and a purple colour formed. Using sludge acclimatized for 10 days or more by the SCAS procedure, consistently high removal values of NMA were achieved by ISO die-away tests. The same was observed when acclimatized effluent

was used as inoculum. These findings were in agreement with those of Gerike and Fischer (1979).

1.5.8 Tetrahydrofuran-2,3,4,5-tetracarboxylic acid (THFTCA).

THFTCA has a molecular formula of $C_8H_8O_9$ and a molecular weight of 248.14. The structure (Figure 11) contains 38.69% carbon and four carboxyl groups.

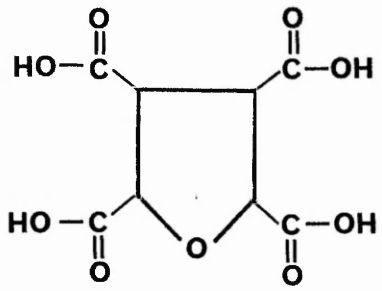


Figure 11. Structure of THFTCA.

THFTCA has, like CPTCA, been proposed as a phosphate substitute. THFTCA is also used to increase high temperature resistance of glass fibres and to reduce smoke production in polyisocyanate and polyurethane foams exposed to flames. THFTCA is also used in dental cements to aid in the setting of these cements. This compound was not degraded in the 28 day ISO die-away test with fresh activated sludge but was completely degraded with an 11-week acclimatized inoculum from a SCAS unit (Painter, 1986). THFTCA was also degraded in

a SCAS test. (Painter, 1986). However, in the simulation test, operated for 9 weeks, no degradation took place. There was evidence of inhibition of COD/TOC removal as there has been during the first 5 weeks of the SCAS test (Painter, 1986).

2.0 MATERIALS AND METHODS.

2.1 Test chemicals reagents and stock solutions.

t-Butanol, 2-chlorobenzoic acid, 3-chlorbenzoic acid, cis, cis, cis, cis-1,2,3,4-cyclopentanetetracarboxylic acid, hexamethylenetetramine, N-methylaniline and tetrahydrofuran-2,3,4,5-tetracarboxylic acid were obtained from, Aldrich Chemical Co. Ltd, Gillingham, Dorset, England, at the purest grade available. Benzene -1,3-disulphonic acid and phenylhydrazine hydrochloride were obtained from Fluka AG, Chemische Fabrik, CH-9470 Buchs, Switzerland, again at the purest grade available. The radioisotope [U-^{14}C]- hexamethylenetetramine at a specific activity of 12.9mCi mmol^{-1} (477 MBq mmol^{-1}) and a purity of 98% was obtained from Amersham International plc, Amersham Laboratories, White Lion Road, Amersham, Buckinghamshire, England. Carbo-sorb and Soluene-350 were obtained from Packard Instruments Ltd, Caversham, Berkshire.

Antibiotic-containing discs were obtained from Oxoid Ltd, Basingstoke, Hants, as were all the proprietary media and purified agar. The API 20E and API 50CH strips were obtained from ; API Laboratory Products Ltd, Grafton Way, Basingstoke, Hampshire. All other chemicals and reagents were of AnalaR grade or of the highest purity commercially available.

Stock solutions of test compounds for use in the SCAS and die-away tests were made by dissolving 400mg of test compound in 1 litre of CO_2-free distilled water. CO_2-free water was used to dissolve the test compounds so that the only carbon present was the test compound. The stock solutions were stored in sealed bottles for 2 to 4 weeks at 4°C except where stated. Both 2 and 3CBA were carefully neutralized with NaOH prior to being dissolved. The NMA stock solution was prepared by stirring 400mg NMA in 1 litre of distilled water at 50°C fo 2-3 hours. The stock solution of NMA was stored at room temperature to prevent separation into two phases.

2.2 Media.

2.2.1 Synthetic sewage.

The synthetic sewage for feeding the SCAS units contained the following components; peptone (16g), Lab Lemco (11g), urea CH_4N_2O(3g), NaCl (0.7g)$CaCl_2$ (0.4g), $MgSO_4.7H_2O$ (0.2g) and K_2HPO_4 (2.8g). A stock solution of synthetic sewage was made up x100 strength in 1 litre of tap water and could be stored for upto 7 days at 4°C.

2.2.2 Die-away test medium.

The basal test medium was made from the following stock solutions (a-f). Fresh test media was made immediately prior to setting up die away test.

a) Ca Cl_2 (27.5g), in 1 litre of CO_2-free distilled water.

b) Mg SO_4 $7H_2O$ (22.5g), in 1 litre of CO_2-free distilled water.

c) $FeCl_3.6H_2O$ (0.25g), in 1 litre of CO_2-free distilled water.

d) KH_2 PO_4 (8.5g), K_2HPO_4 (21.75g), $Na_2HPO_4.2H_2O$ (33.4g) and NH_4Cl (2.5g), in 1 litre of CO_2-free distilled water.

e) Solution of micronutrients

$MnSO_2 4H_2O$ (39.9mg), H_3BO_3 (57.2mg), $ZnSO_4 7H_2O$ (48.8mg); $(NH_4)_6$) Mo_7O_{24} (34.7mg) and $C_{10}H_{12}N_2O_8F_6Na$ (100mg), in 1 litre of CO_2-free distilled water.

f) Yeast extract (15mg) in 100ml, CO_2-free distilled water prepared immediately prior to use.

10ml of solution d) and 1.0ml of each of the other solutions were added in order, to distilled water with shaking between addition to a final volume of 1 litre.

2.2.3 Inhibition test medium.

The phosphate buffer contained KH_2PO_4 (8.5g), K_2HPO_4 (21.75g) $Na_2HPO_4.12H_2O$ (33.4g) in 1 litre of distilled water. The nutrient broth/sodium acetate solution contained nutrient broth (8g) and sodium acetate (6g) made up in 1 litre of distilled water. Test compounds were prepared by dissolving 1g of the test compound in 1

litre of distilled water. The stock reference compound was prepared by dissolving 1g of 3,5-dichlorophenol in 1 litre of distilled water.

2.2.4 Minimal salts medium.

The basal salts medium of Dorn et al., (1974) contained : $Na_2HPO_4.12H_2O$ (7g), KH_2PO_4 (1g), Ferric ammonium citrate (0.01g) $(NH_4)_2SO_4$(1g), Ca $(NO_3)_2$ (0.05g) and $MgSO_4.7H_2O$ (0.2g) per litre of distilled water. The trace element solution contained : $MnSO_4$-$4H_2O$ (400µg) $ZnSO_4$ (200µg), $CuSO_4$-$5H_2O$ (40µg), $CoCl_2$-$6H_2O$ (40µg) KI (300µg), Na $MoO_4.2H_2O$ (50µg) and NaCl (10mg) per litre of distilled water.

The medium was solidified, when necessary, by the addition of Oxoid Purified agar (15g l^{-1}) prior to sterilization at 121°C fo 15 minutes. The carbon sources were sterilized in the medium except where stated. t-Butanol was sterilized by filtration through 0.22µm pore size cellulose acetate filters (Oxoid Ltd, Basingstoke). All carbon sources were added to a final concentration of 0.5g l^{-1} unless otherwise stated, with the exception of THFTCA which was used at a concentration of 1.0g l^{-1}.

2.2.5 Chloride-free minimal salts medium.

The basal salts medium of Brammar and Clarke (1964) was used as the chloride-free minimal salts medium. The

medium contained. K_2HPO_4 (12.5g), KH_2PO_4 (3.8g), $(NH_4)_2SO_4$ (1.0g), $MgSO_4$ (0.1g) and trace element solution (5.0ml) per litre of distilled water. The trace element solution used was that of Kelly and Clarke (1962) and contained : HBO_4 (232.0mg), $ZnSO_4 7H_2O$ (174.0mg), $FeSO_4$ $(NH_4)_2SO_4.6H_2O$ (116.0mg), $CoSO_4.7H_2O$ (95.6mg), $(NH_4)_6Mo_7O_{24}.4H_2O$ (22.0mg), $CuSO_4.5H_2O$ (8.0mg) and $MnSO_4.4H_2O$ (8.0mg) per litre of distilled water. The carbon source (SCBA) was added after being neutralized with NaOH prior to sterilization at 121°C for 15 minutes. The pH of the medium was adjusted to pH 7.2 prior to sterilization.

2.3 Modified semi-continuous activated sludge (SCAS) test.

2.3.1 Principle.

The purpose of this test is to evaluate the potential ultimate biodegradability of water soluble, non-volatile organic substances when exposed to relatively high concentrations of microorganisms over a long time period. The viability of the microorganisms is maintained over this period by daily addition of synthetic sewage.

The conditions provided by the test are highly favourable to the selection and/or adaptation of microorgansisms capable of degrading the test compounds

(ie acclimatization). This procedure is also used to produce acclimatized inocula for use in the die away tests (2.4). In this test, the residual concentration of dissolved organic carbon is used to assess the ultimate biodegradability of the test substances.

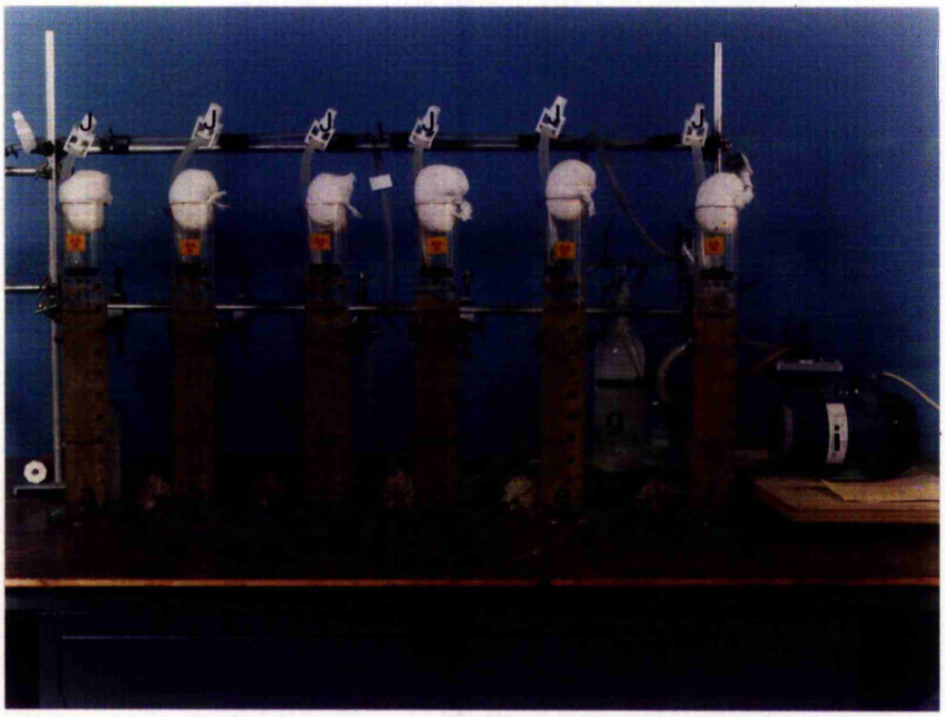
2.3.2 Test procedure.

SCAS tests units were constructed from 500ml measuring cylinders with a plastic aerator placed 2cm from the bottom of the unit. For each test compound three units were initially set up, a control unit, a test unit and a reference unit which was dosed with sodium benzoate, (a known biodegradable compound). Plate 1 shows the units in operation.

For each unit 450ml of activated sludge was obtained from Stoke Bardolph water recla mation works (Severn Trent Water Authority, Trent Division). The activated sludge was placed into the units and aerated at 0.5l air min^{-1} for 23 hours. The aeration was then stopped and the activated sludge allowed to settle for 45 minutes.

At the start of the settling time the walls of each unit were cleaned to prevent accumulation of solids above the level of the liquid. A separate brush was used for each unit to prevent cross contamination of activated sludge. After settling, 300ml of the

Plate 1. SCAS test apparatus



Key :

a : Control SCAS unit

b : Reference SCAS unit

c-f : Test SCAS units

g : Air humidifier

h : Air filter

i : Pump for aeration

j : Regulatory valves

supernatant liquor was removed and replaced with 300ml of synthetic sewage.

This procedure was repeated daily until a clear supernatant was obtained after the 45 minute settling period, (about 3 weeks). The settled sludges remaining after removal of the 300ml supernatant were then mixed, and 150ml of this composite sludge was added to each unit.

To the test unit was added 15ml of stock solution (containing 400mg carbon l^{-1}) and 285ml of synthetic sewage. The control unit received 300ml of synthetic sewage only. The reference unit received 15ml of stock (400mg carbon l^{-1}) sodium benzoate and 285ml of synthetic sewage. The units were operated at a temperature of 22.5°C±2.5°C on a 24 hour cycle. After 23 hours aeration the sludge was allowed to settle for 45 minutes. After settling 300ml of supernatant was drawn off from each unit and 1.25ml samples of each supernatant were taken. The supernatant was replaced in each unit as before, 300ml synthetic sewage to the control unit, 285ml synthetic sewage and 15ml test compound stock solution (400mg carbon l^{-1}) to the test unit with the reference unit receiving 15ml sodium benzoate (400mg carbon l^{-1}) and 285ml synthetic sewage. This fill and draw procedure was continued every 24 hours until the end of the test.

The 1.25ml samples of supernatant were clarified by centrifugation using a pre-cooled motor at 4°C in an MSE Micro-Centaur microfuge, 11600 g for 10 minutes. The resulting supernatants were acidified with 1M HCL (0.1ml) and shaken to remove CO_2 before analysis in triplicate of dissolved organic carbon (DOC) using a Beckman 915A Total Organic Carbon Analyser, the limit of detection for this equipment was approximately 1mg C l^{-1}.

2.3.3 Calculation of degradation in the SCAS unit.

Degradation is expressed as the ratio of test compound removed (degraded) to that added, relative to the control and described as degradation/daily addition. It is calculated as follows:

$$D = 100 \times \frac{C_T - (C_t - C_c)}{C_T} \quad \%$$

where D = degradation/daily addition.

C_T = concentration of test compound added to the synthetic sewage at the start of the aeration period (mg C l^{-1}).

C_t = concentration of dissolved organic carbon found in the supernatant liquor of the test unit at the end of the aeration period (mg C l^{-1}).

C_e = concentration of dissolved organic carbon found in the supernatant liquor of the control unit at the end of the aeration period (mg C l^{-1}).

2.3.4 Deacclimatization of activated sludge.

Deacclimatization was attempted by discontinuing the addition of test compound to the SCAS units after acclimatization of the activated sludge had been achieved. The degradative ability of the activated sludge during deacclimatization was tested by the use of die-away tests as described in section 2.4.2.

2.3.5 SCAS units to test volatility of t-butanol.

A unit similar to the SCAS unit described in section 2.3.2 was set up but without activated sludge. Distilled water containing 20mg C l^{-1} t-butanol replaced the activated sludge and was aerated as before. Samples were taken at intervals over a 48 hour period and the amount of dissolved organic carbon (DOC) measured (in triplicate) using a Beckman 915A Total Organic Carbon Analyser.

2.4 Die-away tests.

2.4.1 Principle.

The purpose of this test is to measure the biodegradability of water soluble organic compounds in an aerobic aqueous medium with a starting concentration of test compounds of 20mg C l^{-1}. Measurements of the dissolved organic carbon are made at the start of the test (day 0) and at 7 days intervals upto 28 days. The percentage removal of DOC at each time interval is calculated and from these data an evaluation of the biodegradability of the test compound is made.

2.4.2 Test Procedure.

Initially and at various times during the performance of the SCAS tests, die-away tests were performed with the activated sludge from the SCAS units as inoculum by the following procedure. Two 250ml conical flasks containing 95ml of the test medium (2.2.2) plus 5ml stock solution of test compound to give 20mg C l^{-1} of test compound (Fr) were set up together with a blank test flask (F_B) containing 100ml of test medium. A further flask was set up containing 95ml of the test medium plus 5ml to give 20mg C l^{-1} of the reference compound (sodium benzoate) to check the activity of the inoculum (F_C). The sodium benzoate was made up freshly as it is easily biodegradable. Abiotic control flasks

(F_s) containing 95ml of test medium 5ml of test compound stock solution to give 20mg C l^{-1} of test compound, sterilized by filtration through 0.22μm pore size cellulose acetate filters (Oxoid Ltd, Basingstoke) into sterile flasks were set up. These flasks were to demonstrate the nature of any degradation either physico-chemical or biological.

Flasks Fr, F_s and F_c were each inoculated with 1ml of activated sludge (30mg l^{-1} final concentration of dry matter) taken from the SCAS unit receiving the appropriate test compound. Flask F_s received 1ml of heat sterilized activated sludge. The flasks were closed with porous bungs and incubated at 22.5°C±2.5°C at 150 rpm in diffused light.

At the beginning of the test (day 0) 1ml samples were taken from flasks Fr, F_s and F_c and centrifuged in an MSE Micro-Centour microfuge, 11600g for 10 minutes, the rotor having previously been cooled to 4°C prior centrifugation. The resulting supernatants were acidified with 1M HCL (0.1ml) and shaken to remove CO_2 before analysis in triplicate for dissolved organic carbon (DOC) using a Beckman 915A Total Organic Carbon Analyser. The flasks were again sampled on days 7, 14, 21 and 28 (the end of the test). The flask Fs was only sampled after 28 days and analysed for DOC.

2.4.3 Calculation of degradation in the die-away tests.

The percentage elimination of dissolved organic carbon (D_t) for each flask was calculated using the following equation:

$$D_t \left(1 - \frac{Q_t - Q_{Bt}}{Q_o - Q_{Bo}} \right) X\ 100\%$$

where;

Q_o is the average concentration of dissolved organic carbon (mg C l^{-1}), at time 0, in each test flask F_T.

Q_{Bo} is the average concentration of dissolved organic carbon (mg C l^{-1}), at time 0, in the blank test flask F_B.

Q_t is the average concentration of dissolved organic carbon (mg C l^{-1}), at time t, in each test flask F_T.

Q_{Bt} is the average concentration of dissolved organic carbon (mg C l^{-1}), at time t, in the blank test flask F_B.

2.4.4 Additional die-away tests performed with t-butanol to assess loss by volatilization.

Test flasks were prepared in duplicate as previously described in section 2.4.2 and 1ml of fresh activated

sludge added. The flasks were incubated for 28 days at 22.5°C±2.5°C under the following conditions. Two flasks were closed with porous bungs and shaken (150 rpm) and two further flasks were sealed with silicone bungs (non porous) and shaken (150 rpm). Identical flasks with both types of bungs were prepared and were not shaken but kept stationary for 28 days. Similar flasks to those described were prepared but the inoculum used was sterilized activated sludge instead of fresh activated sludge. In addition further similar sterile flasks without activated sludge were prepared and incubated under the same conditions as the previous flasks. The amount of dissolved organic carbon (DOC) was measured only at 0 and 28 days.

2.4.5 Use of the hexa SCAS unit as a die-away test to compare results obtained in SCAS units and in die-away tests.

After operating the SCAS unit for 302 days no further synthetic sewage was added and no settlement periods were given. A single dose of hexa giving a final concentation of 20mg C l^{-1} was added to the SCAS unit. The amount of remaining hexa was determined as dissolved organic carbon (DOC) at intervals over the succeeding 28 days and the percentage carbon removal calculated.

2.5 Viability of microorganisms in activated sludge exposed to test compounds in SCAS tests.

At intervals, during the operation of the SCAS units which were being dosed with test compounds samples of suspended activated sludge were taken and homogenised before total viable counts were performed. Then 1ml of sodium tripolyphosphate (3mg ml^{-1}) was added to 9ml of suspended activated sludge taken from the aerated SCAS units and 1g of number 12 silica glass beads. The activated sludge was homogenised in a Mickle Disintegrator for 75 seconds at 30Hz. The resulting supernatant was serially diluted to 10^{-8} in sterile distilled water and 0.1ml aliquots from each dilution were spread over plate count agar (Oxoid) in triplicate. Incubation was performed at 30°C and colonies appearing after 72 hours were enumerated. The number of viable cells in the control SCAS unit were enumerated at the same time. The method for homogenisation of activated sludge was based on the method of Kluczewski (PhD Thesis, 1982).

2.6 Inhibition of activated sludge growth by test compounds.

The method used was that of Painter (1985) based on a previous report by Alsop et al., (1982).

2.6.1 Principle.

Sterile shake flasks containing buffer, nutrients and growth substrate were inoculated with an overnight culture of microorganisms (taken from activated sludge grown in a similar medium) and incubated for up to 6 hours. The growth rate of this culture was determined by periodic measurements of turbidity at a wavelength of 530nm. Sterile test compounds were added to the medium at a range of concentrations and their effect on the rate and amount of bacterial growth determined by measurement of optical density. The percentage inhibition of growth rate was calculated by comparison with the control rate. The sensitivity of activated sludge was tested with a suitable reference compound (eg 3,5-dichlorophenol).

2.6.2 Test procedure.

Approximately 16 hours prior to the commencement of the test, sterile shake flasks containing 26ml distilled water, 4ml of stock buffer solution and 10ml of nutrient broth/sodium acetate were inoculated with a range of volumes of activated sludge, 0.01ml, 0.1ml and 1ml. The flasks were incubated at 22°C±2°C and 150 rpm for 16 hours. After this time each flask was sampled and the optical densities measured at 530nm in a Philips PU8620 spectrophotometer 1cm pathlength.

Table 6 Experimental design for assessment of EC50 for one substance

Flask	Contents*	Water (ml)	Phosphate buffer (ml)	Nutrient broth/ sodium acetate solution (ml)	Inoculum (ml)	Test substance solution (ml)
1	Control	25	4	10	1	-
2,3	Test substance 1 mg/l	24,6	4	10	1	0,4+
4,5	" " 3,2 mg/l	23,7	4	10	1	1,3+
6,7	" " 10 mg/l	21	4	10	1	4+
8,9	" " 32 mg/l	23,7	4	10	1	1,3†
10,11	" " 100 mg/l	21	4	10	1	4+
12	Control	23	4	10	1	-
13,14	3,5-dichlorophenol 2,5 mg/l	24,9	4	10	1	0,1ø
15,16	" 10 mg/l	24,6	4	10	1	0,4ø
17,18	" 100 mg/l	21	4	10	1	4ø

* Total contents in each flask = 40 ml
+ Test Substance stock solution = 100 mg/l
† Test Substance stock solution = 1 g/l
ø Reference substance stock solution = 1 g/l

The flask having an optical density of approximately 0.4 was used as the source of inoculum.

The actual test for inhibition of growth by test compounds was set up according to the experimental design in Table 6. Flasks 1 and 12 were blank controls, flasks 2 to 11 were the test flasks covering a range of test compound concentrates, 1mg l^{-1} to 100mg l^{-1}. The reference compound 3,5-dichlorophenol was prepared over a range of 2.5mg l^{-1} to 100mg l^{-1} in flasks 13 to 18. All the flasks were inoculated with 1ml of precultured inoculum from the appropriate flask. At hourly intervals up to 6 hours, 1ml of sample was taken from each flask and the optical density measured as before at 530nm.

2.6.3 Calculation of inhibition of activated sludge growth by test compounds.

The $\log_{10}$ value for optical density was plotted against time, for each concentration of test substance and the appropriate inoculated controls.

The specific growth rate, μ (h^{-1}) for each concentration of test compound can be calculated from the slopes of the straight line portion of the curves (X 2.303) and percentage inhibition calculated from :

$$\frac{\mu_c - \mu_t}{\mu_c} \quad X\ 100$$

μ_c = specific growth rate of the inoculated control.

μ_t = specific growth rate of the culture containing the test substance.

If suitable data are available, the EC_{50} value may be determined; that is, the concentration of test compound which gives 50% inhibition of growth.

2.7 Isolation (from activated sludge) of microorganisms capable of degrading test compounds.

A sample of activated sludge (9ml) taken from one of the SCAS units was homogenised to break down the flocculent nature of the activated sludge as described in section 2.5. The homogenate was then inoculated into a conical flask containing 99ml minimal salts medium (Dorn et al., 1974) and 1ml of trace elements solution, plus the test compound (0.5g l^{-1}) with whch the SCAS unit sampled was being dosed. The flask was incubated at 30°C and 200 rpm for 21 days. At 7 day intervals the flask was subcultured into fresh liquid medium and observed over a 14 day period for any indication of growth. Any growth was streaked out on solidified medium and incubated at 30°C for up to 14 days to observe colony formation. Isolation for THFTCA-degrading microorganisms was attempted with the test compound present up to 1g l^{-1}. Isolation of hexa-degrading microorganisms was performed in baffled 250ml

conical flasks to prevent flocculation of microorganisms.

2.7.1 Maintenance of isolates.

Isolates were maintained on minimal agar slants containing the appropriate test compound (0.5g l^{-1}) plus 1.5% purified agar. The isolates were stored at 4°C and routinely subcultured onto fresh slants every 3 months. Isolate KCTI was maintained on slants containing 1g l^{-1} of THFTCA.

Long term storage of isolates was achieved by freeze-drying. Freshly grown cells were harvested from slants and freeze-dried in glass ampoules using Oxoid skimmed milk medium as the suspendent. The lyophilised cultures were stored at 4°C.

2.7.2 Characterization and identification of isolates.

2.7.2.1 API 20E test strips.

Duplicate API 20E strips were inoculated with washed nutrient-broth grown cells for each isolate and incubated at 35°C for 24 hours. The API 20E strips were then read after incubation and identifications of isolates made by use of the API 20E Analytical Profile Index, 2nd Edition (1985). Only isolates KCNM1, KC13, KC16, KC20 and KC21 were positively identified by this

method. The other isolates were identified as described in section 2.7.2.2.

2.7.2.2 Identification of isolates by The National Collections of Industrial and Marine Bacteria (NCIMB).

Those isolates which were not identified in section 2.7.2.1, namely KCT1, KCH1 and KCH2, were sent for first stage identification to the NCIMB Ltd, Torry Research Station, 135 Abbey Road, Aberdeen. AB9 8DG. The most important tests performed by NCIMB are those on cell and colonial morphology and the gram reaction.

2.7.2.3 API 50CH test strips.

Duplicate API 50CH test strips containing 49 different carbohydrates were inoculated with washed nutrient broth grown cells for each isolate and incubated at 30°C for 72 hours. After incubation the results for carbohydrate utilization for each isolate were recorded.

2.7.2.4 Growth inhibition by antibiotics.

In order to provide another means of characterising isolates, resistance to antibiotics was investigated. A washed suspension (0.5ml) containing approximately 10^{8} nutrient broth-grown cells was spread and dried on to the surface of nutrient agar plates, on to which were

placed antibiotic-containing discs. The seeded plates were incubated at 30°C for 72 hours and the organism termed resistant if no zone of inhibition was seen. The isolate was tested against ampicillin (25μg), bacitracin (10iu), carbenicillin (100μg), chloromphenicol (10μg), erythromycin (30μg), gentamycin (30μg) kanomycin (30μg), mecillinam (25μg) nalodixic acid (30μg), neomycin (30μg), nitrofurantoin (300μg), penicillin (10iu), streptomycin (25μg), sulphamethoxazole/trimethroprim (25μg), sulphonamide (300μg) and tetracycline (10μg).

2.7.2.5 Shake flask cultures for the determination of growth characteristics.

In order to determine the characteristics for growth of isolates at various temperatures, the isolates were grown in 250ml conical flasks containing 100ml of minimal medium plus-test compound as described in section 2.7. Triplicate flasks were inoculated with washed cells to an optical density of approximately 0.1 at 530nm. After inoculation the flasks were incubated at 25, 30 and 35°C on an orbital shaker at 200rpm in diffused light. Growth was determined by measuring the optical density at 530nm of aseptically removed samples using a Philips PU 8620 spectrophotometer and a 1cm path length cuvette. The pH of the flasks incubated at 30°C was monitored during growth experiments.

2.7.2.6 Plasmid detection in isolates.

Cells of isolates taken from a single colony were resuspended in 50µl buffer E (40mM Tris-acetate and 2mM sodium EDTA, adjusted to pH 7.9 with glacial acetic acid). The cells were lysed by the addition of 100µl of lysing solutions (3% sodium dodecylsulphate SDS and 50mM Tris, pH 12.6), followed by incubation at 55°C for 60 minutes. After incubation 300µl of phenol:chlorform:amyl alcohol (25:24:1v/v) was added to the lysate. The lysate was mixed to obtain an emulsion and centrifuged in a microfuge (11600g for 5 minutes). The supernatant which would contain any plasmid DNA was carefully removed.

To perform agarose gel electrophoresis 20µl of supernatant was mixed with 5µl loading buffer (20% sucrose, 10% ficoll, 10mM EDTA and 1% bromophenol blue) and loaded into a well of a 0.8% agarose gel 5mm thick. Electrophoresis was carried out at 60v using a Bio-Rad Mini sub DNA cell in gel running buffer (89mM Tris, 89mM boric acid and 2.5mM EDTA) for about 2½ hours. The gel was stained with 500µg l^{-1} of ethidium bromide for 30 minutes at 20°C and plasmid observation made using a shortwave U.V. light source.

2.8 Further studies with 3CBA-utilizing isolates.

2.8.1 Growth, dehalogenation and ring cleavage.

The isolates were grown in the basal salts of Brammar and Clarke (1964) plus 0.5g l^{-1} of 3CBA, (see section 2.2.6).

In order to determine the sequence of ring cleavage and chloride-ion release during growth, the cultures KC13, KC16, KC20 and KC21 were grown in Brammar and Clarke's chloride-free salts in 250ml conical flasks containing 100ml medium. The medium was inoculated with a washed cell suspension to give an optical density of approximately 0.1 at 530nm. The cultures were incubated in triplicate at 30°C and 200 rpm. Growth was determined by measuring the optical density at 530nm of an aseptically removed sample (1ml) using a Philips PU 8620 spectrophotometer and a 1cm pathlength cuvette. The sample was then centrifuged at 11600g for 10minutes in an MSE Microfuge. Ring cleavage was determined by observing a decrease in the absorbance of the supernatant at 275nm over a period of time.

Free ionic chloride was estimated using a Corning EEL Chloride Analyser (Model 925) in conjunction with an acid buffer and thymol blue gelatin indicator. The acid buffer contained 100ml glacial acetic acid, 8ml nitric acid and 882ml distilled water. The thymol blue gelatin

indicator contained : 600mg white powdered gelatin and 10mg thymol blue pH indicator per 100ml distilled water.

Acid buffer and thymol blue gelatin indicator were mixed immediately prior to use in a 30:1 ratio. The mixture (15ml) was dispensed into a small beaker and the electrodes of the chloride meter immersed in the liquid. The calibration of the instrument was checked prior to use by titration of a 100mM sodium halide standard. Samples (0.02-0.2ml) were discharged into the acid buffer mixture and the halide titrated automatically. Triplicate readings were obtained for each sample.

2.8.1.1 The effect of 3CBA concentration on dehalogenation.

Each isolate was grown as described in section 2.7.2.5 at 30°C with flasks containing 3CBA at 0.5, 0.75 and 1.0g l^{-1} in Brammar and Clarke's minimal medium. The release of halide by each isolate at the different concentrations of 3CBA was measured in triplicate against time as described in section 2.8.1.

2.8.2 Growth on other compounds as the sole carbon source by 3CBA-utilizing isolates.

To test for growth on other carbon compounds, isolates were spread on to plates containing minimal salts medium of Dorn et al., (1974) containing 0.5g l^{-1} respective carbon substrate solidified with 1.5%

purifie d agar. The carbon sources were 2,3-and 4CBA, acetaldehyde, acetate, chloroacetaldehyde, chloroacetate, chloropropionate, chlorosuccinate fumarate, gentisate, glucose and pyruvate. All the carbon sources were prepared as sterile stock solutions, 2,3, and 4CBA being sterilized at 121°C for 15 minutes. All other carbon sources were sterilized by filtration through 0.22μm pore size cellulose acetate filters (Oxoid Ltd, Basingstoke) and added to cooled, sterilized minimal medium.

Washed cells of each isolate were streak inoculated onto plates containing each carbon source in triplicate. The plates were incubated at 30°C for up to 7 days and growth compared to plates containing no carbon source.

2.8.3 Gilson respirometry with isolates KC13, KC16, KC20 and KC21.

2.8.3.1 Shake flask cultures for preparation of cell pastes.

Isolates were grown as described in section 2.7.2.5, 10ml amounts of the washed suspensions were inoculated into 500ml of minimal media containing 0.5g l^{-1} 3CBA in 1 litre conical flasks. The cultures were incubated at 30°C on an orbital shaker at 200rpm and harvested during exponential growth. Harvesting of the cells was carried out by centrifugation at 9500g for 10 minutes at 4°C.

The pelleted cells were resuspended and washed twice in 0.1M phosphate buffer, pH 7.2, after which the cells were suspended in a small volume of phosphate buffer, pH 7.2, 4°C.

2.8.3.2 Respirometry.

In order to determine the pathway by which 3CBA is degraded by KC13, KC16, KC20 and KC21 the following compounds were examined by Gilson respirometry:- acetaldehyde (10μM), acetate (10μM), β-ketoadipate (10μM), 2CBA (1μM), 3CBA (1μM), 4CBA (1μM), fumarate (10μM), gentisate (1μM), pyruvate (10μM) and sodium benzoate (1μM). The components of the system consisted of : i) a centre well containing 0.2ml 10% potassium hydroxide and folded filter paper to absorb any carbon dioxide evolved. ii) A side arm containing 0.5ml substrate which would give a final concentration the same as indicated above in brackets. iii) A main compartment containing 2.0ml, 0.1M phosphate buffer pH 7.2 and 0.5ml cell suspension to give a total volume of 3ml. Flasks were set up in triplicate and equilibrated before the start of the test. Oxygen uptake was measured after the addition of the substrate every 5-10 minutes for up to 6 hours.

The dry weight of each cell suspension was determined using pre-weighed metal planchets and a vacuum oven used to dry the samples.

2.9 Further studies with KCH1 and KCH2.

2.9.1 Growth with other compounds as the sole carbon source by hexa-utilizing isolates.

Growth on other carbon sources was investigated with the carbon sources present at 0.5g l^{-1} and incubated at 30°C and 200rpm in baffled 250ml conical flasks containing 100ml minimal media (see section 2.2.4). Hexa was investigated both as the sole carbon source and sole carbon and nitrogen source. The cells were grown in the minimal medium of Dorn et al., (1974), without ferric ammonium citrate (the nitrogen source) plus 0.5g l^{-1} hexa. Washed cells were inoculated to an optical density of approximately 0.1 at 530nm, incubated at 30°C and 200rpm in baffled 250ml conical flasks. The flasks set up in triplicate were sampled at intervals, the optical density was determined at 530nm and the culture fluid analysed for ammonia using Nesslers reagent (BDH). The 1ml sample was centrifuged at 11600g for 10 minutes in an MSE microfuge, the supernatant was added to a test tube containing 0.5ml Nesslers reagent together with 10ml of distilled water. The test tube was allowed to stand at room temperature for 15 minutes then the absorbance read at 410nm in a Philips PU 8620 spectrophotometer, against a reagent blank. Calibration was made against ammonium sulphate.

Sodium formate (0.5g l^{-1}) and methanol (0.5g l^{-1}) were investigated as described above. Formaldehyde (0.5g l^{-1}) utilization was investigated by inoculating a number of identical flasks with the isolates and sealing them with silicone bungs. At time intervals triplicate flasks were sacrificed and the optical densities were measured at 530nm.

2.9.1.1 Gilson respirometry with isolates KCH1 and KCH2.

Respirometry was performed as described in sections 2.8.3. to 2.8.3.2 with some exceptions. The cells were grown in baffled flasks and the compounds under test were hexa (2μM) formaldehyde (10μM) methanol (15μM) and sodium formate (10μM).

2.9.2 Removal of hexa by isolates KCH1 and KCH2.

A modified Riker method (Jackson and Stamey, 1971) was used to determine the amount of the formaldehyde present in samples taken during growth of these isolates after hydrolysis. The Riker method is based on a time sensitive reaction of formaldehyde with phenylhydrazine hydrochloride to form phenylhydrazone, which develops a characteristic red colour in the presence of potassium ferricyanide that can be quantified colorimetrically. Shake flasks were prepared as described in section 2.7.2.5 but hexa was presented at 0.1g l^{-1}, and

incubation was at 30°C and 200rpm. At intervals 1ml samples were taken from each flask and the optical density measured at 530nm in a Philips PU 8620 spectrophotometer with a 1cm path length cuvette. The samples were then centrifuged at 11600g for 10 minutes in an MSE Microfuge and the supernatants transferred to fresh microfuge tubes, acidified with 10µl 5N HCl and incubated at 30°C for 1 hour to allow hydrolysis of hexa to be completed. Detection of formaldehyde is described in section 2.9.2.1. In addition to the determination of formaldehyde and optical density the pH of each sample flask was aseptically measured at the time of sampling.

2.9.2.1 Determination of formaldehyde by a modified Riker method after hydrolysis of hexa.

Phenylhydrazine hydrochloride (Fluka) 0.1% (w/v) in 50% isopropanol was prepared fresh daily by suspending 500mg of phenylhydrazine hydrochloride in 500ml of 50% isopropanol and stirring in the dark for 30 minutes. The resulting solution was filtered through Whatman No 2 filter paper.

Potassium ferricyanide was prepared freshly, 5% (w/v) in distilled water and sodium hydroxide 10% (w/v) in distilled water. The reagents were stored in dark glass bottles in the dark when not being used in the assay.

Formaldehyde standard solution 1 to 15µg ml^{-1} were prepared immediately prior to each determination.

Into separate 25ml volumetric flasks were placed 1ml of each standard or 1ml of each sample, and the samples were diluted 1 in 10 times as necessary. To each 25ml volumetric flask, 9ml of distilled water was added and a control blank containing 10ml of distilled water was also set up. To each flask was added 10ml of phenylhydrazine hydrochloride reagent, the flasks were mixed, stoppered and allowed to stand for 5 minutes. Then 1ml of potassium ferricyanide reagent was added to each flask; the flasks were shaken to mix the contents, restoppered and allowed to stand for 5 minutes. Each flask was diluted to volume (25ml) with 10% sodium hydroxide, the flasks were well mixed, restoppered and allowed to stand for 20 minutes. After this time the absorbance at 520nm was determined for each flask against the reagent blank control, in a Philips PH 8620 spectrophotometer with a 1cm path length cuvette.
Note:- Early samples were diluted 1 in 10 due to the high level of formaldehyde; when the level fell below 15$\mu g\ ml^{-1}$ no dilution was made.

2.9.3 Determination of growth using [U-^{14}C] hexa.

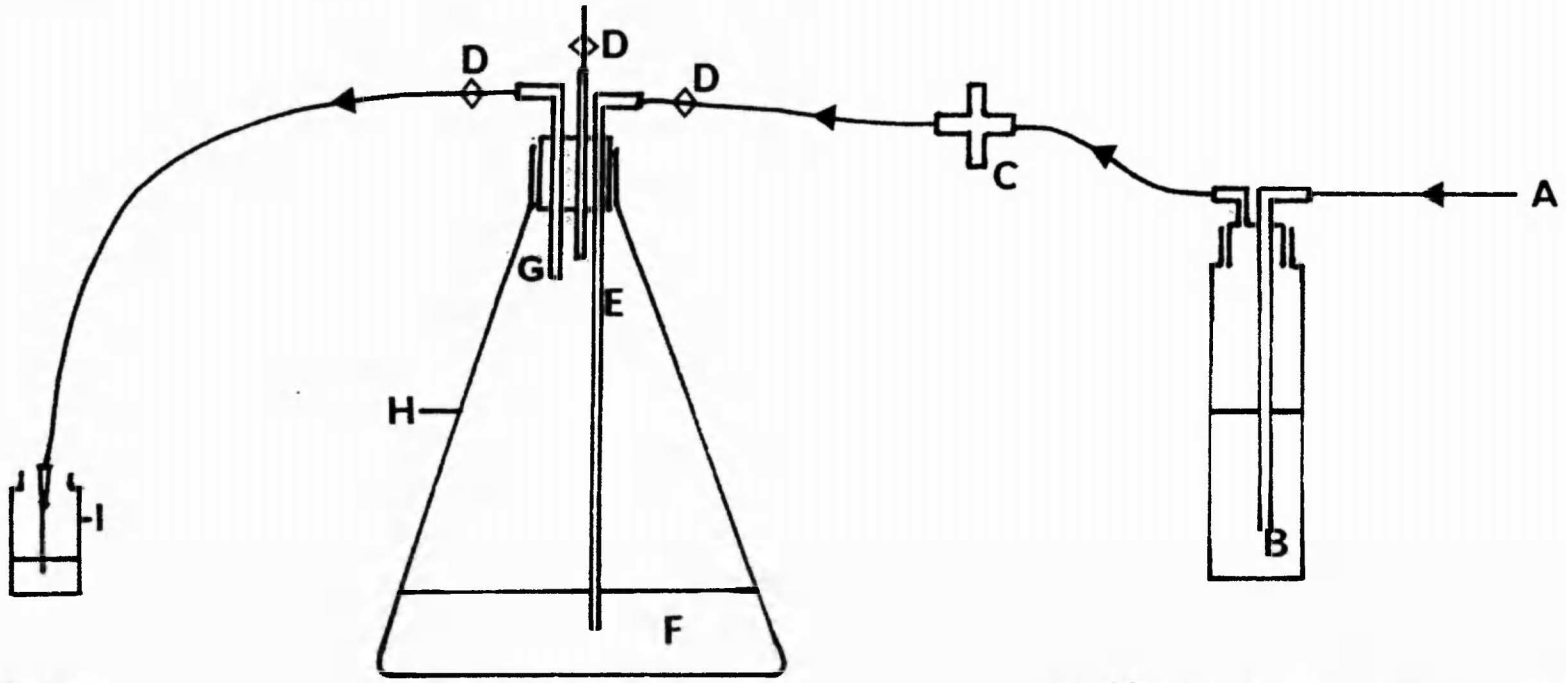
Growth curves of KCH1 and KCH2 were prepared as described previously in section 2.7.2.5, at 30°C and 200 rpm; the inoculum was 10ml of exponentially growing isolate. The flasks for this experiment were 1 litre,

sealed baffled conical flasks (Figure 12), containing 500ml medium (Dorn et al., (1974)) and 0.5g l^{-1} hexa (unlabelled). In addition 0.5ml of stock [U-^{14}C]-hexa was added to each flask. The stock [U-^{14}C]-hexa contained 1mCi (37MBq) in 20ml phosphate buffer (pH 7.2) which was kept frozen at -20°C when not being used.

At intervals the flasks were flushed with sterile CO_2-free air for 2 minutes at 500ml $minute^{-1}$ to displace any CO_2 evolved by the isolates growing on hexa in the flasks. The gas was passed through 1ml of Carbo-sorb (Packard) in a 20ml scintillation vial to collect any $^{14}CO_2$ present in the exhaust gases and the scintillation vial was then sealed. To determine the efficiency of $^{14}CO_2$ collection radiolabelled Na_2 $^{14}CO_3$, 10µl (1mCi ml^{-1}), was added to a sealed conical flask containing 50ml, of 0.1m buffer (pH 4.0) and incubated at 30°C for 60 minutes. After this time $^{14}CO_2$ was collected in 1ml of Carbo-sorb as previously described. This experiment for $^{14}CO_2$ efficiency was performed in triplicate.

After collection of exhaust gases 1ml samples of the culture flasks were aseptically taken and centrifuged at 11600g for 10 minutes in an MSE microfuge. The supernatant was carefully drawn off using a Finn pipette with a fine tip and placed in a 20ml scintillation vial. The cell pellet was resuspended in 200µl of 0.1M phosphate buffer (pH 7.2) and repelleted as before, the

Figure 12. Apparatus to study (U-^{14}C) hexa utilization by isolates KCH1 and KCH2.



A. Air in.
B. KOH to remove CO_2
C. Sterile air filter
D. Closures
E. Air into flask
F. Culture
G. Air out of flask
H. Conical flask
I. $^{14}CO_2$ Collection vial containing Carbo-sorb

supernatant was drawn off as before and added to the original supernatant. The cell pellet was digested by Soluene-350 tissue solubilizer (Packard) for 6 hours at 50°C. To each scintillation vial containing $^{14}CO_2$, supernatant and solubilized cells, was added 10ml of Optiphase 'safe' (LKB) and the samples mixed. The vials were stored for between 48 and 72 hours at 4°C to allow chemiluminescence to subside prior to counting. After this time the samples were again mixed and then counted in a Packard Tri-Carb 300C liquid scintillation counter. All data were corrected for quench by the external channels ratio method and expressed as disintegrations per minute (DPM).

2.9.4. Removal of low levels of hexa.

2.9.4.1 Removal of low levels of hexa by activated sludge.

Previously unexposed fresh activated sludge (500ml, pH 7.2) was placed in duplicate sealed fermentation units (Figure 13). The activated sludge was prefiltered through glass wool to remove large particulate matter prior to the addition of hexa. To each unit was added 0.5ml of [U-^{14}C] stock hexa. The units were magnetically stirred at 30°C, both units were covered in aluminium foil to prevent utilization of $^{14}CO_2$ by photosynthetic organisms present in the activated

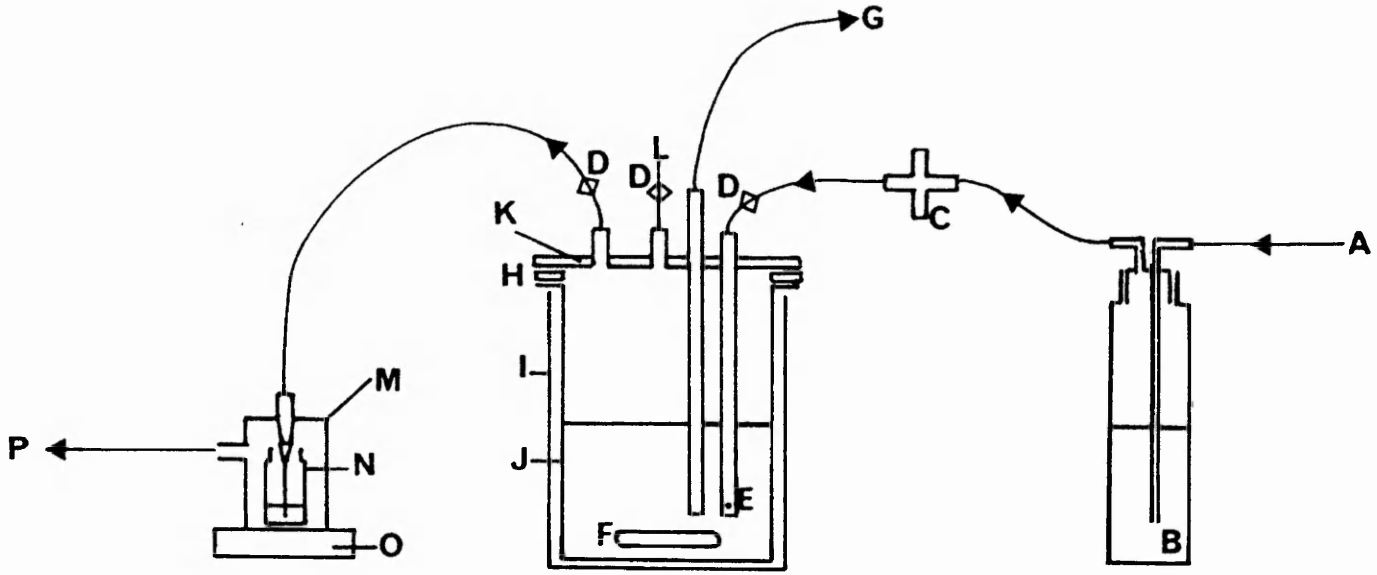
sludge. At intervals the units were sampled for $^{14}CO_2$ by passing sterile CO_2-free air through the units (Figure 13) and collecting any CO_2 in Carbo-sorb as described in section 2.9.3. The activated sludge was also sampled and processed as described in section 2.9.3. for culture fluid during the isotope dilution experiments.

Adsorption of ^{14}C to cell mass was investigated by adding 5µl [U-^{14}C] hexa to 100ml of sterilized activated sludge in duplicate sealed flasks which were incubated for 7 days at 30°C. The activity of the cell mass was determined as described in section 2.9.3. Adsorption was determined as the activity found in the digested cell debris. In addition the pH values of the units were monitored.

Two similar units were prepared as above but only 50µl of [U-^{14}C] hexa stock solution was added to each duplicate unit. The units were sampled as described above. The efficiency of $^{14}CO_2$ was determined as previously described in section 2.9.3. with a collection time of 15 minutes.

The number of viable microorganisms ml^{-1} of activated sludge at time zero was determined as previously described in section 2.5.

Figure 13.



Apparatus to study the behaviour of low levels of [U- ^{14}C] hexa with activated sludge,soil and river water.

Figure 13 Apparatus to study the behaviour of low levels of [U-^{14}C] hexa with activated sludge, soil and river water.

A. Air in.

B. KOH to remove CO_2.

C. Air filter.

D. Closures.

E. Sparger.

F. Magnetic stirrer.

G. Cooling system.

H. Seal.

I. Aluminium foil jacket.

J. Reaction vessel.

K. Head plate.

L. Sample port.

M. $^{14}CO_2$ Collected system.

N. Scintillation vial containing Carbo-Sorb.

O. Plasticine seal.

P. Vent to outside.

2.9.4.2 Removal of low levels of hexa by soil.

In two duplicate units, similar to those used in section 2.9.4.1 was added 500ml of 0.1M phosphate buffer (pH 7.5) containing 10g l^{-1} loam type soil pH 7.5, prefiltered through glass wool prior to the addition of hexa to remove large particulate matter. To each unit was added 0.5ml of [U-^{14}C] stock hexa. The units were magnetically stirred at 30°C and sampled as described in section 2.9.4.1, in addition, the pH values of the units were monitored at intervals.

Two similar units were prepared as above but only 50µl of [U-^{14}C] hexa stock solution was added to each duplicate unit. The units were sampled as described above. The level of adsorption of [U-^{14}C] hexa to soil cell mass was measured as described in section 2.9.4.1.

The number of viable microorganisms per gram of soil was determined by suspending 10g soil in 1 litre of pH 7.5 phosphate buffer (0.1M) followed by filtration through glass fibre to remove humus and then plating serial dilations of the filtrate on to plate count agar (Oxoid).

2.9.4.3 Removal of low levels of hexa by river water.

To two duplicate units, similar to those used in section 2.9.4.1 was added 500ml of river water pH 7.6 (River Trent) prefiltered through glass wool prior to the addition of hexa to remove large particulate matter.

To each unit was added 0.5ml of [U-^{14}C] stock hexa. The units were magnetically stirred at 30°C and sampled as described in section 2.9.4.1; in addition the pH values of the units were monitored at intervals. The level of adsorption of [U-^{14}C] hexa to river water cell mass was measured as described in section 2.9.4.1.

The number of viable microorganisms ml^{-1} of river water was determined by plating onto plate count agar (Oxoid).

2.9.5 Acid Hydrolysis of hexa.

Duplicate flasks containing 0.1M phosphate buffer and 0.1g l^{-1} hexa at pH values 5.6, 6.3, 6.8, 7.2 and 8 were prepared. Samples were taken from each flask at time 0 and after 6 hours incubation. The amount of the formaldehyde for each sample was determined as described in section 2.9.2.1.

2.9.6 Cell-free extract preparation.

2.9.6.1 Growth of isolates KCH1 and KCH2

Cultures were routinely grown in 250ml conical flasks containing 100ml of medium, Dorn et al., (1974) and 0.5g l^{-1} of hexa. When larger volumes were required cells were grown in 1 litre baffled conical flasks containing 500ml of medium. The isolates were grown as described in section (2.7.2.5) then 10ml of the washed inoculum

cultures were inoculated into 500ml of minimal media containing 0.5g l^{-1} hexa in baffled conical flasks. The cultures were incubated at 30°C on an orbital shaker at 200rpm and harvested during exponential growth. Harvesting of the cells was carried out by centrifugation at 4°C and 9500g for 10 minutes. The pelleted cells were resuspended and washed twice in 0.1M phosphate buffer, pH 7.2, and repelleted.

2.9.6.2 Preparation of cell-free extracts.

All subsequent operations were carried out at 4°C. Cells were washed once in 1/5th volume of 0.1M phosphate buffer, pH 7.2, centrifuged as above and resuspended in 1/25th volume for the same buffer. Cells were broken by sonication for 90 seconds (6 x15 seconds with equal periods of cooling) using an MSE 100W disintegrator. Unbroken cells and cell debris were removed from the sonicate by centrifugation at 38000g for 30 min. The resultant supernatant was stored at 4°C until required.

2.9.6.3 Cell-free extract activity.

Cell-free extract activity was investigated by Gilson respirometry (see section 2.3.3.2) with the cell paste suspension being replaced by 0.5ml cell-free extract. Cell-free extract was investigated for oxygen uptake with hexa (2μM) and formaldehyde (10μM). The amount of protein was determined by Lowry assay (section 2.9.6.4).

2.9.6.4 Protein determination.

Protein was estimated in cell-free extracts by the method of Lowry et al., (1951) according to Schleif and Wensink (1981).

To samples (0.5ml) containing up to 200µg protein was added 4ml of reagent A, containing : 3% (w/v), Na_2CO_3 in 0.1M NaOH plus 2ml of 2% (w/v) $CuSO_4.5H_2O$ and 2ml of NaK tartrate (4% w/v) per 100ml; added immediately prior to use. Mixtures were incubated at room temperature for 10 minutes. Following incubation, 0.5ml of Folin-Ciocalteau reagent (reagent B) (50% v/v) was added and each sample immediately mixed by vortexing for 10 seconds. After a further 30 min incubation at room temperature, the absorbance of each sample was measured at 650nm using a Philips PU8620 spectrophotometer against a distilled water blank, containing no protein. The protein content was determined by relating the corrected absorbance values to a standard curve using bovine serum albumin as the reference protein.

2.9.7 Vertical polyacrylamide gel electrophoresis (PAGE) of cell-free extracts.

2.9.7.1 Preparation of polyacrylamide gels.

Gels (10%) were prepared in the following way 30% Acrylamide (10ml), 1% methylene bisacrylamide (1.73ml); Tris-HCl buffer (1M) pH 8.8 (11.2ml) and distilled water

(6.87ml) were mixed. Immediately prior to pouring, the following were added in order, 10% Sodium dodecyl sulphate SDS (300µl) ammonium persulphate solution 100mg ml^{-1} (100µl) and Temed (10µl). The gels were poured, topped with n-butanol and left overnight to set. On the day of running the gels, the stacking gel (5%) was prepared as follows : 30% Acrylamide (1.67ml), 1% methylene bisacrylamide (1.5ml), 1M Tris-HCl buffer, pH 6.8 (1.75ml) and distilled water (5.6ml) were mixed. Immediately prior to pouring the stacking gel, the following were added in order, 10% SDS (100µl), ammonium persulphate 100mg ml^{-1} (50µl) and Temed (5µl).

2.9.7.2 Loading and running vertical PAGE gels.

The water layer was removed immediately prior to electrophoresis. The slab gel was loaded into a Hoefer SE400 vertical electrophoresis system. Samples (20µl) containing approximately 100µg cell-free extract, were applied to the well of the gel and overlaid with 10µl of sample buffer glycerol (10%) and bromphenol blue (0.2%). The reservoir was filled with running buffer, glycine (28.8g l^{-1}), Tris (6g l^{-1} and 10% SDS (10ml l^{-1}) at pH8.5. The electrophoresis was performed at 4°C at a constant voltage of 150v (Pharmacia power supply, EPS 500/400) until the bromophenol blue marker was 5mm from the bottom of the gel.

2.9.7.3 Staining PAGE gels for protein.

The gel was stained for 2 hours in stain, 5% methanol, 7% acetic acid and 0.1% Coomassie blue in distilled water. After staining, the gel was fixed in a solution of methanol (500ml) acetic acid (100ml^{-1}) and distilled water (500ml) for 1 hour. The gel was then destained with changes of destain overnight. The destain was the same as the stain but without Coomassie blue.

3.0 RESULTS.

3.1 Degradation of cis, cis, cis, cis-1,2,3, 4-cyclopentanetetracarboxylic acid (CPTCA).

3.1.1. Removal of CPTCA in the SCAS test.

CPTCA was added to a SCAS unit at the rate of 20mg l^{-1} (as total organic carbon) per day. The amount of CPTCA remaining in the unit after 23 hours aeration was determined as dissolved organic carbon (DOC). The change in DOC in both the test unit (with CPTCA) and the control (without CPTCA) with time is shown (Figure 14a). If the two curves approach close to each other then degradation may well be occurring, but apart from one probably spurious result at day 94 no such curve proximity is evident. Degradation is also expressed as percentage degradation/daily addition. Calculated values of this parameter plotted against time are shown (Figure 14b). Degradation of the test compound can be postulated if consistent positive values are observed. Over the first 84 days the values are low or negative. After day 85 there was a rapid rise to a peak of 90% on day 94. This was however followed by an equally rapid fall to near 15% on day 105. Examination of the DOC curves shows that this corresponds to the abnormally low result in the test unit. The data show no evidence

Figure 14a. Removal of CPTCA in the SCAS test.

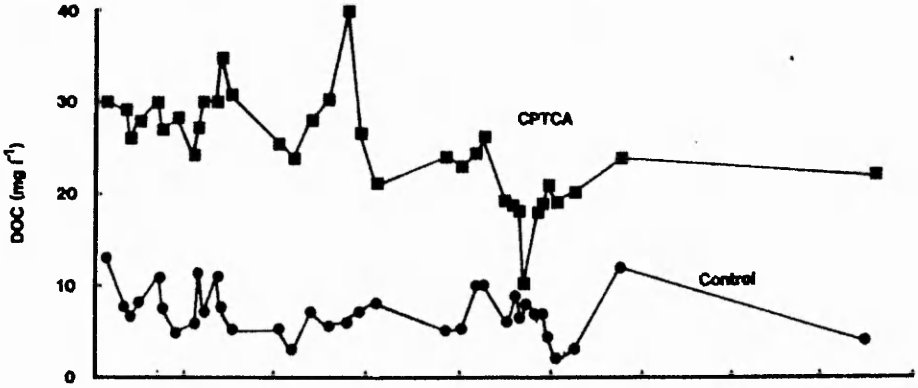
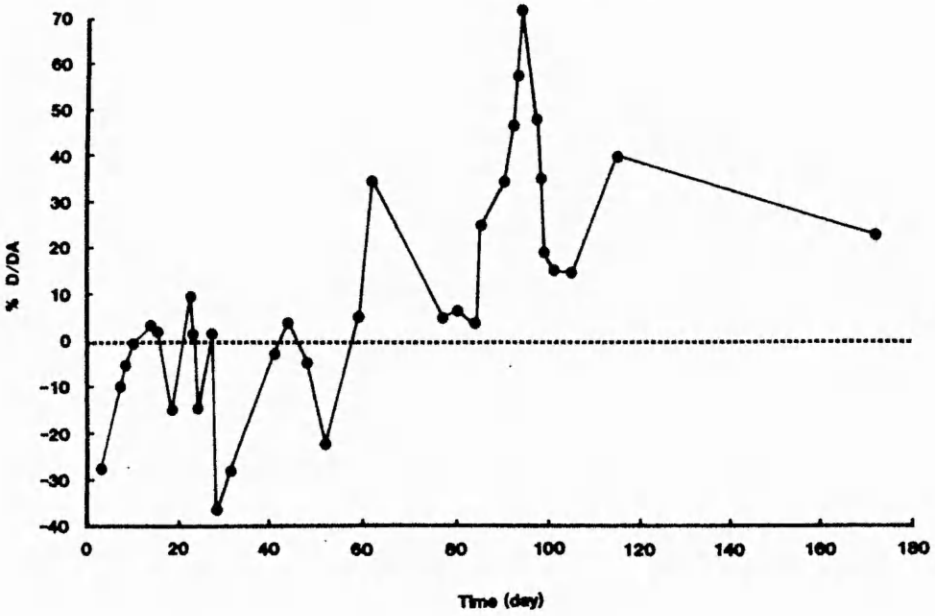


Figure 14b. Removal of DOC on a daily basis.



that this compound was being degraded in the SCAS unit even after 175 days of the test.

3.1.2 Removal of CPTCA in die-away tests.

Die-away tests were set up using inocula taken from the SCAS unit dosed with CPTCA at intervals up to 170 days. Degradation in the die-away tests was estimated from measurements of DOC at 7 day intervals up to 28 days.

Table 7 gives the summary of the die-away test results. Both positive and negative values are possible because of the method of calculation, but only positive values indicate degradation. It can be clearly seen that this compound was not degraded in the die-away tests. It was seen that a negative value for the abiotic die-away test was obtained and this shows that no degradation took place in 28 days. No change in pH was measured during the die-away tests performed with CPTCA as the test compound.

3.1.3 Viability of microorganisms exposed to CPTCA in the SCAS unit.

Viability of microorganisms present in the activated sludge in the SCAS unit exposed to CPTCA remained constant at about $2x10^7$ microorganisms ml^{-1} (Table 8). The number of microorganisms found in the SCAS control

Table 7 CPTCA : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to CPTCA (days)	Days of sampling the die-away tests and % biodegradation observed,			
	7	14	21	28
2	0,21 (10,5±14,8)	2,-4 (-1±4,2)	-17,-14 (-15,5±2,1)	-26,-18 (-22±5,6)
30	-14,-14 (-14±0)	-14,-7 (-10,5±4,9)	-18,-18 (-18±0)	-41,-8 (-24,5±23,3)
57	-12,-8 (-10±2,8)	0,2 (1±1,4)	-8,-5 (-6,5±2,1)	-17,-17 (-17±0)
85	-13,-13 (-13±0)	-21,-27 (-24±4,2)	-7,-7 (-7±0)	-27,-22 (-24,5±3,5)
114	-15,-8 (-11,5±4,9)	N,D,	-31,-17 (-24±9,9)	-15,19 (17±2,8)
142	N,D,	-42,-30 (-36±8,5)	N,D,	-30,30 (-30±0)
170	N,D,	N,D,	N,D,	-13,-17 (-15±2,8)

Abiotic Control - 23,5% removal

N,D, = Not determined

Values in brackets = $(x \pm \sigma_{n-1})$

unit were on the whole slightly higher but of the same order of magnitude (Table 8), indicating little if any effect of CPTCA on cell viability.

3.1.4 Inhibition of activated sludge growth by CPTCA.

Table 9 shows the effect of CPTCA on the ability of activated sludge microorganisms to grow on acetate/nutrient broth medium. Growth was measured by optical density and inhibition evaluated as the % reduction in optical density of the exposed cultures compared with the control. It can be seen that values of percentage reduction are low and therefore CPTCA has little if any inhibitory effect on activated sludge at levels up to 100mg l^{-1}. The EC50 for this compound was greater than 100mg l^{-1}.

3.1.5 Isolation of CPTCA-degrading organisms.

No CPTCA utilizing organisms were isolated from activated sludge previously exposed to CPTCA, when isolation was attempted by batch culture methods at 30°C, 200 rpm and 0.5g l^{-1} of CPTCA present.

3.2 Degradation of t-butanol.

3.2.1 Removal of t-butanol in the SCAS test.

t-Butanol was added to the SCAS unit at a rate of 20 mg l^{-1} (as total organic carbon) per day. The amount of

Table 8. Number of viable microorganisms ml^{-1} found in activated sludge after increasing periods of exposure to CPTCA in the SCAS unit.

Day of SCAS	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	CPTCA dosed unit
0	2.62	2.62
28	1.32	3.20
56	2.20	2.00
85	3.00	1.60
115	2.10	1.40
149	6.85	3.30
160	3.33	3.00

Table 9. Inhibition of activated sludge growth by CPTCA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration (mg l^{-1})				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	10	7	13	7	2

t-butanol remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). Figure 15a shows the DOC concentration in both the test and control SCAS units over 118 days the duration of the experiment. It can be seen that the data points from the two units follow similar patterns and are quite close, indicating that t-butanol is indeed disappearing from the test unit.

The same data were used to calculate degradation/daily addition. Figure 15b shows the daily percentage removal observed in the control SCAS unit over a period of 118 days and it can be seen that there is some scatter in the data. The addition of t-butanol to the test unit was discontinued after 118 days to observe deacclimatization.

3.2.2 Removal of t-butanol in die-away tests.

Removal of t-butanol in 28 day die-away tests reached almost 100% even when the inoculum was taken from the SCAS unit after only two days exposure to the compound (see Table 10 and Figure 16). A drop in the observed maximum removal of t-butanol was observed with the inoculum taken on day 57 but maximum removal again occurred with inocula taken at day 87 and day 114. The lower value observed with inoculum taken at day 57 is thought not to be significant.

Figure 15a. Removal of t-butanol in the SCAS test.

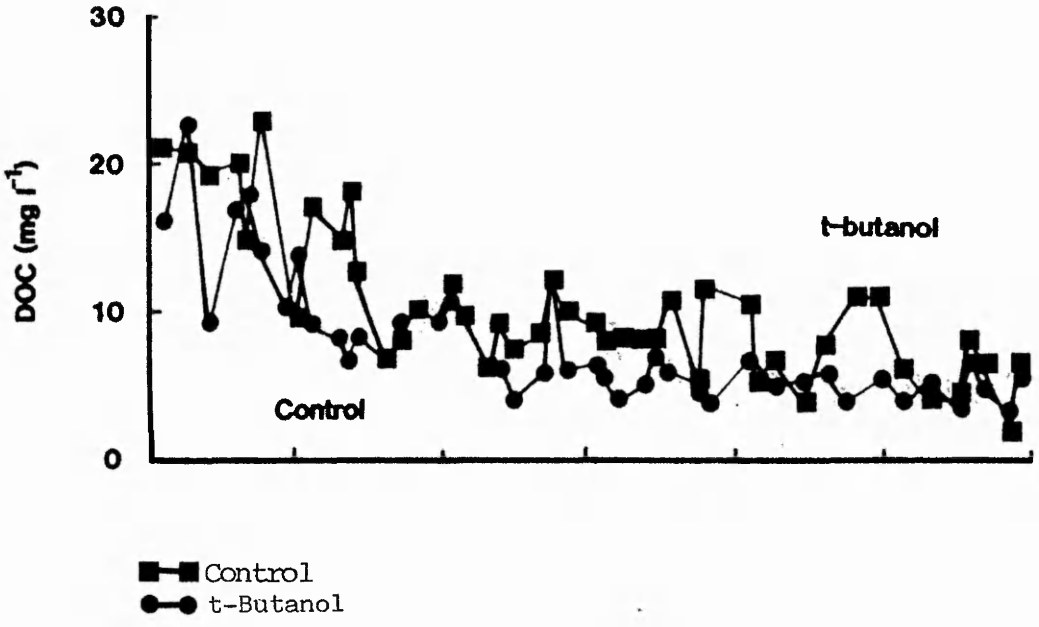
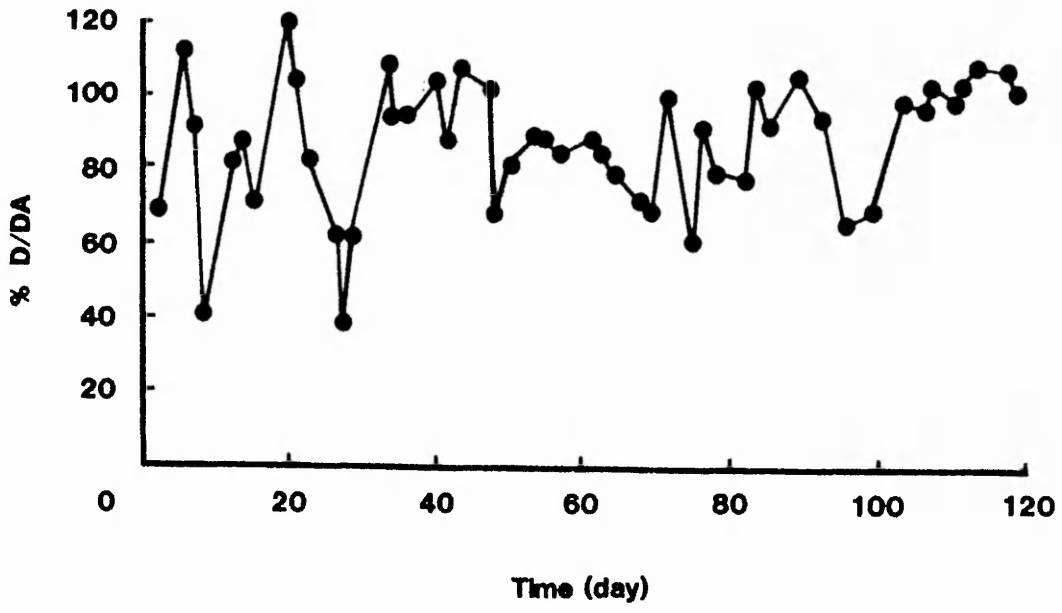


Figure 15b. Removal of DOC on a daily basis.



After 118 days the addition of t-butanol to the SCAS unit was discontinued. Inoculum taken from the SCAS unit after 200 days (ie 82 days after the addition of t-butanol was discontinued) still gave die-away test results which showed 100% removal of t-butanol after 28 days (Table 10).

Further consideration of the data (Table 10) shows the maximum removal measured was only achieved on one occasion before day 21 of a die-away test, (that was with inoculum taken on day 85 of the SCAS test). The pH of the die-away test medium remained at pH 7.2 throughout the 28 days of the test.

3.2.2.1 Physico-chemical removal of t-butanol.

When abiotic control flasks containing sterile activated sludge and t-butanol (20mg C l^{-1}) were shaken at 150 rpm and 22.5±2.5°C, 63% of the t-butanol originally present had disappeared by day 28 of the test (Table 11). Approximately the same degree of removal was observed in abiotic control flasks with no activated sludge present. In similar flasks which were stationary during incubation 40% removal was observed during the die-away test. With sealed flasks 18 and 19% removal was observed for abiotic flasks containing sterile activated sludge and no sludge respectively; slightly less removal (12%) was observed in sealed flasks which were stationary.

Table 10. t-Butanol : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to t-butanol (days)	Days of sampling die-away tests and % biodegradation observed			
	7	14	21	28
2	81,80 (80,5±0,7)	59,61 (60±1,4)	81,79 (80±1,4)	97,97 (97±0)
30	54,27 (40,5±19,1)	59,59 (59±0)	70,86 (78±11,3)	94,96 (94±0)
57	33,30 (31,5±2,1)	52,5 (53±1,4)	55,61 (58±4,2)	81,82 (81,4±0,7)
85	61,60 (61±0)	83,83 (83±0)	100,100 (100±0)	100,100 (100±0)
114	30,45 (37,5±10,6)	55,58 (56,5±2,1)	74,92 (83±12,7)	100,100 (100±0)
142	N,D.	90,90 (90±0)	N,D.	100,100 (100±0)
170	N,D.	N,D.	N,D.	100,100 (100±0)
200	N,D.	N,D.	N,D.	100,100 (100±0)

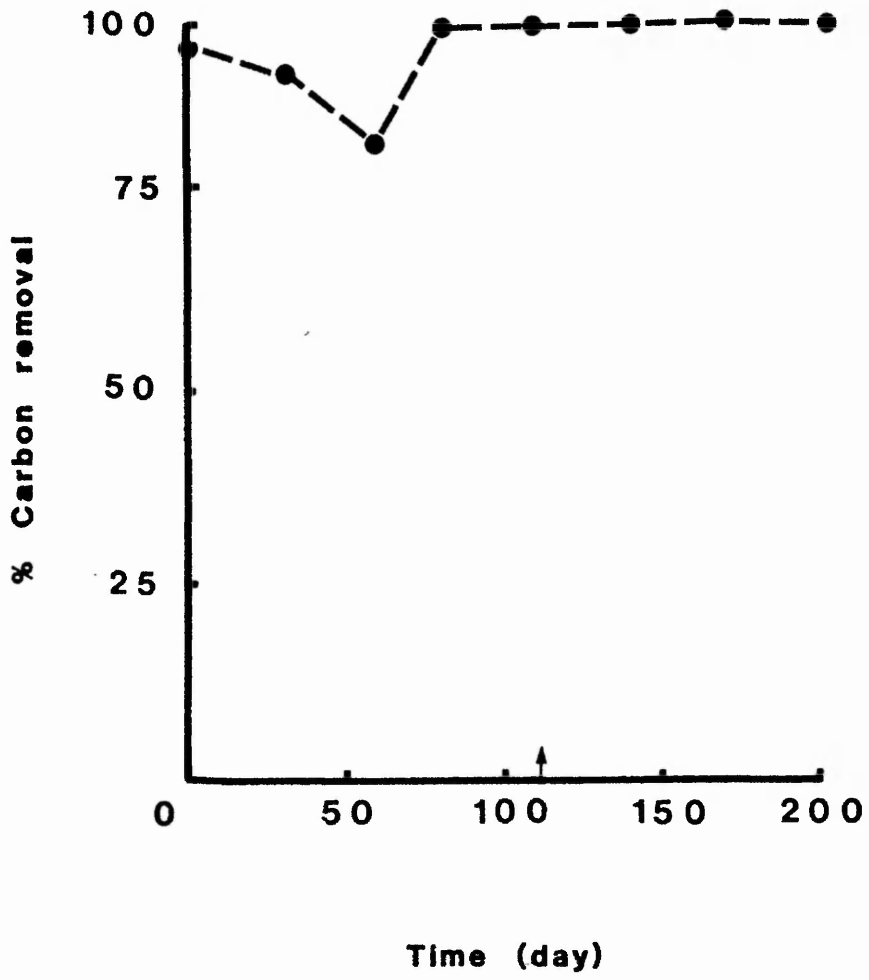
Abiotic control 63% removal

Deacclimatization started on day 118 of SCAS test.

N,D. = Not determined

Values in brackets = ($x \pm \sigma_{n-1}$)

Figure 16. Maximum measured removal of carbon from die-away tests performed with t-butanol, determined as DOC.



↑ Addition of NMA to test SCAS unit discontinued.

In flasks containing fresh unacclimatized activated sludge 89% removal was seen when the flasks were shaken (150 rpm) and 81% removed in stationary flasks. Sealed flasks showed over twice the amount of removal (29,35%) of t-butanol than the equivalent abiotic flasks (12-19%) (Table 11).

3.2.2.2 Removal of t-butanol by volatilization.

Removal of t-butanol was also observed when added to a system similar to that reported in section 3.2.1 but in the absence of activated sludge. After 24 hours aeration at 0.5 l min^{-1}, 92% of the added t-butanol had disappeared from the test unit (Figure 17). It can also be seen that 86% of the t-butanol had disappeared after only 3 hours aeration (0.5 l min^{-1}).

3.2.3 Viability of microorganisms in activated sludge treated with t-butanol.

The number of viable microorganisms present in the activated sludge exposed to t-butanol remained fairly constant at about 10^7 viable microorganisms ml^{-1}. The actual counts ranged from $1x10^7$ microorganisms ml^{-1} to $7x10^7$ microorganisms ml^{-1} during the period of the SCAS test. The number of microorganisms found in the control SCAS unit fell within similar limits although they were on the whole slightly higher but of the same order of magnitude as shown (Table 12).

Table 11. % Removal of t-butanol after 28 days incubation.

Unadapted activated sludge

Conditions	% Removal
Shaken - open	89
Static - open	81
Shaken - closed	35
Static - closed	29

Abiotic control - sterilized activated sludge

Conditions	% Removal
Shaken - open	63
Static - open	40
Shaken - closed	18
Static - closed	12

Abiotic sterilized control - no activated sludge present

Conditions	% Removal
Shaken - open	65
Static - open	37
Shaken - closed	19
Static - closed	12

3.2.4 Inhibition of activated sludge growth by t-butanol.

Table 13 shows the effect of t-butanol on the ability of activated sludge to grow in acetate/nutrient broth medium. Inhibition was shown to some extent at all concentrations tested and appears to be independent of t-butanol concentration. The EC50 value for t-butanol is greater than 100mg l^{-1}.

3.2.5 Isolation of t-butanol-degrading organisms.

Although removal of t-butanol was observed, attempts to isolate by batch culture any organisms capable of growing on t-butanol as the sole carbon source were unsuccessful.

3.3 Biodegradation of benzene-1,3-disulphonic acid (BDSA).

3.3.1 Removal of BDSA in the SCAS test.

BDSA was added to a SCAS unit at the rate of 20mg l^{-1} (as total carbon) per day. The amount of BDSA remaining in the unit after 23 hours aeration was determined as dissolved organic carbon (DOC). The change in DOC in the test unit together with that of the control (without BDSA) is shown (Figure 18a). Consideration of the two curves shows little evidence to suggest that biodegradation is taking place.

Figure 17. Removal of t-butanol by volatilization, measured as DOC.

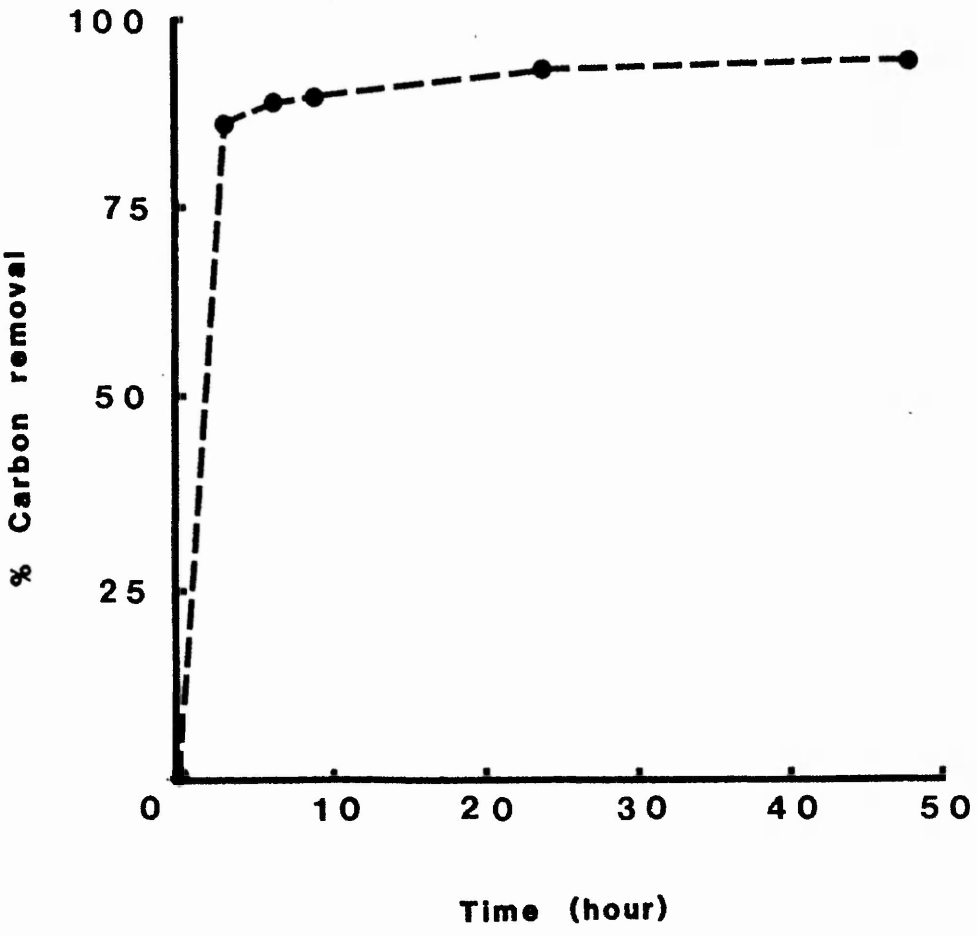


Table 12. Number of viable microorganisms ml^{-1} found in activated sludge after increasing periods of exposure to t-butanol in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	t-butanol dosed unit
0	2.62	2.62
23	1.32	1.20
42	3.20	1.70
85	3.00	2.30
115	2.10	7.00
149	6.85	1.00
170	3.00	1.70

t-Butanol withdrawn at day 118.

Table 13. Inhibition of activated sludge growth by t-butanol. Values represent % reduction in O.D. (530 nm) relative to the control after 6 hours incubation.

	Concentration (mg l^{-1})				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	22	26	29	16	7

(t-Butanol was filter sterilized)

The same data were used to calculate the parameter, degradation/daily addition. The plot of degradation/ daily addition is shown (Figure 18b). It can be seen that the data treated in this way gives very large fluctuations with time, with values ranging from -48% to + 75%, but the values are erratic and no trend with time is observed and therefore there is no biodegradation.

3.3.2 Removal of BDSA in die-away tests.

Degradation of BDSA was assessed in die-away tests by measuring the residual DOC at seven day intervals over a period of 28 days and expressing the data as % removal over the control. Table 14 shows the results of die-away tests carried out using inocula from SCAS units receiving BDSA. The negative values obtained with inocula exposed for 21 days and over indicate that degradation is not occurring. Positive values were obtained with inocula taken directly from the SCAS unit without exposure, though the % degradation remained fairly low (25% after 28 days). No degradation was observed in the abiotic control set up for this compound. The pH of the die-away test medium did not change during the 28 days of the test remaining at pH 7.2.

Figure 18a. Removal of BDSA in the SCAS test.

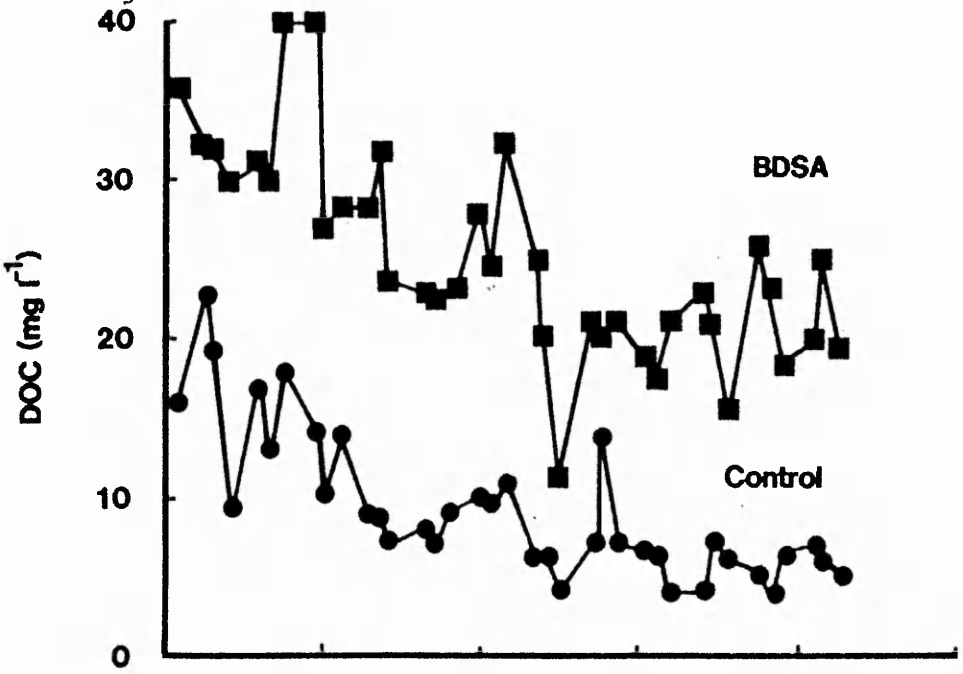


Figure 18b. Removal of DOC on a daily basis.

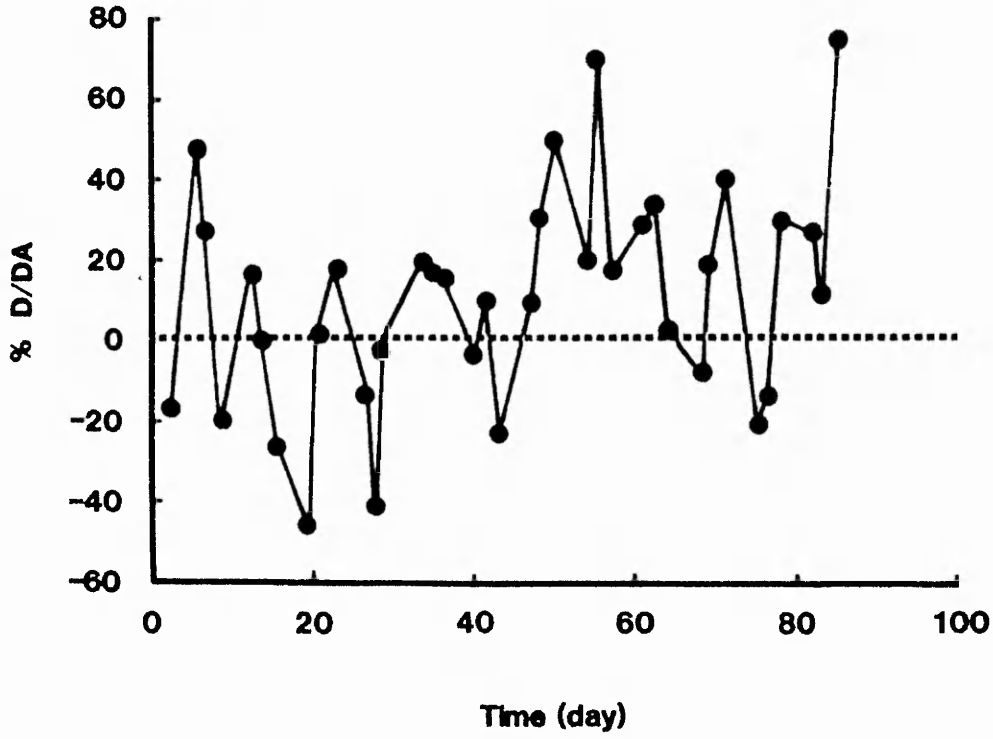


Table 14. BDSA: die-away test results.

Period for which SCAS test sludge inoculum had been exposed to BDSA (days)	Days of sampling die-away tests and % biodegradation observed			
0	7	14	21	28
	33,14	28,10	31,12	23,27
	(23,5±13,4)	(19±12,7)	(21,5±13,4)	(25±2,8)
21	-77,-62	-43,-3	-32,-5	-26,-14
	(-69,5±10,6)	(-23±28,3)	(-18,5±19,1)	(20±8,5)
42	-34,-31	-35,-40	-39,-42	-30,-42
	(-32,5±2,1)	(-37,5±3,5)	(-40,5±2,1)	(-36±8,5)
68	-35,-30	-39,-36	-43,-36	-39,-70
	(-32,5±3,5)	(-37,5±2,1)	(-39,5±4,9)	(-54,5±21,9)

Abiotic control 3,0% removal

Values in brackets = $(x \pm \sigma_{n-1})$

3.3.3 Viability of microorganisms in activated sludge treated with BDSA.

The number of viable microorganisms present in activated sludge exposed to BDSA in a SCAS unit fell from 2.1×10^7 microorganisms ml^{-1} to 0.081×10^7 microorganisms ml^{-1} during the first 28 days of the SCAS test (Table 15). The number of microorganisms present in the activated sludge exposed to BDSA decreased further from 0.081×10^7 microorganisms ml^{-1} at day 28 down to 0.030×10^7 microorganisms ml^{-1} by day 85. The number of microorganisms in the control activated sludge remained relatively constant compared to time zero throughout the period of the test.

3.3.4 Inhibition of activated sludge growth by BDSA.

Table 16 shows the effect of BDSA on the ability of activated sludge to grow on acetate/nutrient broth medium. Inhibition due to BDSA is expressed as % reduction in O.D. at 530nm compared to the control. There was some reduction in growth at all concentrations in this test (Table 16) although the level of inhibition was small and did not increase with concentration from 10mg l^{-1} to 100mg l^{-1}. The EC50 value (50% measured inhibition) for BDSA is greater than 100mg l^{-1}.

Table 15. Number of viable microorganisms ml^{-1} found in activated sludge after increasing periods of exposure to BDSA in the SCAS unit.

Day of SCAS unit	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	BDSA dosed unit
0	2.62	2.10
28	1.32	0.081
42	3.20	0.057
85	3.00	0.030

Table 16. Inhibition of activated sludge growth by BDSA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration (mg l^{-1})				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	13	N.D.	25	19	28

3.3.5 Isolation of BDSA-degrading organisms.

No microorganisms could be isolated from previously exposed activated sludge, by batch culture at 30°C, 200 rpm and a concentration of BDSA of 0.5g l^{-1} capable of utilizing this compound as its sole carbon source.

3.4 Degradation of 2-chlorobenzoic acid (2CBA).

3.4.1 Removal of 2CBA in the SCAS test.

2CBA was added to the SCAS unit at a rate of 20mg l^{-1} (as total organic carbon) per day. The amount of 2CBA remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). Consideration of the two curves shown (Figure 19a) indicates little evidence of degradation. No convergence of the two graphs, (indicating biodegradation) was observed over the 275 days of the test.

The same data were used to calculate the parameter degradation/daily addition. Figure 19b shows that over the first 100 days the amount of degradation in the SCAS unit fell from approximately zero to the region of -70%. After 100 days a general increase in the amount of degradation was observed up to a level of approximately +40% by day 285 although this is not thought to be significant.

3.4.2 Removal of 2CBA in die-away tests.

Predominantly negative values for biodegradation were observed for the die-away tests performed with 2CBA (Table 17). The data for the die-away tests became less negative as the SCAS test (the inoculum source) progressed. It can be seen that the only die-away test which gave positive mean values for each sampling time was the one set up with inoculum taken at day 245 of the SCAS test. A slightly negative value for biodegradation was observed in the abiotic control.

3.4.3 Viability of microorganisms in activated sludge treated with 2CBA.

The number of viable microorganisms present in the activated sludge exposed to 2CBA remained constant at approximately $2x10^7$ microorganisms ml^{-1} (Table 18). The number of microorganisms found in the control SCAS unit were on the whole slightly higher but the same order of magnitude (Table 18).

3.4.4 Inhibition of activated sludge by 2CBA.

Table 19 shows the effects of 2CBA on the ability of activated sludge to grow on acetate/nutrient broth medium. It can be seen that 2CBA has only a weak inhibitory effect ie 30% at a concentration of 100mg l^{-1}. The EC50 for 2CBA is therefore greater than 100mg l^{-1}.

Figure 19a. Removal of 2CBA in the SCAS test.

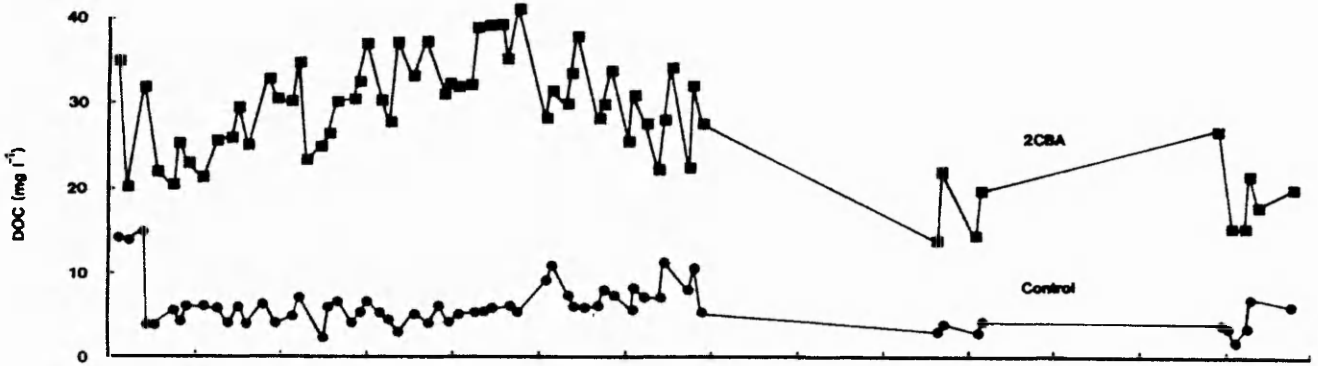


Figure 19b. Removal of DOC on a daily basis.

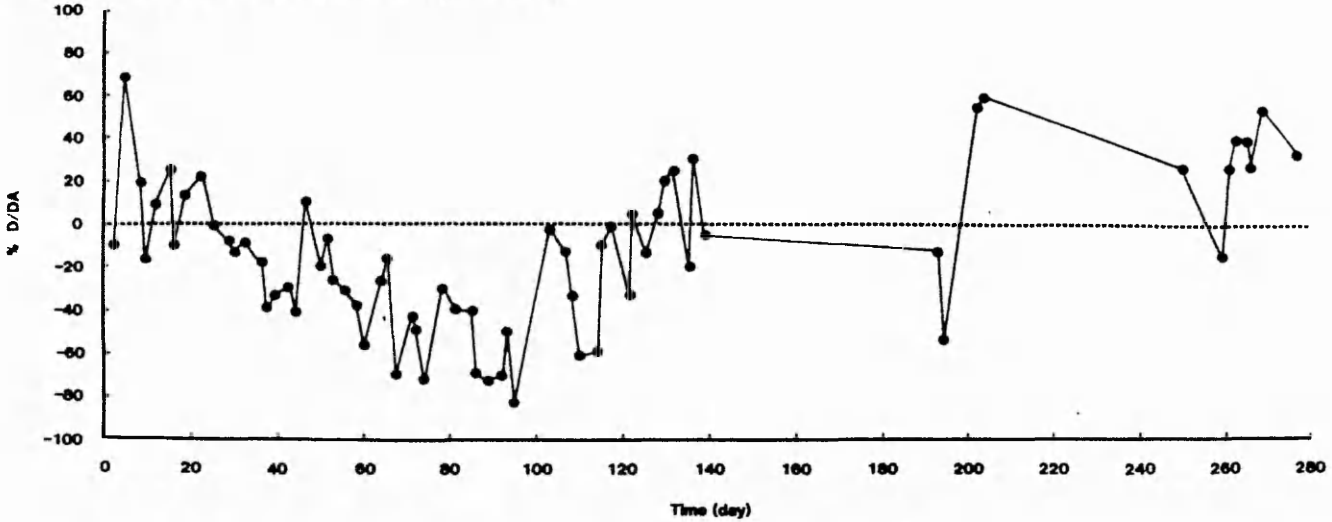


Table 17. 2CBA : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to 2CBA (days)	Day of sampling die-away tests and % biodegradation observed			
	7	14	21	28
0	-17,-12 (-14,5±3,5)	-56,-42 (-49±10,0)	-45,-41 (-43±2,8)	-79,-75 (-77±2,8)
28	-20,-20 (-20±0)	-53,-48 (-50,5±3,5)	-53,-64 (-58,5±7,8)	-37,-40 (-38,5±2,1)
51	-1,-5 (-3±2,8)	-15,-17 (-16±1,4)	-15,-14 (-14,5±0,7)	-1,-3 (-2±1,4)
79	-61,-46 (-53,5±10,6)	-77,-84 (-80,5±4,9)	-68,-77 (-72,5±6,4)	-50,-61 (-55,5±7,8)
107	-17,-20 (-18,5±2,1)	2,5,-4,5 (-1±4,9)	15,11 (13±2,8)	1,9 (5±5,7)
135	N,D,	N,D,	N,D,	-5,-8 (-6,5±2,1)
173	1,1 (1±0)	-18,5,-18,5 (-18,5±0)	N,D,	-16,5,-31,5 (-24±10,6)
245	25,8 (16,5±12,0)	26,10 (18±11,3)	31,3 (17±19,8)	30,-3 (13,5±23,3)

Abiotic control - 19,0% removal

N,D, = Not determined

Values in brackets = ($x \pm \sigma_{n-1}$)

Table 18. Number of viable microorganisms ml^{-1} found in activated sludge after increasing periods of exposure to 2CBA in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	2CBA dosed unit
0	2.10	2.10
56	2.20	3.64
110	4.27	3.60
149	6.85	2.28
212	4.87	2.00
270	3.50	2.00

Table 19. Inhibition of activated sludge growth by 2CBA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration ($mg\ l^{-1}$)				
	1.0	3.2	10.0	32.0	100.0
% inhibition	0	1	5	5	30

3.4.5 Isolation of 2CBA degrading organisms.

No organisms capable of utilizing 2CBA as the sole source of carbon were isolated by incubation of activated sludge with 0.5g l^{-1} 2CBA at 30°C and 200 rpm.

3.5 Degradation of N-methylaniline (NMA).

3.5.1 Removal of NMA in the SCAS test.

NMA was added to the SCAS unit at a rate of 20mg l^{-1} (as total organic carbon) per day. The amount of NMA remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). The levels of DOC in the test unit and control (undosed) unit are shown (Figure 20a). Up to day 62 of the test, the DOC level in the test unit remained higher than would have been expected. After day 62 however, the DOC level in the test fell rapidly (by day 77) to approach that in the control unit, indicating that significant degradation was taking place. The two levels remained similar until day 118 when addition of NMA was discontinued.

The same data were used to calculate the parameter degradation/daily addition. Figure 20b shows the daily percentage removal above that observed in the control SCAS unit over a period of 115 days. The data shows negative degradation during each 23 hour aeration period up to day 62 of the SCAS test. No analysis of effluent

Figure 20a. Removal of NMA in the SCAS test.

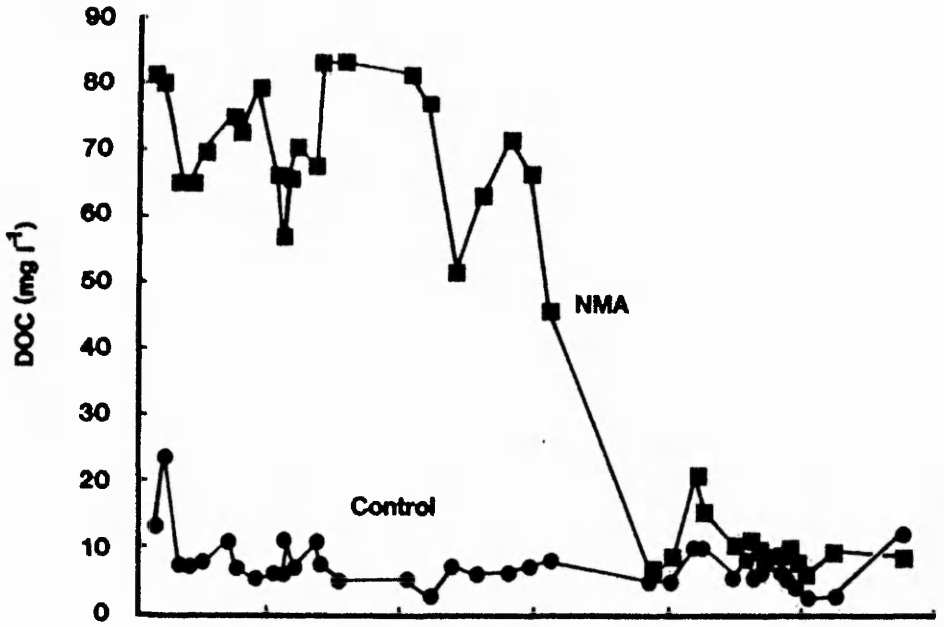
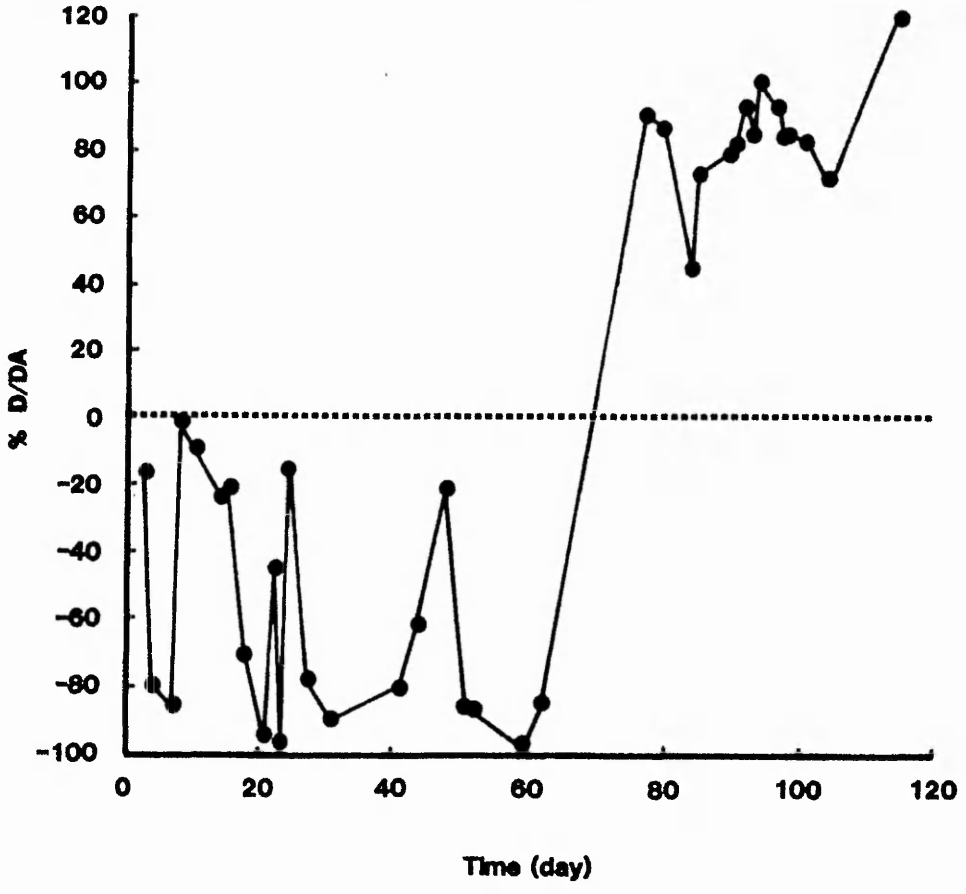


Figure 20b. Removal of DOC on a daily basis.

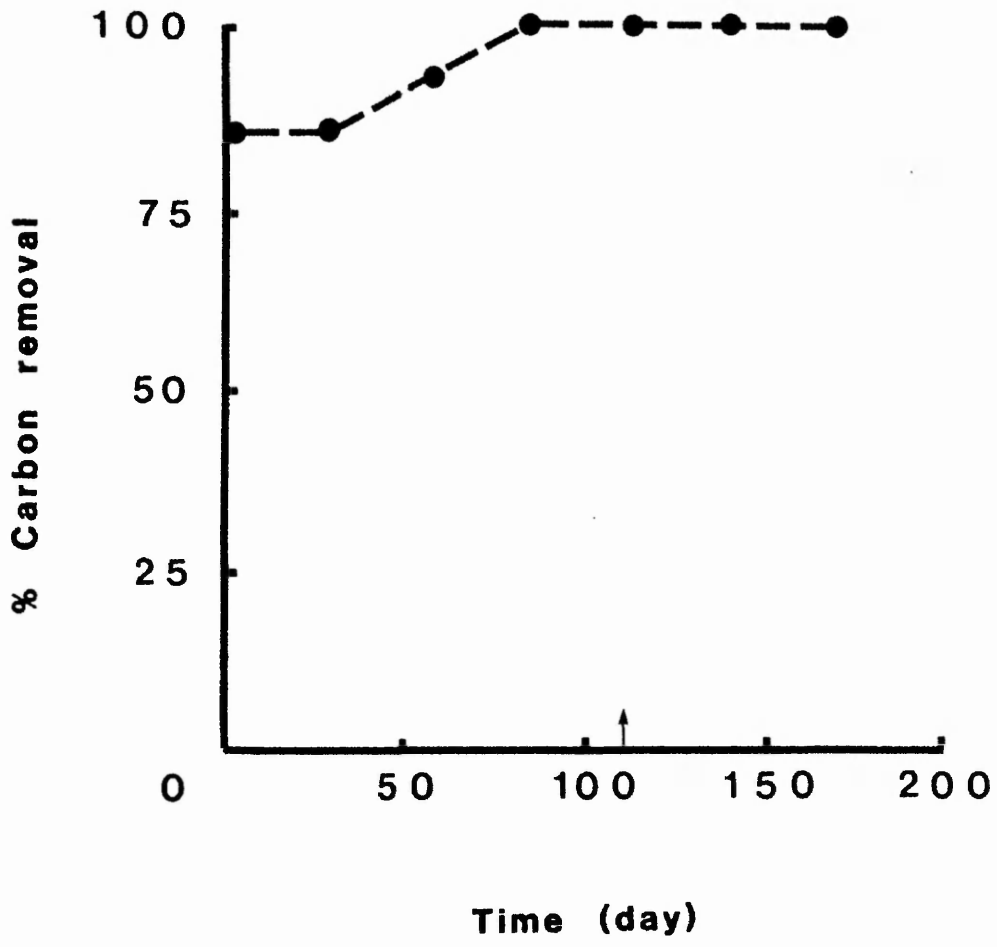


was carried out between day 62 and 77. By day 77, removal of carbon was calculated to be 100% and remained at this level until the addition of NMA was discontinued at day 115. After this time no further measurements of the total organic carbon in the SCAS effluent were made.

3.5.2 Removal of NMA in die-away tests.

Using inocula taken at intervals from a SCAS unit being dosed with NMA 28 day die-away tests were conducted. Removal of NMA was observed in die-away tests set up with an inoculum which had previously been exposed to NMA for only 2 days (Table 20). In this die-away test, 70% removal was observed after 7 days increasing to 86% after 28 days. It was not until the die-away test performed with inoculum taken from the SCAS unit at day 57 that almost 100% removal of DOC was measured during a 28 day die-away test (Table 20). All subsequent die-away tests exhibited 100% removal of DOC by day 28. Figure 21 shows the maximum measured removal of DOC in each die-away test. Maximum degradation in 28 day die-away tests occurred even when inocula taken after discontinuation of NMA addition to the SCAS unit were used. Reference to Table 20 shows that 100% degradation occurred in the die-away test with inocula taken from a SCAS unit starved of NMA for 83 days, indicating that the sludge population in the SCAS unit did not deacclimatize during this time. In the abiotic

Figure 21. Maximum measured removal of carbon from die-away tests performed with NMA, determined as DOC.



↑ Addition of NMA to test SCAS unit discontinued.

control containing heat sterilised sludge 55% removal of NMA as DOC was measured during the 28 days of the test (Table 20).

3.5.3 Viability of microorganisms in activated sludge treated with NMA.

The microbial population was determined in accordance with the method outlined in section 2.6.3. The number of viable microorganisms present in the activated sludge exposed to NMA remained fairly constant and of the order of 10^{-7} viable microorganisms ml^{-1} (Table 21). The fluctuations in the microbial populations of the control and exposed activated sludges remained very similar throughout the duration of the test with the control SCAS unit having marginally greater numbers.

3.5.4 Inhibition of activated sludge growth by NMA.

Table 22 shows the effect of NMA on the ability of activated sludge to grow in acetate/nutrient broth medium. It can be seen that a small inhibitory effect caused by NMA was observed below 32mg l^{-1} (6%) and only 19% inhibition was recorded at 100ml l^{-1} NMA. Levels of NMA higher than this were not tested; the EC50 value for NMA must be greater than 100mg l^{-1}.

Table 20. NMA : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to NMA (days)	Days of sampling die-away tests and % biodegradation observed			
	7	14	21	28
2	72,69 (70,5±2,1)	80,79 (79,5±0,7)	84,86 (85±1,4)	83,90 (86,5±4,9)
30	93,93 (93±0)	89,90 (89,5±0,7)	83,90 (89±1,4)	84,88 (86±2,8)
57	81,81 (81±0)	89,87 (88±1,4)	98,100 (99±1,4)	93,93 (93±0)
85	88,88 (88±0)	94,94 (94±0)	97,97 (97±0)	100,100 (100±0)
115	70,70 (70±0)	87,80 (83,5±4,9)	90,90 (90±0)	100,100 (100±0)
142	N,D.	94,92 (93±1,4)	N,D.	100,100 (100±0)
170	N,D.	N,D.	N,D.	100,100 (100±0)
198	N,D.	N,D.	N,D.	100,100 (100±0)

Abiotic control, 55% removal in the presence of sterile activated sludge.

N,D, = Not determined

Values in brackets = $(x \pm \sigma_{n-1})$

Deacclimatization started at day 115

Table 21. Number of viable microorganisms ml^{-1} found in activated sludge after varying periods of exposure to NMA in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	NMA dosed unit
0	2.62	2.62
28	1.32	6.00
56	2.20	2.50
85	3.00	3.00
115	2.10	2.00
149	6.85	2.80
160	3.33	2.40
200	3.10	2.30

Table 22. Inhibition of activated sludge growth by NMA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration (mg l^{-1})				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	0	1	3	6	19

3.5.5 Isolation of an NMA degrading microorganism.

One microorganism (KCNM1) capable of growing on NMA as its sole source of carbon was isolated from activated sludge by enrichment with 0.5g l^{-1} NMA at 30°C and 200rpm in batch culture.

3.5.6 Identification and characterization of KCNM1 an NMA degrading microorganism.

3.5.6.1 API 20E-biochemical tests performed at 35°C.

Table 23, lists the results of biochemical tests performed on KCNM1 using the API 20E test system. The isolate KCNM1 is a Gram negative, non spore forming rod, in which motility was not demonstrated. The isolates could utilize glucose, citrate and urea in addition to utilizing sodium pyruvate to produce acetoin. KCNM1 was found to convert nitrate to nitrogen and also to be catalase and cytochrome oxidase-positive. Using the API identification system KCNM1 was identified with 86.6% reliability as being Alcaligenes spp; though motility, a characteristic of this genus, was never observed.

3.5.6.2 Carbohydrate utilization by isolate KCNM1.

KCNM1 could utilize 10 of the 49 carbohydrates it was supplied with, at a temperature of 30°C after 72 hours incubation (Table 24). The carbohydrates utilized by KCNM1 were L-arabinose, ribose, D-xylose, galactose,

Table 23. API 20E-biochemical tests performed on isolate KCNM1.

Test	Test Substrates	Isolate KCNM1
ONPG	ortho-nitro-phenyl-galactoside	-
ADH	arginine	-
LDC	lysine	-
ODC	ornithine	-
CIT	sodium citrate	+
H_2S	sodium thiosulfate	-
URE	urea	+
TDA	tryptophane	-
IND	tryptophane	-
UP	sodium pyruvate	+
GEL	Kohn's gelatin	-
GLU	glucose	+
MAN	mannitol	-
INO	inositol	-
SOR	sorbitol	-
RHA	rhamnose	-
SUC	sucrose	-
MEL	melibiose	-
AMY	amygdalin	-
ARA	arabinose	-
OX	H_2S or ONPG tube	+
NO_3-NO_2	GLU tube	-
NO_3-N_2	GLU tube	+
Motility		?
MAC	MacConkey medium	-
CAT	in any negative sugar	+

Table 24. Utilization of carbohydrates by isolate KCNM1.

Carbohydrate	Isolate KCNM1
Glycerol	-
Erythritol	-
D-Arabinose	-
L-Arabinose	+
Ribose	+
D-Xylose	+
L-Xylose	-
Adonitol	-
β-methylxyloxide	-
Galactose	+
D-Glucose	+
D-Fructose	+
D-Mannose	-
L-Sorbose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Mannitol	-
Sorbitol	-
α Methyl-D-amoside	-
α Methyl-D-glucoside	-
N-Acetyl glucosamine	+
Amygdaline	-

Carbohydrate	Isolate KCNM1
Arbutine	-
Esculine	-
Salicine	-
Cellobiose	-
Maltose	-
Lactose	-
Melibiose	-
Saccharose	-
Trehalose	-
Inuline	-
Melezitose	-
D-Raffinose	-
Amidon	-
Glycogene	-
Xylitol	-
β Gentiobiose	-
D-Turanose	-
D-Lyxose	-
D-Tagatose	-
D-Fucose	-
L-Fucose	-
D-Arabitol	-
L-Arabitol	-
Gluconate	-
2 ceto-gluconate	-
5 ceto-gluconate	-

D-glucose, D-mannose, N-acetyl glucosamine, esculin, trehalose and D-fucose.

3.5.6.3 Growth inhibition of KCNM1 by antibiotics.

In order to provide another criterion for characterisation of KCNM1, antibiotic resistance was investigated. A washed cell suspension (0.5 ml) containing approximately 10^{8} nutrient broth grown cells was spread and dried onto the surface of nutrient agar plates, onto which were placed absorbent discs containing specified doses of antibiotics. The seeded plates were incubated at 30°C for 48 hours and the isolate was termed resistant if no zone of inhibition was seen. The isolate KCNM1 was found to be resistant only to sulphamethoxazole/trimethroprim (25μg), nitrofurantoin (300μg) and mecillinam (25μg). KCNM1 was found to be sensitive to penicillin G (10iu), ampicillin (25μg), tetracycline (10μg), kanomycin (30μg), sulphonamide (300μg), bacitracin (10iu), gentamycin (30μg), chloramphenicol (10μg), streptomycin (25μg), carbenicillin (100μg), erythromycin (30μg), nalodixic acid (30μg) and neomycin (30μg). KCNM1 was found to be very sensitive to the action of antibiotics and was resistant to only 3 of the antibiotics tested.

3.5.7 The effect of temperature on the growth of KCNM1.

Isolate KCNM1 was grown in shake flask cultures at 25, 30 and 35°C in minimal medium containing 0.5g l^{-1} NMA and at 200rpm. Growth was followed by measuring the change in the optical density of the cultures at 530nm against an uninoculated blank. Table 25 gives the specific growth rates at the temperatures investigated. There was very little difference in the specific growth rates for KCNM1 at either 25, 30 or 35°C. The pH of the culture fluid at 30°C remained fairly constant at about pH 7.1 throughout the 72 hours of the growth curve. Although stationary phase was achieved the isolate did not enter death phase during the 72 hours of the growth curves. It was noticed that discarded flasks if not autoclaved immediately began to develop a purple pigment in the culture medium.

3.5.8 Plasmid detection in KCNM1.

Detection of plasmids was attempted by the method of Kado and Liu (1981), but no plasmids were detected from KCNM1 by this method.

Table 25. The effect of temperature on the specific growth rate of KCNM1.

Temperature (°C)	Specific Growth rate (h^{-1})
25	0.056
30	0.055
35	0.055

3.6 Degradation of tetrahydrofuran-2,3,4,5-tetracarboxylic acid (THFTCA).

3.6.1 Removal of THFTCA in the SCAS test.

THFTCA was added to the SCAS unit at a rate of 20mg l^{-1} (as total organic carbon) per day. The amount of THFTCA remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). Figure 22a shows no convergence of the test and control DOC values until day 90 of the test. After this time the DOC values for both units were very similar indicating biodegradation of THFTCA by activated sludge.

The same data were used to calculate the parameter degradation/daily addition. Figure 22b shows the daily percentage removal above that observed in the control SCAS unit over a period of 115 days. The data show little degradation during the 23 hour aeration period over the first 60 days of the test. After this time an increase in degradation was observed during each period of aeration until the addition of THFTCA was discontinued at day 115. Afer this time no further THFTCA was added to the SCAS unit and no further measurements of DOC made. The SCAS unit was periodically used as a source of inoculum for die-away tests after day 115 to observe decacclimatization.

Figure 22a. Removal of THFTCA in the SCAS unit.

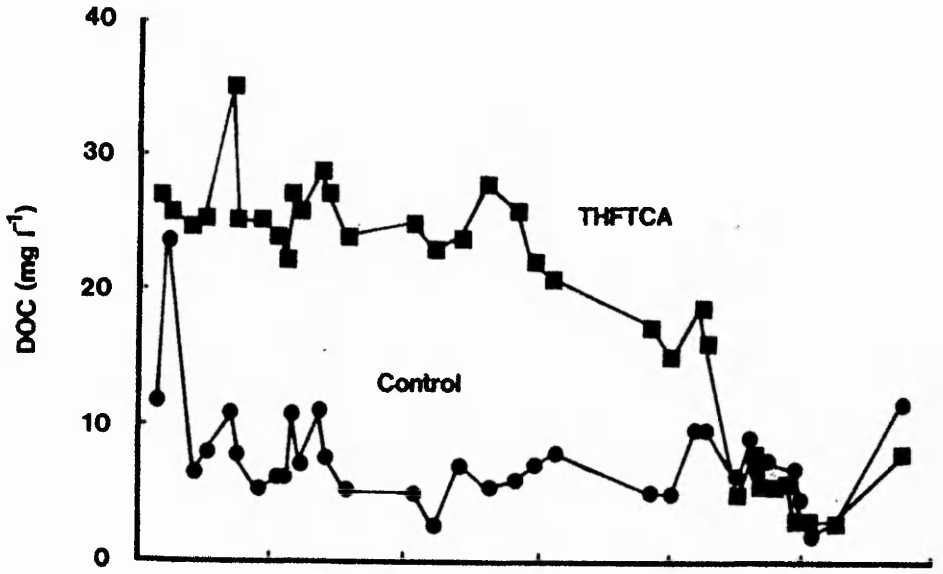
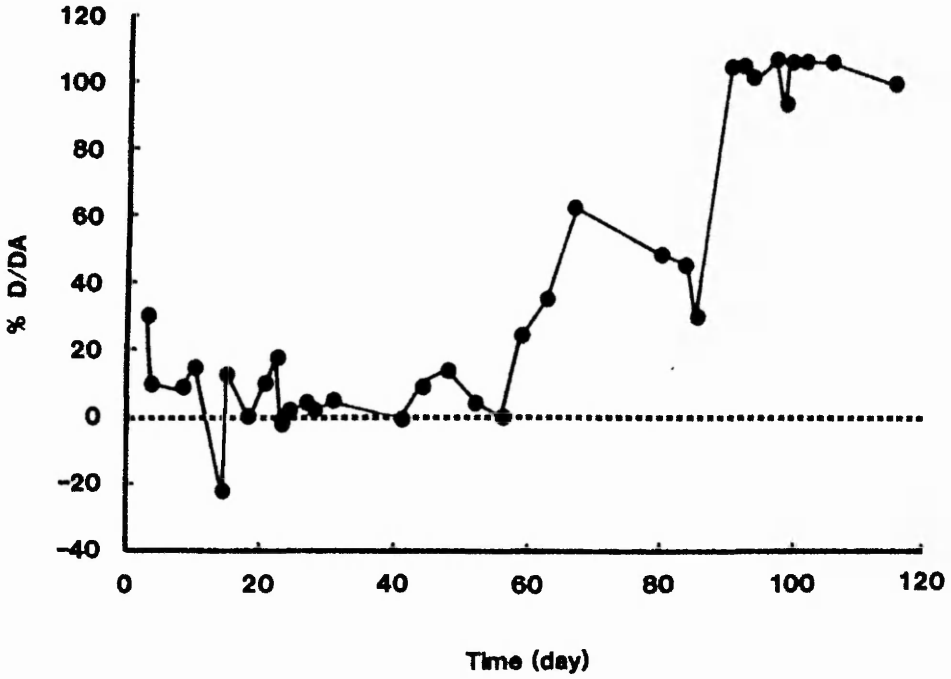


Figure 22b. Removal of DOC on a daily basis.



3.6.2 Removal of THFTCA in die-away tests.

Negative removal of THFTCA (-19%) was observed for the die-away test set up with an inoculum which had previously been exposed to THFTCA for only 2 days (Table 26). The die-away test set up with SCAS inoculum taken at day 30 showed negative degradation between day 7 of the die-away test and day 21, but by day 28, 89% degradation was observed. The day 57 inoculum brought about removal (18%) after 14 days and 100% by day 21, and the day 85 inoculum was even more active, since by day 7 19% was removed and 50% by day 14. After this time 100% degradation was observed by day 28 of each die-away test. Complete degradation by day 28 of the die-away test continued to be observed with inocula taken from the SCAS unit up to 83 days after withdrawal of THFTCA (Day 198 of test). No degradation was observed at the THFTCA abiotic control. Figure 23 shows the maximum degradation measured during each die-away test, 100% degradation was measured in the die-away test set up with inoculum taken from the SCAS unit on day 57 of the SCAS test. No decrease in the 100% level of degradation was measured. No deacclimatization of the sludge inoculum was observed in the die-away tests up to 83 days after the last exposure to THFTCA in the SCAS test (the source of inoculum).

Table 26. THFTCA : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to THFTCA (days)	Days of sampling die-away tests and % biodegradation observed			
	7	14	21	28
2	0,-2 (-1±1,4)	-9,3 (-3±8,5)	-6,0 (-3±4,2)	-24,-14 (-19±7,1)
30	-8,30 (11±26,9)	-13,-22 (-17,5±6,4)	-34,-22 (-28±8,5)	95,83 (89±8,5)
57	-15,-10 (12,5±3,5)	15,20 (17,5±3,5)	100,100 (100±0)	100,100 (100±0)
85	26,13 (19,5±9,2)	51,48 (49,5±2,1)	81,81 (81±0)	100,100 (100±0)
115	27,11 (19±11,3)	54,33 (43,5±14,8)	78,82 (80±2,8)	100,100 (100±0)
142	N,D,	100,100 (100±0)	N,D,	100,100 (100±0)
170	N,D,	N,D,	N,D,	100,100 (100±0)
198	N,D,	N,D,	N,D,	100,100 (100±0)

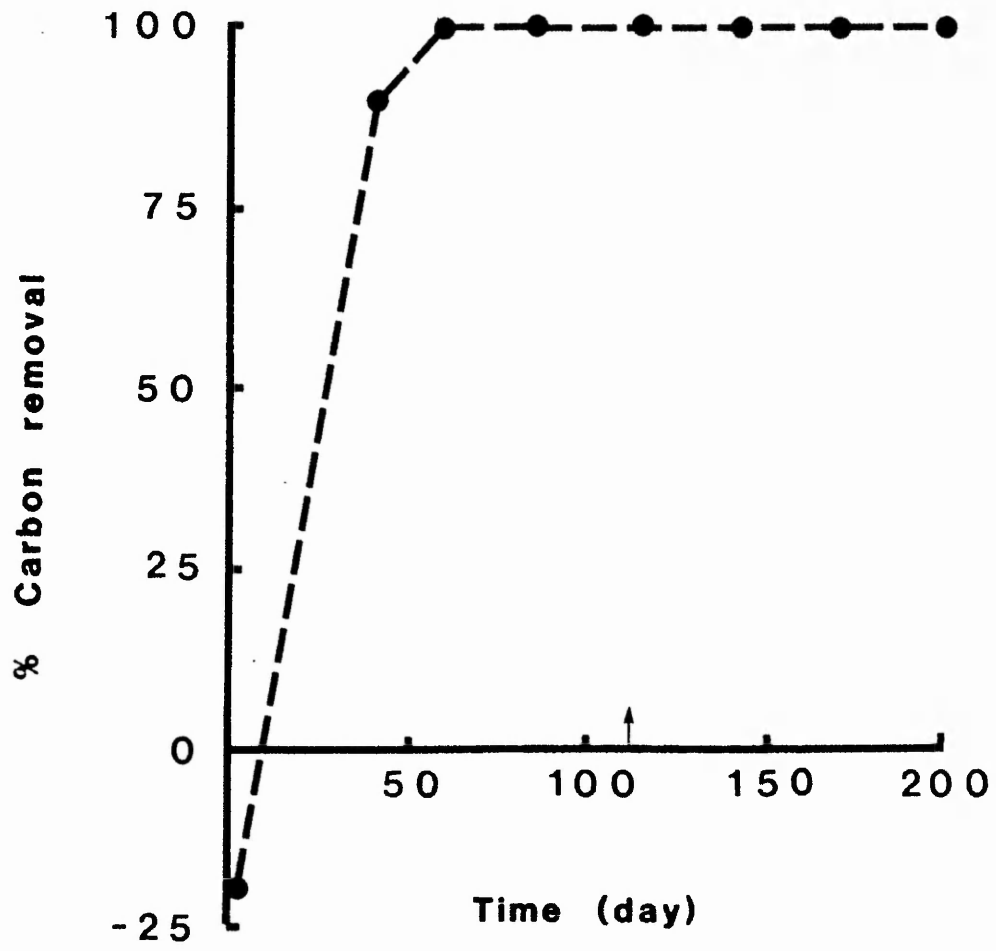
Abiotic control - 2% removal

N,D, = Not determined

Values in brackets = ($x \pm \sigma_{n-1}$)

Deacclimatization started on day 115 of the SCAS test.

Figure 23. Maximum measured removal of carbon from die-away tests performed with THFTCA, determined as DOC.



↑ Addition of THFTCA to test SCAS unit discontinued.

Table 27. Number of viable microorganisms ml^{-1} found in activated sludge after varying periods of exposure to THFTCA in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	THFTCA dosed unit
0	2.62	2.62
28	1.32	6.00
56	2.20	2.50
85	3.00	3.00
115	2.10	2.00
149	6.85	2.80
160	3.33	2.40
200	3.10	2.30

Table 28. Inhibition of activated sludge growth by THFTCA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration ($mg\ l^{-1}$)				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	0	1	3	6	19

3.6.3 Viability of microorganisms in activated sludge treated with THFTCA.

The numbers of viable microorganisms present in the activated sludge exposed to THFTCA remained relatively constant at 2-6x10^7 viable microorganisms ml^{-1} (Table 27). The fluctuation in total microorganisms present in the control and exposed activated sludge were very similar throughout the duration of the test.

3.6.4 Inhibition of activated sludge growth by THFTCA.

Table 28 shows the effect of THFTCA on the ability of activated sludge to grow on acetate/nutrient broth medium. It can be seen that THFTCA was not inhibitory at low levels (1-3.2mg l^{-1}) although the maximum inhibition was only 19% at 100mg l^{-1}. The EC50 for THFTCA is greater than 100mg l^{-1}.

3.6.5 Isolation of a THFTCA degrading microorganism.

One species of microorganism (KCT1) was isolated by enrichment of activated sludge with 1.0g l^{-1} THFCTA at 30°C and 200 rpm in batch culture.

3.6.6 Identification and characterization of KCT1 : a THFTCA degrading microorganism.

3.6.6.1 Identification of KCTI by NCIMB.

Table 29 lists the results obtained from the NCIMB for the identification of KCTI. The isolate appears to be a Gram-positive, non-motile, coryneform rod which does not grow at 37°C and appears to have a rod/coccus life cycle.

3.6.6.2 API 20E-biochemical tests performed at 35°C.

Table 30 lists the results obtained for the biochemical tests using API 20E strips for the isolated organism KCTI. The isolate was found to use sodium pyruvate to produce acetoin, and was cytochrome oxidase-positive and converted nitrate to nitrogen gas. KCTI utilized only glucose of those sugars present in the API 20E strip.

3.6.6.3 Carbohydrate utilization by isolate KCTI.

Of the 49 carbohydrates tested only 10 were found to be utilized by KCTI at a temperature of 30°C after 72 hours incubation. The utilized carbohydrates were : L-arabinose, D-xylose, galactose, D-glucose, D-mannose, N-acetyl glucosamine, esculine, melibiose, saccharose and D-fucose (Table 31).

Table 29. NCIMB first stage identification of isolate KCTI.

Isolate	KCT1
°C incubation	30°C
Gram	+
Spores	-
Motility	-
Colonial morphology - 4 days	Confluent growth only. Off-white, smooth, raised opaque. Growth positive on modified cytophaga and glucose agar.
°C, growth	37°C 41°C
Catalase	+
Oxidase, Kovacs	-
O-F glucose	-
First stage identification	Coryneform rod, showing a rod/coccus life cycle.

Morphological descriptions are from growth on LABM nutrient agar except as stated.

Table 30. API 20E-biochemical tests performed on isolate KCT1.

Test	Test Substrate	Isolate KCT1
ONPG	ortho-nitro-phenyl-galactoside	-
ADH	arginine	-
LDC	lysine	-
ODC	ornithine	-
CIT	sodium citrate	+
H_2S	sodium thiosulfate	-
URE	urea	+
TDA	tryptophane	-
IND	tryptophane	-
UP	sodium pyruvate	+
GEL	Kohn's gelatin	-
GLU	glucose	+
MAN	mannitol	-
INO	inositol	-
SOR	sorbitol	-
RHA	rhamnose	-
SUC	sucrose	-
MEL	melibiose	-
AMY	amygdalin	-
ARA	arabinose	-
OX	H_2S or ONPG tube	+
NO_3-NO_2	GLU tube	-
NO_3-N_2	GLU tube	+
Motility		-
CAT	in any negative sugar	+

Table 24. Utilization of carbohydrates by isolate KCT1.

Carbohydrate	Isolate KCT1
Glycerol	−
Erythritol	−
D-Arabinose	−
L-Arabinose	+
Ribose	−
D-Xylose	+
L-Xylose	−
Adonitol	−
β-methylxyloxide	+
Galactose	+
D-Glucose	−
D-Fructose	+
D-Mannose	−
L-Sorbose	−
Rhamnose	−
Dulcitol	−
Inositol	−
Mannitol	−
Sorbitol	−
α Methyl-D-amoside	−
α Methyl-D-glucoside	−
N-Acetyl glucosamine	+
Amygdaline	−

Carbohydrate	Isolate KCT1
Arbutine	-
Esculine	+
Salicine	-
Cellobiose	-
Maltose	-
Lactose	-
Melibiose	+
Saccharose	+
Trehalose	-
Inuline	-
Melezitose	-
D-Raffinose	-
Amidon	-
Glycogene	-
Xylitol	-
β Gentiobiose	-
D-Turanose	-
D-Lyxose	-
D-Tagotose	-
D-Fucose	-
L-Fucose	+
D-Arabitol	-
L-Arabitol	-
Gluconate	-
2 ceto-gluconate	-
5 ceto-gluconate	-

3.6.6.4 Growth inhibition of KCT1 by antibiotics.

In order to provide another criterion for characterization of KCT1, antibiotic resistance was investigated. A washed cell suspension (0.5ml) containing approximately 10^{8} nutrient broth-grown cells was spread and dried onto the surface of nutrient agar plates, onto which were placed antibiotic-containing discs. The seeded plates were incubated at 30°C for 48 hours and the isolate KCT1 was termed resistant if no more inhibition was seen. The isolate KCT1 was found to be resistant to kanomycin (30μg), streptomycin (25μg) nitrofurantoin (300μg), erythromycin (30μg) and mecillinam (25μg). KCT1 was found to be sensitive to penicillin G (10iu), ampicillin (25μg), tetracycline (10μg), sulphonamide (300μg), bacitracin (10iu), sulphamethoxazole/trimethroprim (25μg), gentamycin (30μg), chloramphenicol (10μg), carbenicillin (100μg), nalodixic acid (30μg) and neomycin (30μg).

3.6.5 The effect of temperature on the growth of KCT1.

Isolate KCT1 was grown in shake flask cultures at 25, 30 and 35°C in minimal medium containing 1.0g l^{-1} THFTCA and at 200 rpm. Growth was followed by measuring the change in the optical density of the cultures at 530nm against an uninoculated blank. Table 32 gives the specific growth rates of KCT1 at each of the

temperatures investigated at the time of isolation. As the temperature of incubation increased the specific growth rate of KCT1 decreased. The isolate grew fastest at 25°C with a specific growth rate of 0.013h^{-1} and grew slowest at 35°C with a specific growth rate of 0.008h^{-1}. The specific growth rate of KCT1 at 30°C was 0.01h^{-1}.

3.6.8 Change of pH during growth of KCT1 at 30°C.

After 18 months subculturing on minimal agar slants containing 1.0g l^{-1} THFTCA, the specific growth rate of KCT1 had become 0.014h^{-1}. Figure 24 shows the increase in pH of the culture fluid from pH6.4 to pH 7.1 over an 82 hour period at 30°C, 200 rpm and 1.0g l^{-1} THFTCA in the presence of isolate KCT1. The change in pH appears to be directly proportional to the growth of the isolate.

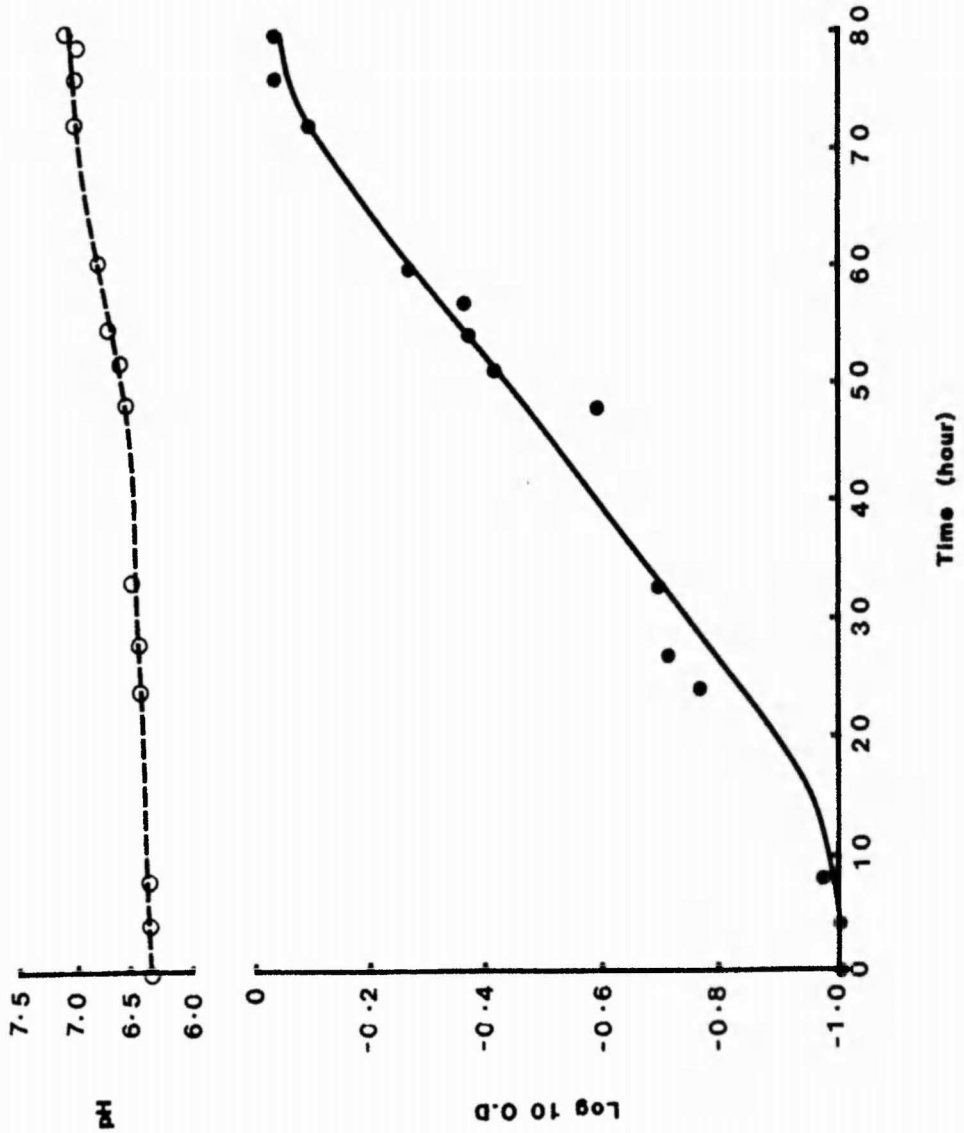
3.6.7 Plasmid detection in KCT1.

Detection of plasmids was attempted by the method of Kado and Liu (1981), but it was found that KCT1 did not contain any plasmids which could be isolated by this method.

Table 32. The effect of temperature on the specific growth rate of KCT1.

Temperature (°C)	Specific growth rate (h^{-1})
25	0.013
30	0.010
35	0.008

Figure 24. Change of pH during growth of KCTI at 30°C.



3.7 Degradation of 3-chlorobenzoic acid (3CBA).

3.7.1. Removal of 3CBA in the SCAS test.

3CBA was added to the SCAS unit at a rate of 20mg l^{-1} (as total organic carbon) per day. The amount of 3CBA remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). Figure 25a shows that the DOC values for the test and control units did not converge until day 54. After this time the control and test units gave very similar values, showing removal of 3CBA indicating biodegradation of 3CBA by activated sludge.

The same data were used to calculate the parameter degradation/daily addition. Figure 25b shows that little degradation/daily addition was observed during the first 28 days of the SCAS test. Between day 30 and day 60 of the SCAS test the amount of degradation increased dramatically and after day 60 up to 100% degradation was seen during each 23 hour period. The level of degradation remained at 100% until addition of 3CBA to the SCAS unit was discontinued at day 68. After this time no further measurements of DOC in the SCAS unit were made. The SCAS unit was periodically used as a source of inoculum for the die-away tests after the

Figure 25a. Removal of 3CBA in the SCAS test.

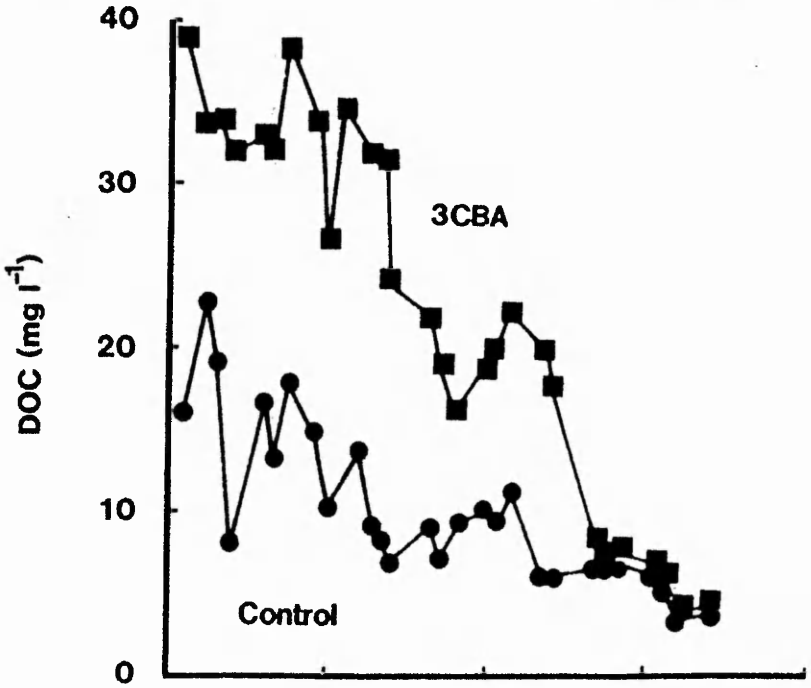
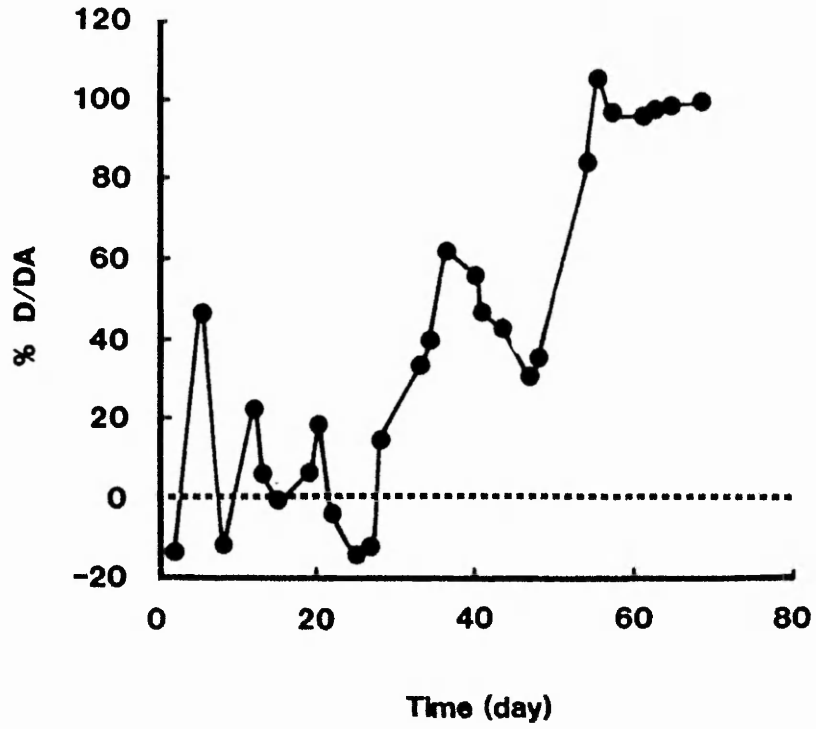


Figure 25b. Removal of DOC on a daily basis.



discontinued addition of 3CBA.

3.7.2 Removal of 3CBA in die-away tests.

It can be seen from Table 33 that in all the die away tests a high level of 3CBA removal was measured. This high level of removal was observed with an inoculum of activated sludge which had never previously been exposed to 3CBA (Table 33). High levels of 3CBA removal were measured even after the activated sludge used as an inoculum had not been exposed to 3CBA for 178 days. Figure 26 shows how the maximum level of degradation remained fairly constant at between 90 and 100% in all the die-away tests performed. The abiotic control showed only 20% removal of 3CBA during a 28 day die-away test (Table 33). No change in pH was detected during the 28 day test period.

3.7.3 Viability of microorganisms in activated sludge exposed to 3CBA.

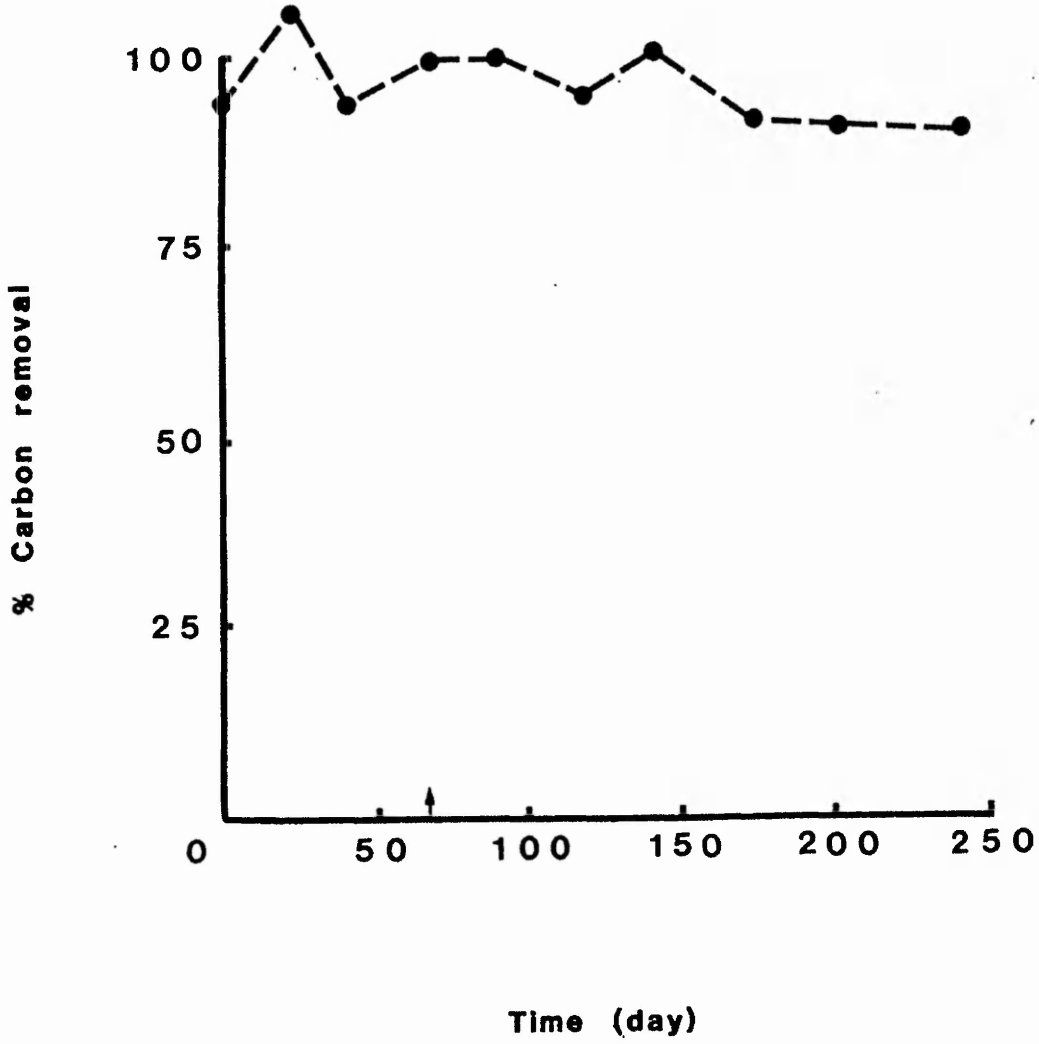
The number of viable microorganisms present in the activated sludge exposed to 3CBA remained relatively constant at 2-6x10^7 viable microorganisms ml^{-1} (Table 34). The fluctuation in total microorganisms present in the control and exposed activated sludge were very similar throughout the duration of the test.

Table 33. 3CBA : die-away test results.

Period for which SCAS test sludge inoculum had been exposed to 3CBA (days)	Days of sampling die-away tests and % biodegradation observed			
	7	14	21	28
0	34,38 (36±2,8)	94,92 (93±1,4)	91,91 (91±0)	93,96 (94,5±2,1)
21	85,32 (83,5±2,1)	91,91 (91±0)	91,103 (97±8,5)	106,106 (106±0)
42	81,83 (82±1,4)	100,100 (100±0)	96,96 (96±0)	94,98 (96±2,8)
68	92,93 (92,5±0,7)	91,93 (92±1,4)	100,100 (100±0)	87,91 (89±2,8)
91	104 -	97 -	101 -	94 -
119	70 -	94 -	94 -	95 -
143	96 -	99 -	98 -	100 -
176	84,83 (83,5±0,7)	90,91 (90,5±0,7)	92,91 (91,5±0,7)	85,88 (86,5±2,1)
204	N.D.	N.D.	N.D.	88,90 (89±1,4)
246	100,100 (100±0)	87,87 (87±0)	96,96 (96±0)	88,88 (88±0)

Abiotic control 20±2% removal
Deacclimatization started on day 68 of SCAS test
N.D. = Not determined
Values in brackets = ($x \pm \sigma_{n-1}$)

Figure 26. Maximum measured removal of carbon from die-away tests performed with 3CBA, determined as DOC.



↑ Addition of 3CBA to test SCAS unit discontinued.

3.7.4 Inhibition of activated sludge growth by 3CBA.

Table 35 shows the effect of 3CBA on the ability of activated sludge to grow on acetate/nutrient broth medium. It can be seen that no inhibition of growth of activated sludge was observed. The EC50 for 3CBA is therefore greater than 100mg l^{-1}.

3.7.5 Isolation of 3CBA degrading microorganisms.

Four different microorganisms capable of utilising 3CBA as their sole source were isolated by enrichment of activated sludge with 0.5g l^{-1} 3CBA at 30°C and 200rpm in batch culture. These isolates were designated KC13, KC16, KC20 and KC21 respectively.

3.7.6 Identification and characterization of KC13, KC16, KC20 and KC21.

3.7.6.1 API 20E-biochemical tests performed at 35°C.

Table 36 lists the results of biochemical tests performed on the four 3CBA utilizing isolates using the API 20E test system.

KC13 is a Gram-negative motile rod, which is catalase - positive, converts nitrate to nitrite, is cytochrome oxidase-positive and can grow in MacConkey medium at 37°C. The isolate can utilize the following compounds as substrates : ortho-nitro-phenyl-galactoside,

Table 34. Number of viable microorganisms ml^{-1} found in activated sludge after varying periods of exposure to 3CBA in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	3CBA dosed unit
0	2.10	2.10
28	1.32	4.87
42	3.20	4.00
85	3.00	4.78
110	4.87	6.85
160	3.33	3.09
212	4.87	2.15

Table 35. Inhibition of activated sludge growth by 3CBA. Values represent % reduction in O.D. (530nm) relative to the control after 6 hours incubation.

	Concentration (mg l^{-1})				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	-17	-11	-3	-3	-15

arginine, lysine, ornithine, sodium citrate urea and glucose. KC13 was unable to utilize any other sugars apart from glucose in this test. This isolate has been identified as Pseudomonas putrefaciens with a 92.9% reliability of identification.

KC16 is a Gram-negative motile rod which is catalase-positive, converts nitrate to nitrite, is cytochrome oxidase-positive and can grow in MacConkey medium at 37°C. Isolate KC16 was found to be able to utilize the following compounds as substrates, arginine, sodium citrate, urea and glucose. No other sugars in this test were utilized by KC16. The isolate has been identified as Pseudomanas fluorescens/putida with an 87% reliability of identification.

KC20 has been identified as being a Gram-negative, motile rod which is catalase-positive, converts nitrate to nitrite, is cytochrome oxidase-positive and can grow in MacConkey medium at 37°C. The only substrates this organism could utilize were sodium citrate and the two sugars, glucose and melibiose. This isolate was identified only to genus level and was found to be a Pseudomonas spp.

The last of the isolates which could utilize 3CBA as its sole carbon source was KC21. This organism is a Gram-negative, motile rod which is catalase-positive, converts nitrate to nitrite and is cytochrome oxidase-positive. KC21 was found to utilize the following

compounds as substrates, arginine, sodium citrate and three sugars, glucose, melibiose and arabinose. KC21 has been identified as a Pseudomonas fluorescens/putida with a 91% reliability of identification.

3.7.6.2 Carbohydrate utilization by the 3CBA-utilizing isolates.

All four isolates were tested for carbohydrate utilization to help differentiate further between similar isolates, and to observe the diversity of carbohydrate utilization. KC13 could utilize 9 of the 49 carbohydrates, against which it was tested, after 72 hours incubation at 30°C (Table 37). The carbohydrates utilized by KC13 were : L-arabinose, D-xylose, galactose, D-glucose, D-mannose, N-acetyl glucosamine, esculine, melibiose and D-fucose.

KC16 could also utilize 9 of the 49 carbohydrates it was tested against (Table 37). The 9 carbohydrates utilized were : L-arabinose, ribose, D-xylose, galactose, D-glucose, D-mannose, N-acetyl glucosamine, esculine and D-fucose. The third isolate KC20 could utilize 10 of the 49 carbohydrates it was tested against after 72 hours incubation at 30°C (Table 37). The carbohydrates utilized were D-arabinose, L-arabinose, ribose, galactose, D-glucose, D-mannose, N-acetyl glucosamine, esculine, trehalose and D-fucose. The

Table 36. API 20E-biochemical tests performed on isolate KC13, KC16, KC20 and KC21 which utilize 3CBA.

Test	Test Substrates	Isolates			
		KC13	KC16	KC20	KC21
ONPG	ortho-nitro-phenyl-galactoside	+	-	-	-
ADH	arginine	+	+	-	+
LDC	lysine	+	-	-	-
ODC	ornithine	+	-	-	-
CIT	sodium citrate	+	+	+	+
H_2S	sodium thiosulfate	-	-	-	-
URE	urea	+	+	-	-
TDA	tryptophane	-	-	-	-
IND	tryptophane	-	-	-	-
UP	sodium pyruvate	-	-	-	-
GEL	Kohn's gelatin	-	-	-	-
GLU	glucose	+	+	+	+
MAN	mannitol	-	-	-	-
INO	inositol	-	-	-	-
SOR	sorbitol	-	-	-	-
RHA	rhamnose	-	-	-	-
SUC	sucrose	-	-	-	-
MEL	melibiose	-	-	+	+
AMY	amygdalin	-	-	-	-
ARA	arabinose	-	-	-	+
OX	H_2S or ONPG tube	+	+	+	+
NO_3-NO_2	GLU tube	+	+	+	+
Motility		+	+	+	+
CAT	in any negative sugar	+	+	+	+

final isolate KC21 could utilize 10 of the 49 carbohydrates it was tested against, they were L-arabinose, ribose, D-xylose, galactose, D-glucose, D-mannose, esculine, melibiose, saccharose and D-fucose.

The differences found between the four isolates with respect to carbohydrate utilization were as follows : KC13 was the only isolate which did not utilize ribose; only KC21 was unable to utilize N-acetyl glucosamine but was the only isolate which could utilize saccharose; melibiose was utilized by KC13 and KC21 but not by KC16 and KC20; trehalose was utilized only by isolate KC20. All the other results obtained for each isolate were identical to one another.

3.7.6.3 Growth inhibition of the 3CBA utilizing isolates by antibiotics.

Antibiotic resistance was investigated in order to provide a further method of characterizing and distinguishing the four 3CBA-degrading isolates from one another. A washed cell suspension (0.5ml) containing approximately 10^{8} nutrient broth-grown cells was spread and dried onto the surface of nutrient agar plates, onto which were placed antibiotic-containing discs. The seeded plates were incubated at 30°C for 48 hours and resistance recorded if no zone of inhibition was seen. Table 38 represents the results obtained for growth inhibition by antibiotics of KC13, KC16, KC20 and KC21.

Table 37. Utilization of carbohydrates by isolates KC13, KC16, KC20 and KC21.

	Isolates			
Carbohydrate	KC13	KC16	KC20	KC21
Glycerol	-	-	-	-
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	+	+	+	+
Ribose	-	+	+	+
D-Xylose	+	+	+	+
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β-Methylxyloxide	-	-	-	-
Galactose	+	+	+	+
D-Glucose	+	+	+	+
D-Fructose	-	-	-	-
D-Mannose	+	+	+	+
L-Sorbose	-	-	-	-
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
α Methyl-D-amoside	-	-	-	-
α Methyl-D-glucoside	-	-	-	-
N-Acetyl glucosamine	+	+	+	-
Amygdaline	-	-	-	-

Carbohydrate	KC13	KC16	KC20	KC21
Arbutine	-	-	-	-
Esculine	+	+	+	+
Salicine	-	-	-	-
Cellobiose	-	-	-	-
Maltose	-	-	-	-
Lactose	-	-	-	-
Melibiose	+	-	-	+
Saccharose	-	-	-	+
Trehalose	-	-	+	-
Inuline	-	-	-	-
Melezitose	-	-	-	-
D-Raffinose	-	-	-	-
Amidon	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
ß Gentiobiose	-	-	-	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	+	+	+	+
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
L-Arabitol	-	-	-	-
Gluconate	-	-	-	-
2 ceto-gluconate	-	-	-	-
5 ceto-gluconate	-	-	-	-

Table 38. Growth of isolates KC13, KC16, KC20 and KC21 in the presence of antibiotics.

	Isolates			
Antibiotic	KC13	KC16	KC20	KC21
Pencillin G 10iu	-	-	-	+
Ampicillin 25µg	-	-	-	+
Tetracycline 10µg	-	-	-	-
Kanomycin 30µg	+	-	+	-
Sulphonamide 300µg	+	+	+	-
Bacitracin 10iu	+	+	+	+
Sulphanethoxazole/ Trimethroprim 25µg	+	+	+	+
Gentamycin 30µg	+	+	+	+
Chloramphenicol 10µg	+	-	-	-
Streptomycin 25µg	+	+	+	-
Nitrofurantoin 300µg	+	+	+	+
Carbenicillin 100µg	-	-	-	-
Erythromycin 30µg	-	-	-	+
Mecillinam 25µg	+	+	+	-
Naladoxic acid 30µg	+	+	+	-
Neomycin 30µg	+	+	+	-

+ = Resistance (growth)

- = Inhibition (no growth)

Only KC21 was resistant to pencillin G (10iu), ampicillin (25μg) erythromycin (30μg) and sensitive to sulphonamide (300μg), gentamycin (30μg), streptomycin (25μg), nalodixic acid (30μg) and neomycin (30μg). All isolates were sensitive to tetracycline (10μg), carbenicillin (100μg) and mecillinam (25μg). All isolates were found to be resistant to bacitracin (10iu), sulphamethozamole/trimethroprim (25μg) and nitrofurantoin (300μg). KC13 was the only isolate resistant to chloramphenicol (10μg), and only KC13 and KC20 were resistant to kanomycin (30μg).

3.7.7 The effect of temperature of the growth of the 3CBA-utilizing isolates.

The isolates were grown in shake flask cultures at 25, 30 and 35°C in minimal medium containing 0.5g l^{-1} 3CBA and at 200 rpm. Growth was measured by following the change in the optical density of the cultures at 530nm against an uninoculated blank. Table 39 gives the specific growth rates of the 3CBA-utilizing isolates at the temperatures investigated at the time of isolation. For KC13 the specific growth rates at 25 and 30°C were identical but the rate was lower at 35°C. The change in incubation temperature had no effect on the specific growth rate of KC16, which was higher than KC13 at each temperature investigated. KC20 had the lowest specific growth rates of all the isolates at each temperature, it

Table 39. The effect of temperature on the specific growth rate (h^{-1}) of the 3CBA utilizing isolates.

Isolate	Temperature	Specific growth rate (h^{-1})
KC13	25	0.067
	30	0.067
	35	0.043
KC16	25	0.075
	30	0.075
	35	0.075
KC20	25	0.060
	30	0.060
	35	0.037
KC21	25	0.077
	30	0.077
	35	0.060

Table 40. The effect of storage on 3CBA minimal media slants (4°C) on the specific growth rate (h^{-1}) at 30°C of the 3CBA utilizing isolates.

Time (months) after isolation that specific growth rates were determined	Specific growth rate (h^{-1}) of isolates			
	KC13	KC16	KC20	KC21
0	0.067	0.075	0.060	0.077
20	0.080	0.080	0.080	0.150

grew equally well at 25 and 30°C but more slowly at 35°C. KC21 had the highest specific growth rate of all the isolates at 25 and 30°C, but its specific growth rate was reduced at 35°C.

After the isolates had been subcultured and stored on minimal medium agar plus 0.5g l^{-1} 3CBA slopes at 4°C for 20 months the specific growth rates of each organism at 30°C had increased. The specific growth rates of KC13, KC16 & KC20 had risen to the same value namely 0.080h^{-1} at 30°C (while the specific growth rate of KC21 had almost doubled to 0.150h^{-1} at 30°C (Table 40). No change in the pH of culture fluids was detected during any growth determination on isolates KC13, KC16, KC20 and KC21 with 3CBA.

3.7.8 Plasmid detection in isolates KC13, KC16, KC20 and KC21.

Detection of plasmids was attempted by the method of Kado and Liu (1981). Isolates KC13, KC16 and KC20 contained one plasmid each which appeared to be the same one. KC21 contained 3 plasmids, two with smaller molecular weights than those found in the other isolates and one heavier. The plasmid with the middle molecular weight appeared to be the most prevalent of the three.

3.7.9 Growth, dehalogenation and ring cleavage of 3CBA.

Ring cleavage was measured by following the change in absorbance of U.V. light at 275nm in culture fluid against time. A decrease in the amount of U.V. light absorbed corresponded to a decrease in the amount of aromatic ring structures present in the culture fluid. Dehalogenation could be followed by monitoring halide-ion liberation (chloride) from 3CBA in the culture fluid.

Figure 27 shows that as KC13 grew a corresponding reduction in the U.V. absorbance is seen. After 14 hours incubation a 50% reduction in U.V. absorption of the maximum observed decrease was observed. The liberation of halide-ions into the culture fluid did not reach 50% of the maximum value observed until the culture had been growing for 29 hours. Figure 28 shows that as KC16 grows a corresponding reduction in absorbance at 275nm is observed as with KC13. After 13½ hours incubation a 50% reduction in U.V. absorbance of the maximum observed decrease was observed. The liberation of halide-ions into the culture fluid did not reach 50% of the maximum value observed until the culture had been growing for 20 hours.

It can be seen from Figure 29 that as with KC13 and KC16 as KC20 grows there is a corresponding reduction in absorbance at 275nm. As with KC16 50% reduction in U.V. absorbance was observed 13½ hours after the start of

Figure 27. Ring cleavage and chloride release during growth of KC13 on 3CBA (0.5g l^{-1}) at 30°C and 200 rpm.

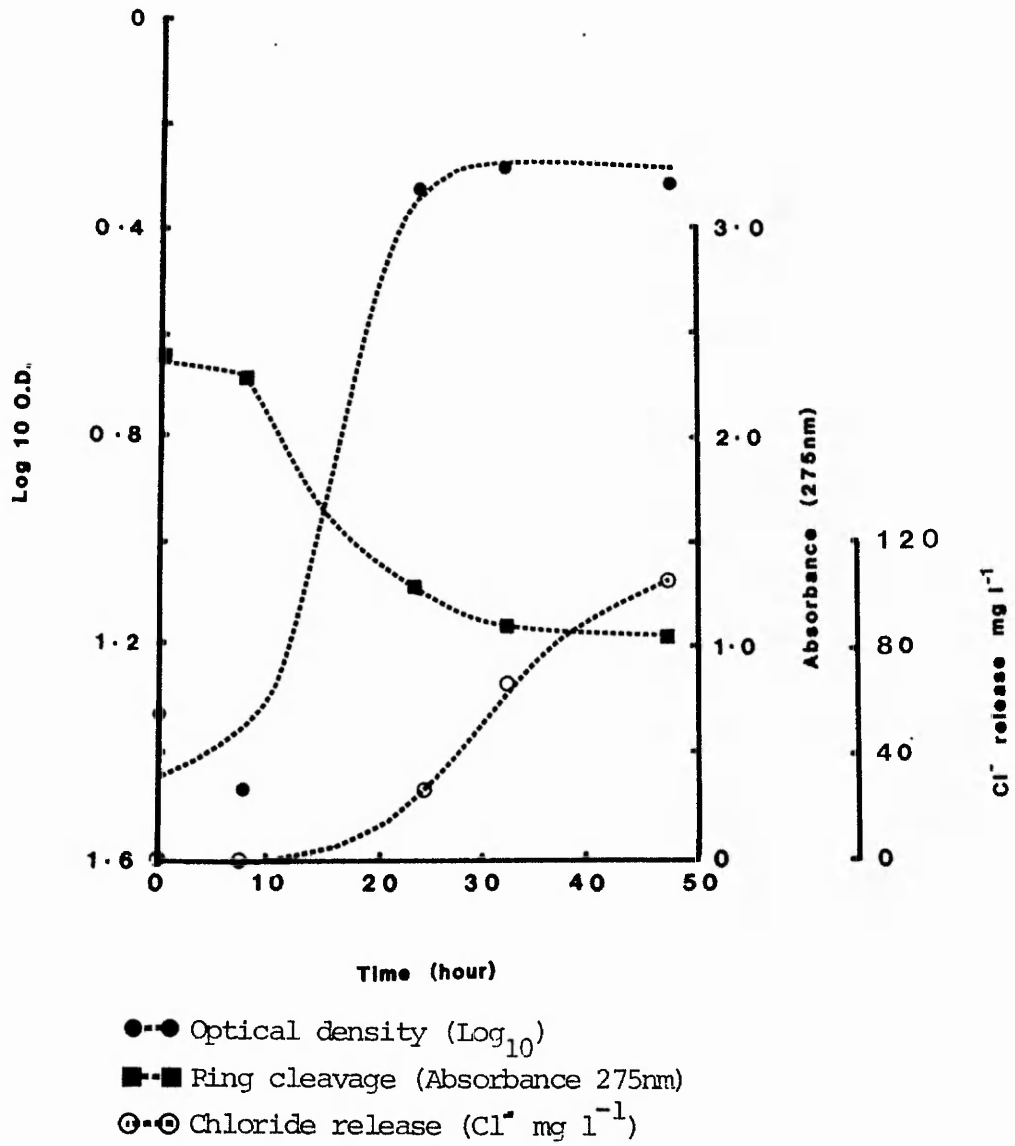


Figure 28. Ring cleavage and chloride release during growth of KC16 on 3CBA (0.5g l^{-1}) at 30°C and 200 rpm.

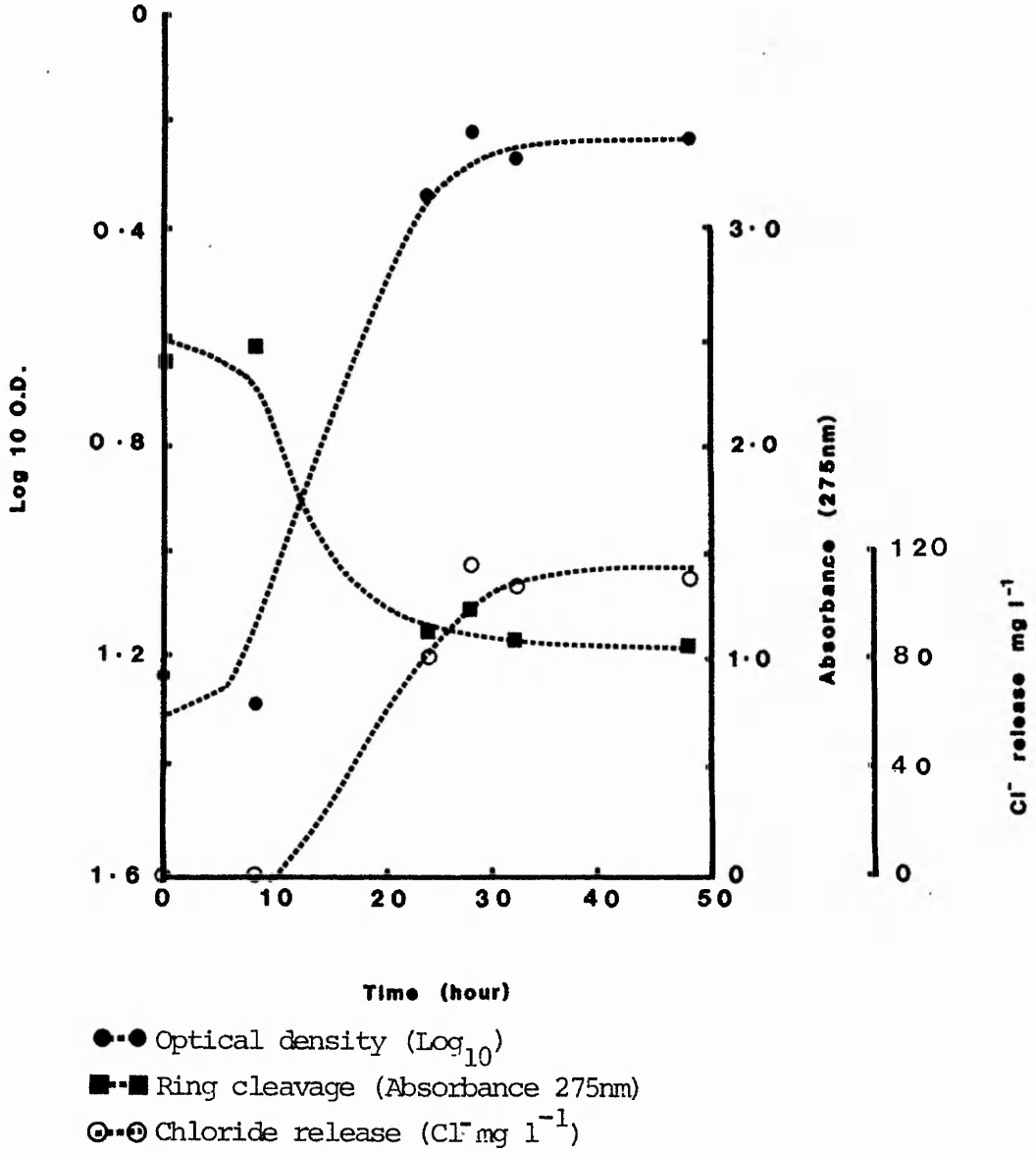


Figure 29. Ring cleavage and chloride release during growth of KC20 on 3CBA (0.5g l^{-1}) at 30°C and 200 rpm.

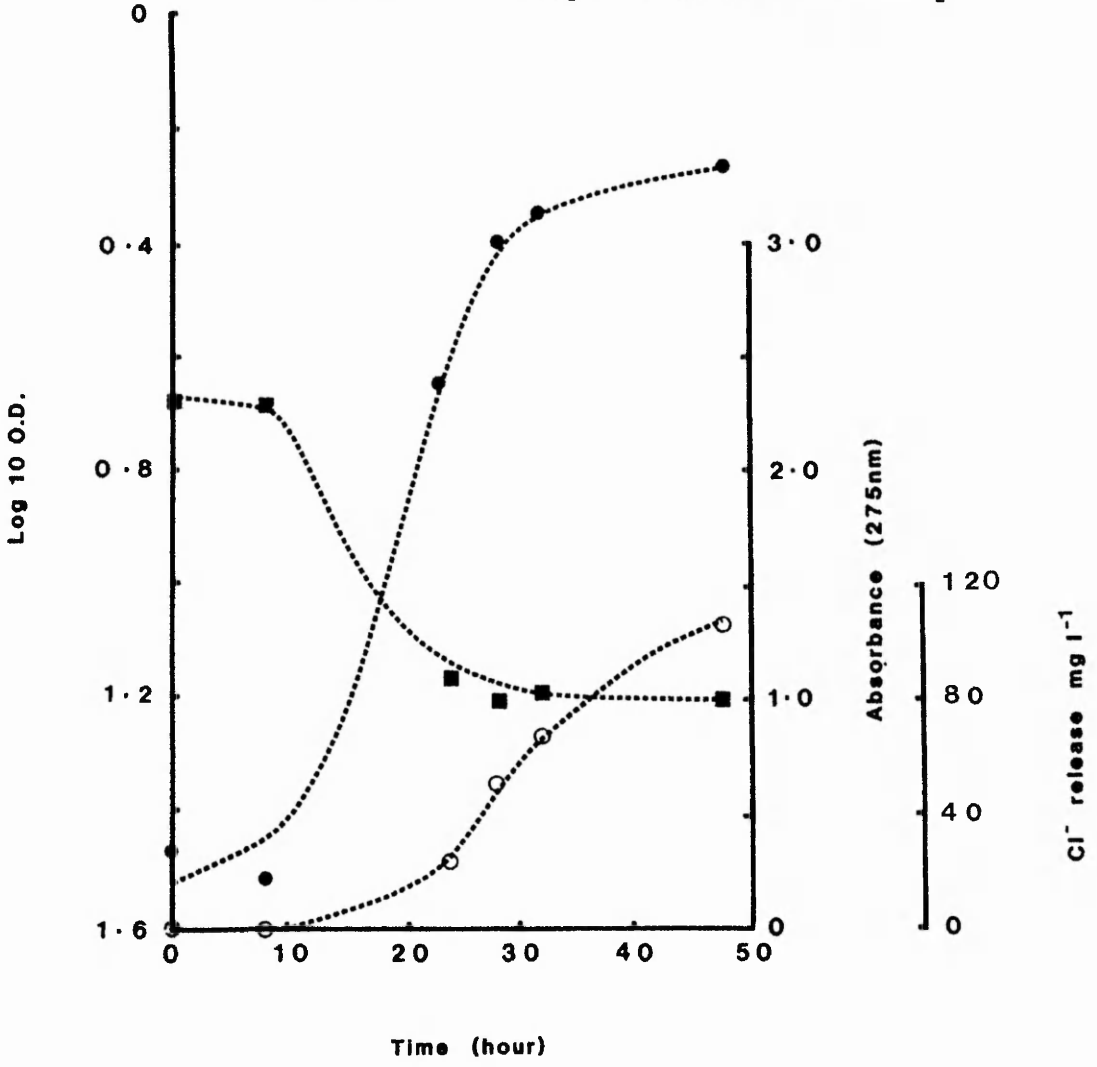
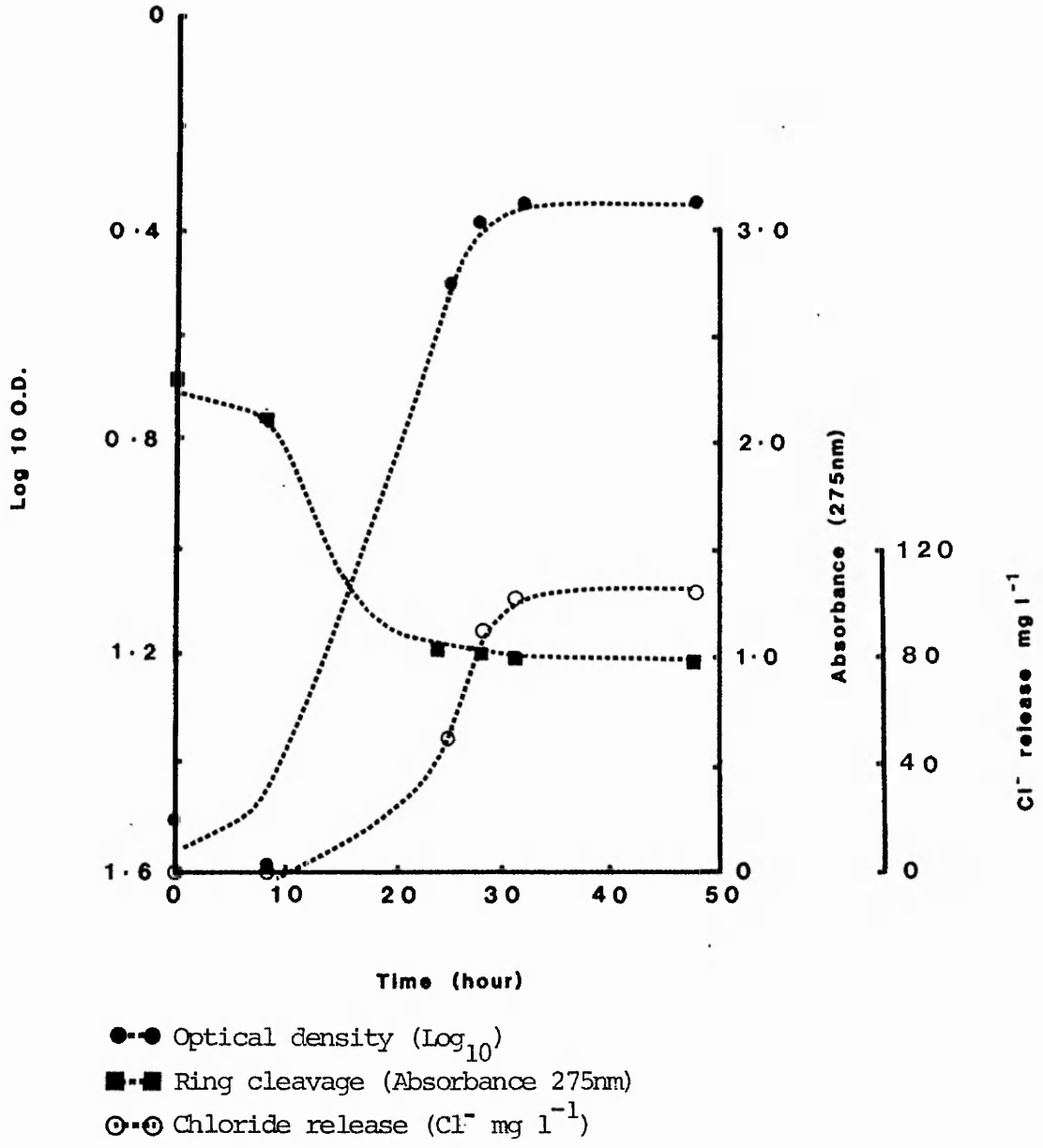


Figure 30. Ring cleavage and chloride release during growth of KC21 on 3CBA (0.5g l^{-1}) at 30°C and 200 rpm.



incubation but 50% halide-ion liberation was not observed until 30 hours after the start of incubation.

KC21 gave a 50% reduction in absorbance at 275nm of the maximum observed decrease within 11½ hours of the start of incubation and 50% of the maximum halide release within 24 hours (Figure 30).

3.7.10 The effect of 3CBA concentration on dehalogenation.

Each isolate was incubated at three different concentrations of 3CBA namely, 0.5, 0.75 and 1.0g l^{-1} and the halide-ion liberation measured. Figure 31 shows the results with isolate KC13 and it can be seen that as the concentration of 3CBA increases so does the lag before dehalogenation begins. The theoretical maximum values for halide-ion release from 0.5, 0.75 and 1.0g l^{-1} 3CBA were achieved by this isolate.

With KC16 (Figure 32), as the concentration of 3CBA increased the lag before halide ion release did not increase as was observed with isolate KC13. Maximum halide release from all concentrations was observed when KC16 was incubated with 3CBA.

KC20 (Figure 33) behaved similarly to KC13 but the lag before halide ion release was less at 0.75 and 1.0g l^{-1} 3CBA. As for the previous two isolates maximum halide ion release from all concentrations was observed when KC20 was incubated with 3CBA.

Figure 31. Chloride release against time for different 3CBA concentrations with KC13.

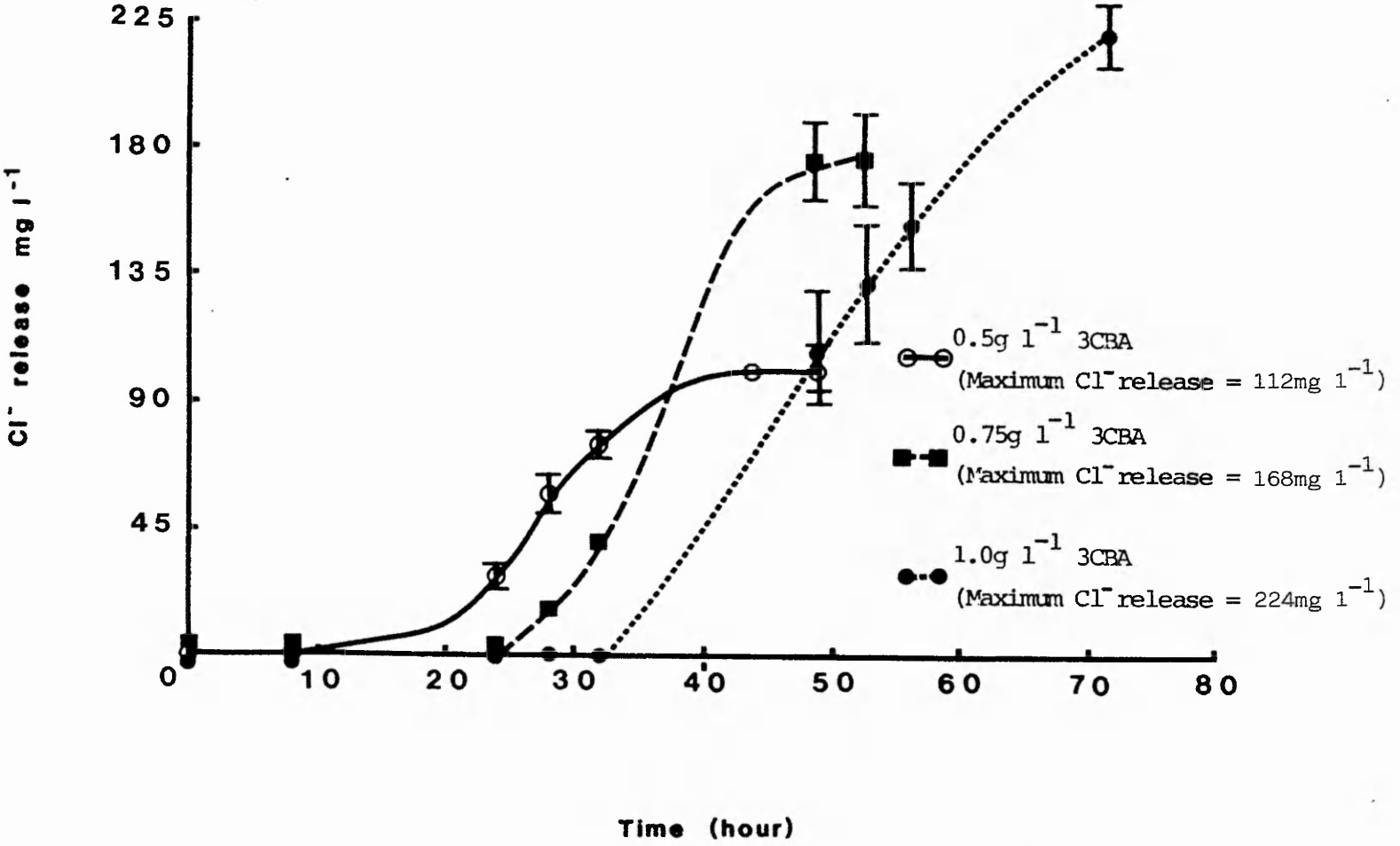


Figure 32. Chloride release against time for different 3CBA concentrations with KC16.

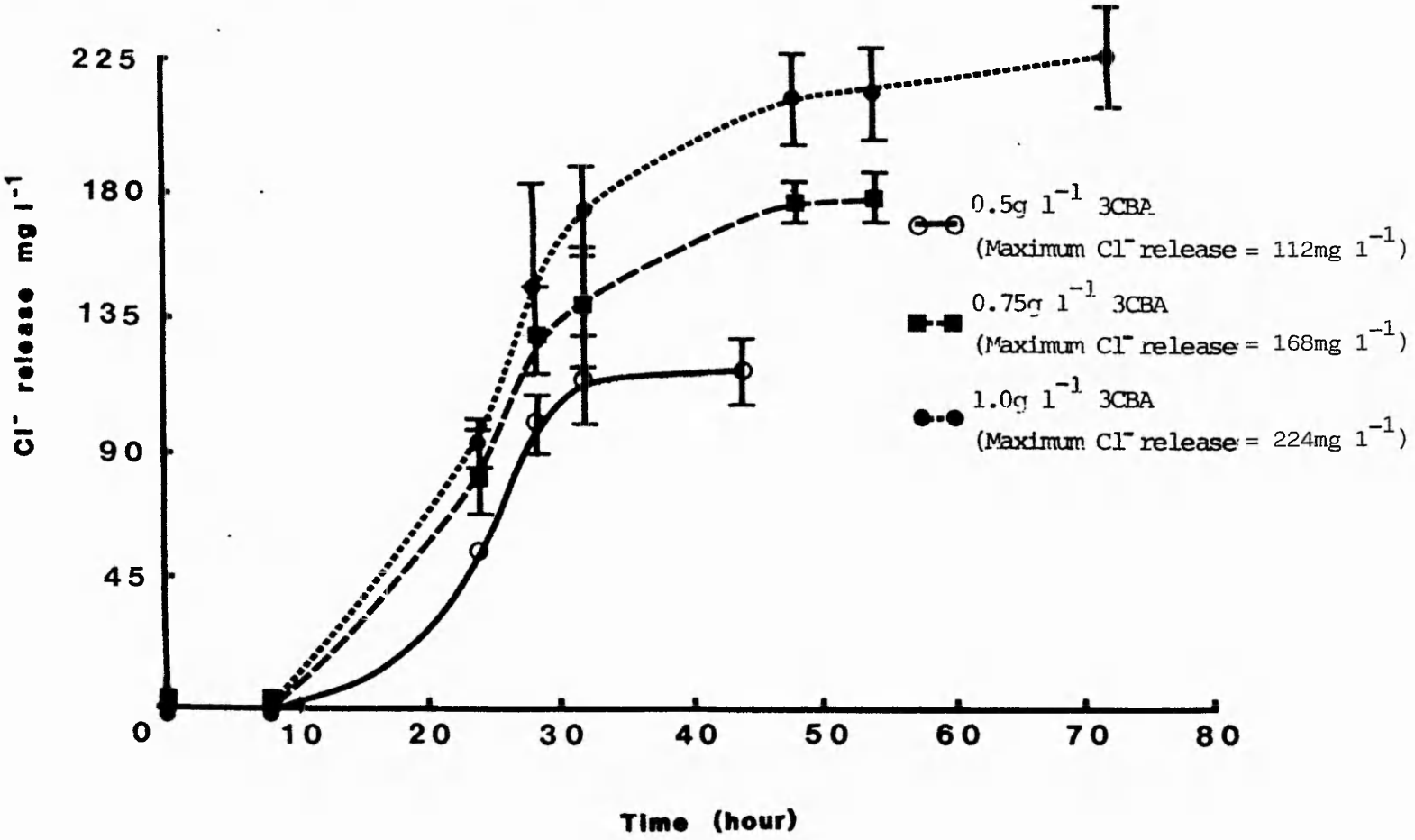


Figure 33. Chloride release against time for different 3CBA concentrations with KC20.

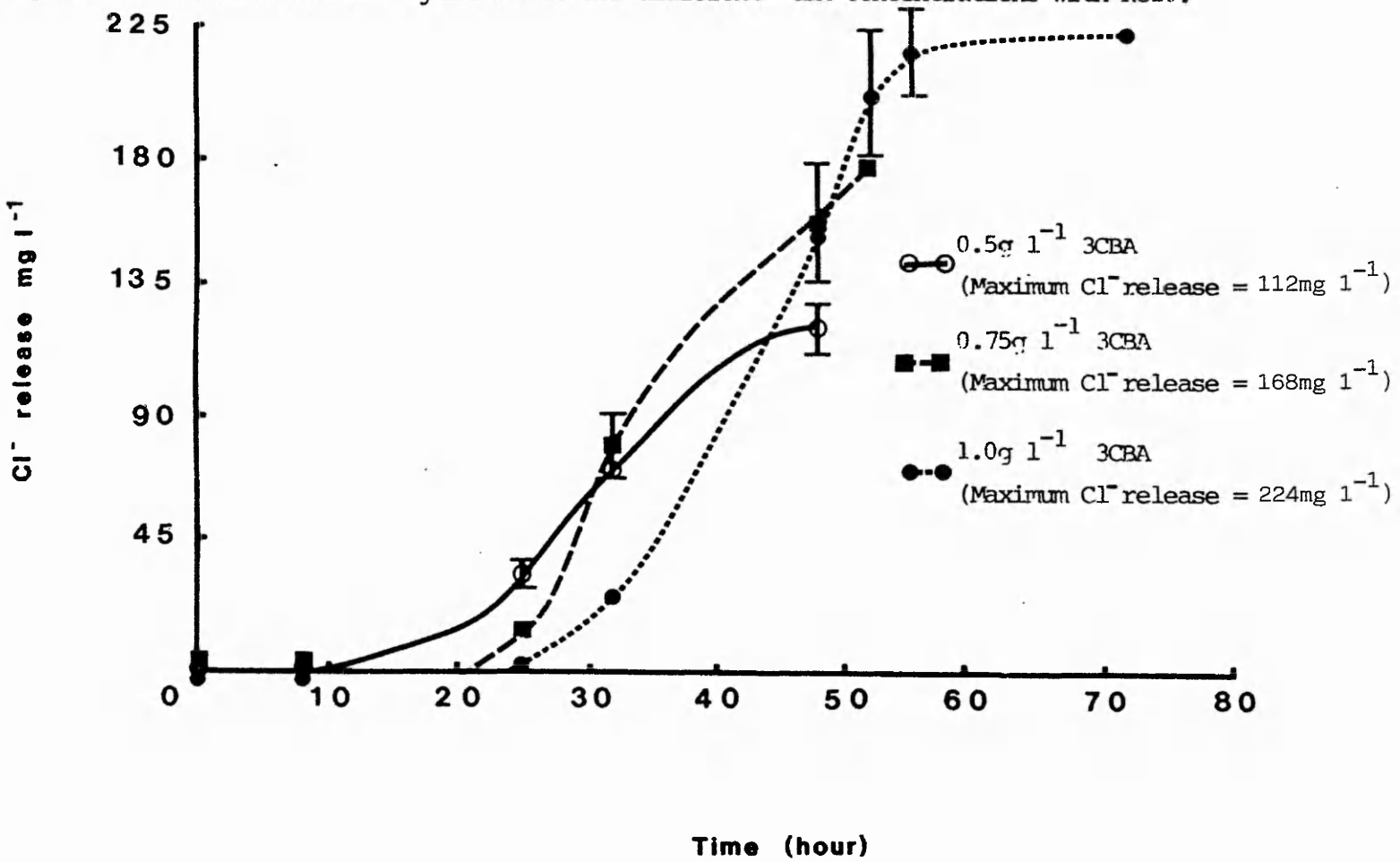
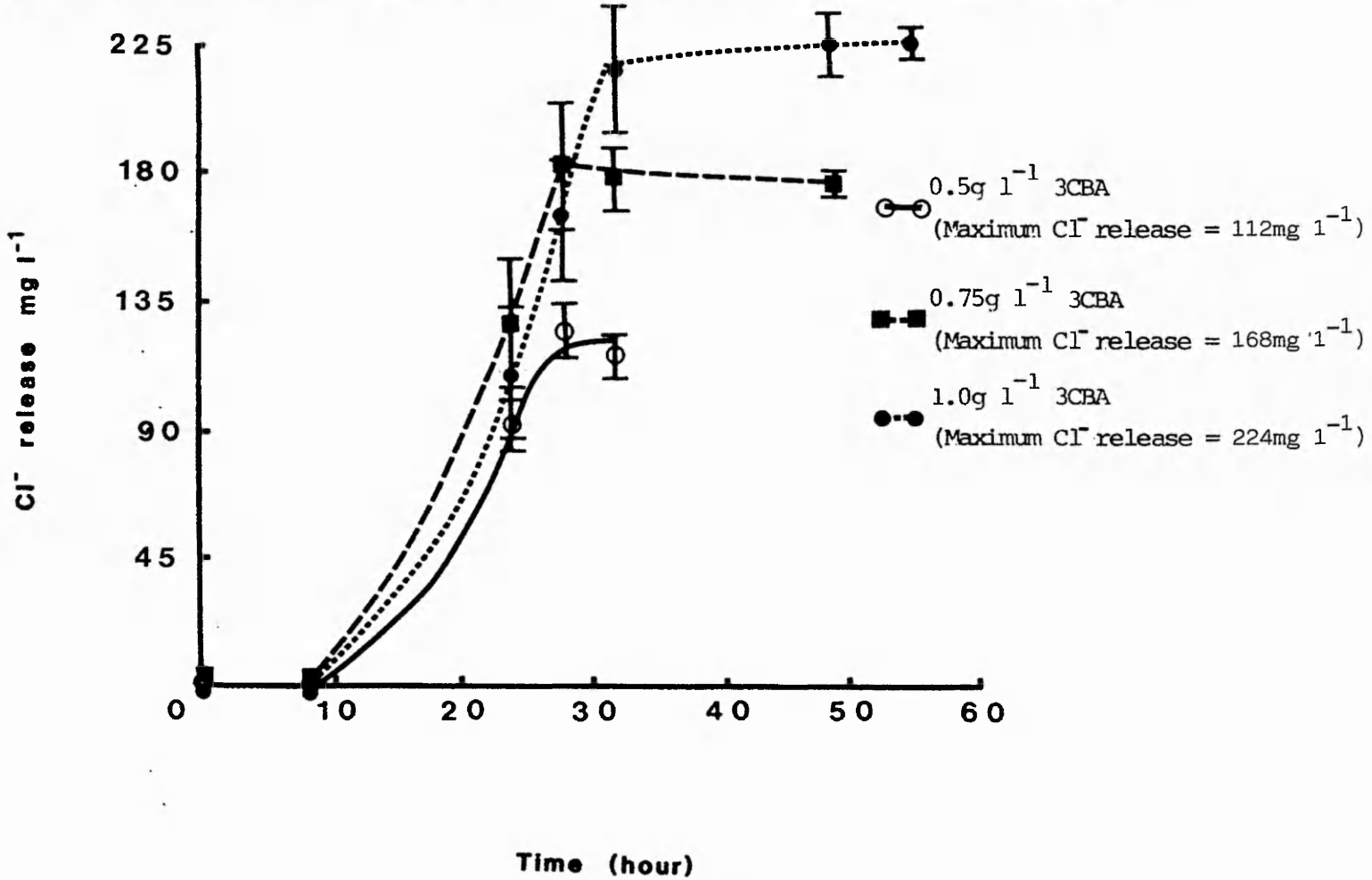


Figure 34. Chloride release against time for different 3CBA concentrations with KC21.



KC21 behaved in a very similar manner to KC16 when incubated with increasing concentrations of 3CBA. KC21 (Figure 34) achieved maximum theoretical halide release faster than KC16.

3.7.11 Growth of 3CBA utilizing isolates on other compounds as their sole carbon source.

Table 41 lists the isolates which could grow on the compounds used in the respirometry experiments. The isolates were plated out onto minimal agar plates plus test compound and incubated at 30°C for upto 7 days. The only compounds on which all the isolates were observed to grow, were 3CBA, acetate, chlorosuccinate, fumarate, glucose and pyruvate. In addition KC13 could grow on chloropropionate, the other isolates only showed weak growth on this compound. Similar weak growth was observed by all isolates on chloroacetate. None of the isolates grew on plates containing 2CBA, 4CBA, acetaldehyde, chloroacetaldehyde and gentisate. No growth was observed in the absence of carbon source.

3.7.12 Gilson respirometry with KC13, KC16, KC20 and KC21.

Respirometric studies were performed with all the isolates capable of utilizing 3CBA in a Gilson respirometer. Various possible intermediates were supplied as substrates in an attempt to identify the

Table 41. Growth of 3CBA utilizing isolates on other compounds as sole carbon sources in minimal agar.

Carbon source	Isolates			
	KC13	KC16	KC20	KC21
2CBA	-	-	-	-
3CBA	+	+	+	+
4CBA	-	-	-	-
Acetaldehyde	-	-	-	-
Acetate	+	+	+	+
Chloroacetaldehyde	-	-	-	-
Chloroacetate	-/+	-/+	-/+	-/+
Chloropropionate	+	-/+	-/+	-/+
Chlorosuccinate	+	+	+	+
Fumarate	+	+	+	+
Gentisate	-	-	-	-
Glucose	+	+	+	+
Pyruvate	+	+	+	+
None	-	-	-	-

+ Growth

-/+ Doubtful growth

- No growth

Table 42. Maximum oxygen uptake rates for isolates KC13, KC16 KC20 and KC21.

Substrate	Max. oxygen uptake rate ($\mu l O_2$ min^{-1} mg^{-1} dry weight cells.			
	KC13	KC16	KC20	KC21
2CBA	0.0	0.0	0.0	0.009
3CBA	0.037	0.052	0.054	0.127
4CBA	0.0	0.025	0.026	0.022
Acetaldehyde	0.109	0.093	0.049	0.103
Acetate	0.366	0.269	0.296	0.352
Fumarate	0.589	0.560	0.496	0.391
Gentisate	0.0	0.0	0.0	0.0
β-Ketoadipate	0.543	0.756	0.607	0.599
Pyruvate	0.366	0.366	0.338	0.307
Sodium benzoate	0.360	0.414	0.248	0.301

mechanism of 3CBA degradation. All cells were initially grown on 3CBA and harvested in mid-exponential phase, concentrated and washed in 0.1M phosphate buffer, pH 7.2. Table 42, shows the initial (maximum) uptake rates corrected for dry weight variations by the isolates KC13, KC16, KC20 and KC21 when attempting to metabolize various organic compounds. The rate with which the isolates took up oxygen in the presence of gentisate and 2CBA was zero with one exception, KC20 had a very low oxygen uptake rate with 2CBA. KC13 did not take up oxygen in the presence of 4CBA but KC16, KC20 and KC21 all exhibited low oxygen uptake rates in the presence of 4CBA. All four isolates had uptake rates at least twice as fast in the presence of 3CBA than in the presence of 4CBA. Of the remaining compounds tested only acetaldehyde had low uptake rates for each of the four isolates. The other compounds acetate, fumarate, β-ketoadipate, pyruvate and sodium benzoate all had faster oxygen uptake rates than those compounds already mentioned.

The theoretical and total observed oxygen uptake can be seen and compared (Table 43). Only KC21 had any measured oxygen uptake in the presence of 2CBA but this was only 22% of the maximum possible. Some oxygen uptake was observed with KC16, KC20 and KC21 in the presence of 4CBA, 23%, 13% and 23% of the theoretical total respectively. With 3CBA between 83% (KC13) and

99% (KC21) of the theoretical maximum oxygen uptake was observed for all the isolates, KC16 and KC20 having oxygen uptake levels of 88% and 94% respectively. All the isolates took up oxygen in the presence of acetaldehyde up to 47% for KC21, but not as much as when incubated with acetate, although the levels of oxygen uptake by KC21 in the presence of acetaldehyde (47%) and acetate (51%) were similar. Oxygen uptake was high by all isolates for fumarate up to 83% for KC21. Only KC21 showed any oxygen uptake in the presence of gentisate, 43% of the theoretical oxygen uptake. All isolates showed oxygen uptake in the presence of β-ketoadipate between 36 and 50% of the theoretical maximum as they did for pyruvate 60-73%. Sodium benzoate showed almost maximum theoretical oxygen uptake for all isolates.

3.8 Degradation of hexamethylenetetramine (hexa).

3.8.1 Removal of hexa in the SCAS test.

Hexa was added to the SCAS unit at a rate of 20mg l^{-1} (as total organic carbon) per day. The amount of hexa remaining after 23 hours aeration was determined as dissolved organic carbon (DOC). Figure 35a shows the DOC values for the hexa test unit to be very erratic and, with only a few exceptions, high compared to the control unit. No convergence of the DOC values for

Table 43. Experimental and theoretical total oxygen uptake values for isolates KC13, KC16, KC20 and KC21.

Substrate	Experimental oxygen uptake ($\mu l O_2$)				Theoretical oxygen uptake ($\mu l O_2$)
	KC13	KC16	KC20	KC21	
2CBA	0	0	0	34(22)	156,8
3CBA	130(83)	138(88)	148(94)	155(99)	156,8
4CBA	0	36(23)	20(13)	36(23)	156,8
Acetaldehyde	231(41)	200(36)	220(39)	264(47)	560
Acetate	291(65)	290(65)	295(66)	228(51)	448
Fumarate	453(67)	480(71)	440(65)	560(83)	672
Gentisate	0	0	0	62(43)	145,6
β-ketoadipate	491(44)	634(57)	521(46)	678(60)	1120
Pyruvate	350(62)	350(62)	407(73)	335(60)	560
Sodium Benzoate	130(100)	127(98)	133(102)	127(98)	130

Values in brackets = oxygen uptake as % of the theoretical maximum.

the two units was observed, indicating no removal of hexa by activated sludge over a 24 hour aeration period.

Degradation was also expressed as degradation/daily addition (Figure 35b). The data points show a great deal of scatter and range from values of +100% degradation down to -50% over the 205 day period of the experiment. The average observed removal of hexa was 16.8±43% during the SCAS test. There is little evidence to suggest that hexa may be degrading in the SCAS test. After 205 days, addition of hexa was discontinued and the SCAS unit continued to be used as a source of inoculum for die-away tests until day 302.

3.8.2 Removal of hexa in die-away tests.

No degradation of hexa was observed in the abiotic die-away test. For all the die-away tests the amount of degradation increased steadily throughout the period of the tests (Table 44); although on no occasion was 100% removal achieved. The maximum degradation observed was 81.5% by day 28 of the die-away test set up with inoculum taken from the SCAS unit on day 208.

Figure 36 shows the maximum observed degradation in each of the die-away tests performed. It can be seen that a general increase in degradation is observed until addition of hexa to the SCAS unit was discontinued at day 208, the only exception being the die-away test set

Figure 35a. Removal of hexa on the SCAS test.

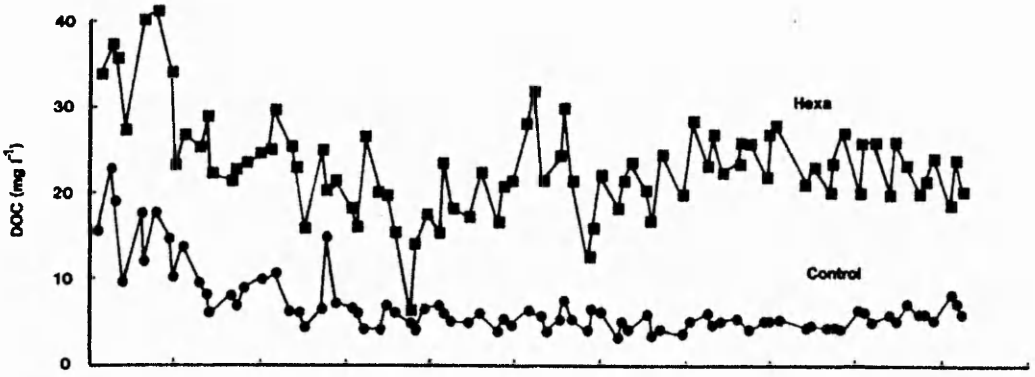
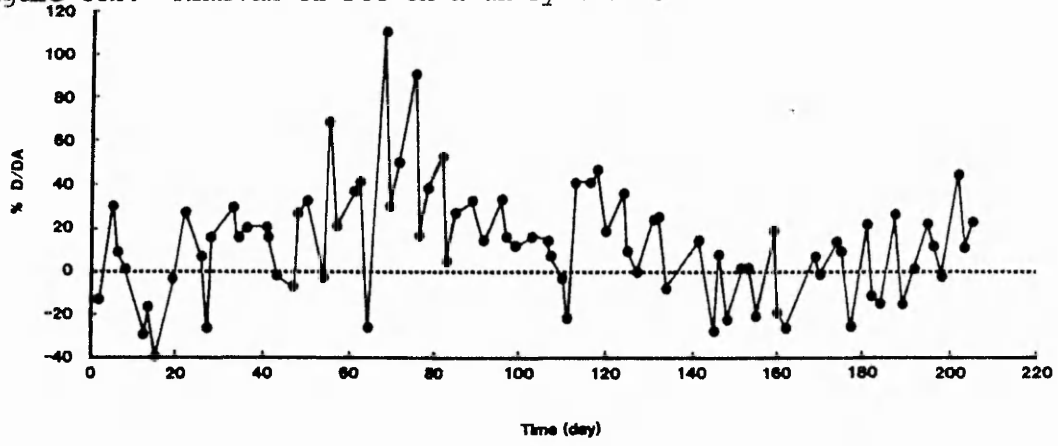


Figure 35b. Removal of DOC on a daily basis.



up at day 68 of the SCAS test which only showed 45% removal of hexa. This was the only deviation in the trend of increased degradation.

After addition of hexa to the SCAS unit was discontinued, a decrease in degradation was observed in the die-away tests performed. Even though the inoculum used for the die-away tests set up at day 246 and 302 of the SCAS test had not been exposed to hexa for 38 and 94 days respectively, degradation was observed in the die-away tests between 66.5 and 62%. The pH of the die-away test flasks decreased from pH 7.2 down to pH 6.95 over the 28 days of the test.

3.8.3 Use of the SCAS unit as a die-away test.

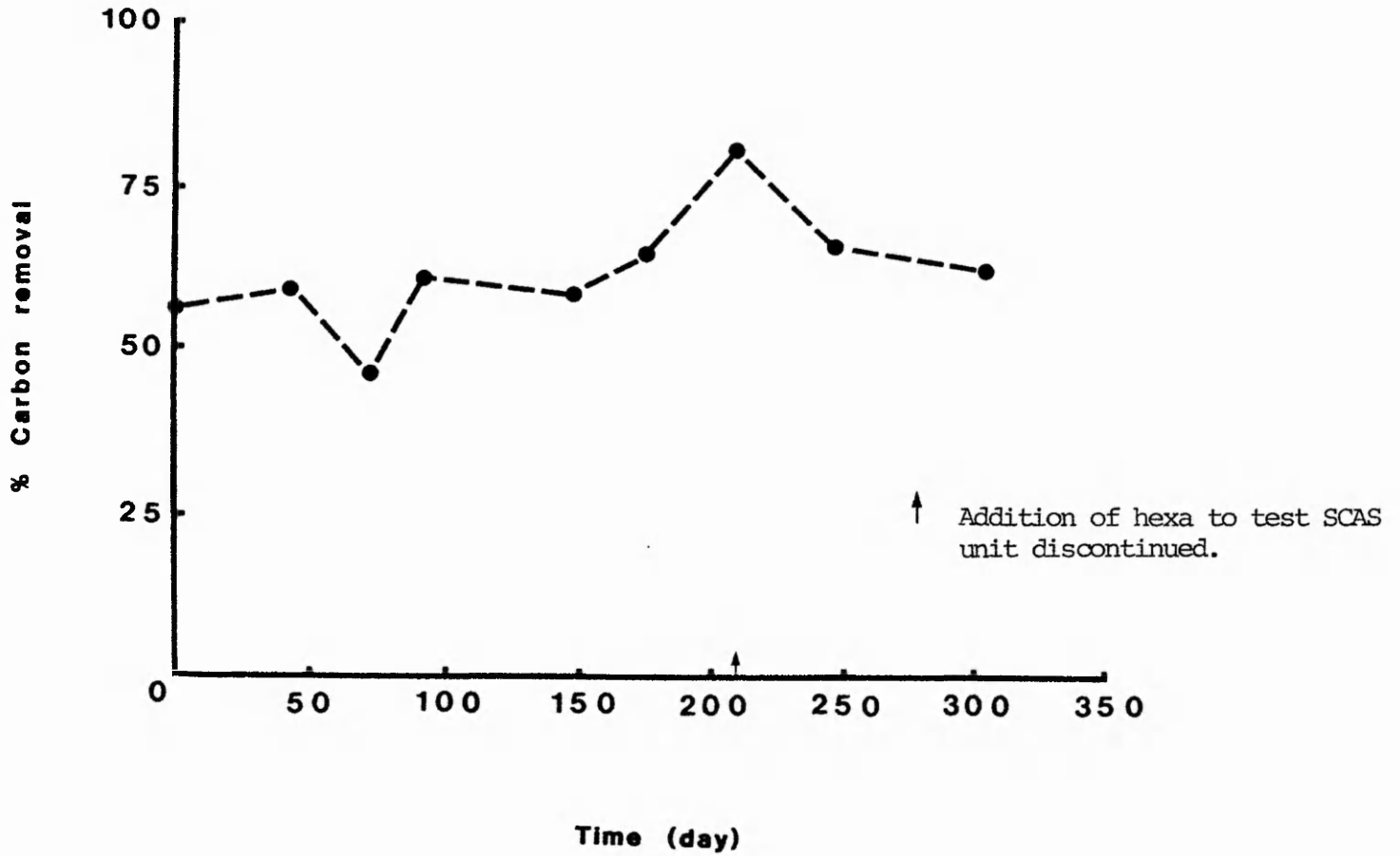
At day 302, 94 days after the addition of hexa had been discontinued, a single dose of hexa (20mg l^{-1}) was added to the SCAS unit. No further synthetic sewage was added to the unit. The SCAS unit was sampled at various times upto 28 days, the amount of hexa remaining determined as DOC, and the percentage carbon removal calculated. Figure 37 shows the percentage carbon removal plotted against time. A steady increase in the amount of carbon removed was observed until day 10 at which point the maximum observed removal of carbon (75%) was measured and a plateau reached. No further carbon was removed from the test before it was stopped after 28

Table 44. Hexa : die-away test results.

Period of which SCAS test sludge inoculum had been exposed to hexa (days),	Days of sampling of die-away tests and % biodegradation observed,			
	7	14	21	28
0	28,29 (28,5±0,7)	35,35 (35±0)	47,44 (45,5±2,1)	54,59 (56,5±3,5)
42	-28,-18 (-23±7,1)	23,23 (23±0)	44,46 (45±1,4)	54,64 (59±7,1)
68	-33,-33 (-33±0)	15,12 (13,5±2,1)	15,13 (14±1,4)	45,45 (45±0)
91	18,22 (20±2,8)	29,33 (31±2,8)	44,43 (43,5±0,7)	64,58 (61±4,2)
147	39,30 (34,5±6,4)	31,22 (26,5±6,4)	38,44 (41±4,2)	58,56 (57±1,4)
176	41,35 (38±4,2)	38,31 (34,5±4,9)	47,49 (48±1,4)	64,64 (64±0)
208	N,D,	N,D,	N,D,	82,81 (81,5±0,7)
246	28,32 (30±2,8)	28,33 (30,5±3,5)	63,65 (64±1,4)	63,70 (66,5±4,9)
302	32,43 (37,5±7,8)	41,52 (46,5±7,8)	48,58 (53±7,1)	61,63 (62±1,4)

Abiotic control 0 % removal
N,D, = Not determined
Values in brackets = $(x \pm \sigma_{n-1})$
Deacclimatization started on day 208 of SCAS test,

Figure 36. Maximum measured removal of carbon from die-away tests performed with hexa, determined as DOC.



days. The 75% removal of carbon corresponds to 15mg C l^{-1}, (29.2mg l^{-1} of hexa).

3.8.4 Viability of microorganisms in activated sludge exposed to hexa.

The number of viable microorganisms present in activated sludge exposed to hexa fell from an initial level of $2.1x10^{7}$ microorganisms ml^{-1} before exposure to $3.8x10^{5}$ microorganisms ml^{-1} during the first 28 days of exposure (Table 45). The level of microorganisms present in the activated sludge remained relatively constant after this initial large decrease in numbers. The number of microorganisms present in the control unit remained relatively constant at the order of 10^{7} microorganisms ml^{-1} although the actual number fluctuated between 1.32 and $4.87x10^{7}$ microorganisms ml^{-1}.

3.8.5 Inhibition of activated sludge growth by hexa.

Table 46 shows the effect of hexa on the ability of activated sludge to grow in acetate/nutrient broth medium. At 32.0mg l^{-1}, 37% inhibition was observed and 46% inhibition at 100.0mg l^{-1} of hexa though no inhibition was observed, at 10mg l^{-1} of hexa or below. Although inhibition was observed at the higher concentrations after 6 hours incubation it was observed that after 24 hours there was no difference in optical

Figure 37. Removal of carbon (as DOC) from the SCAS unit when used as a die-away test.

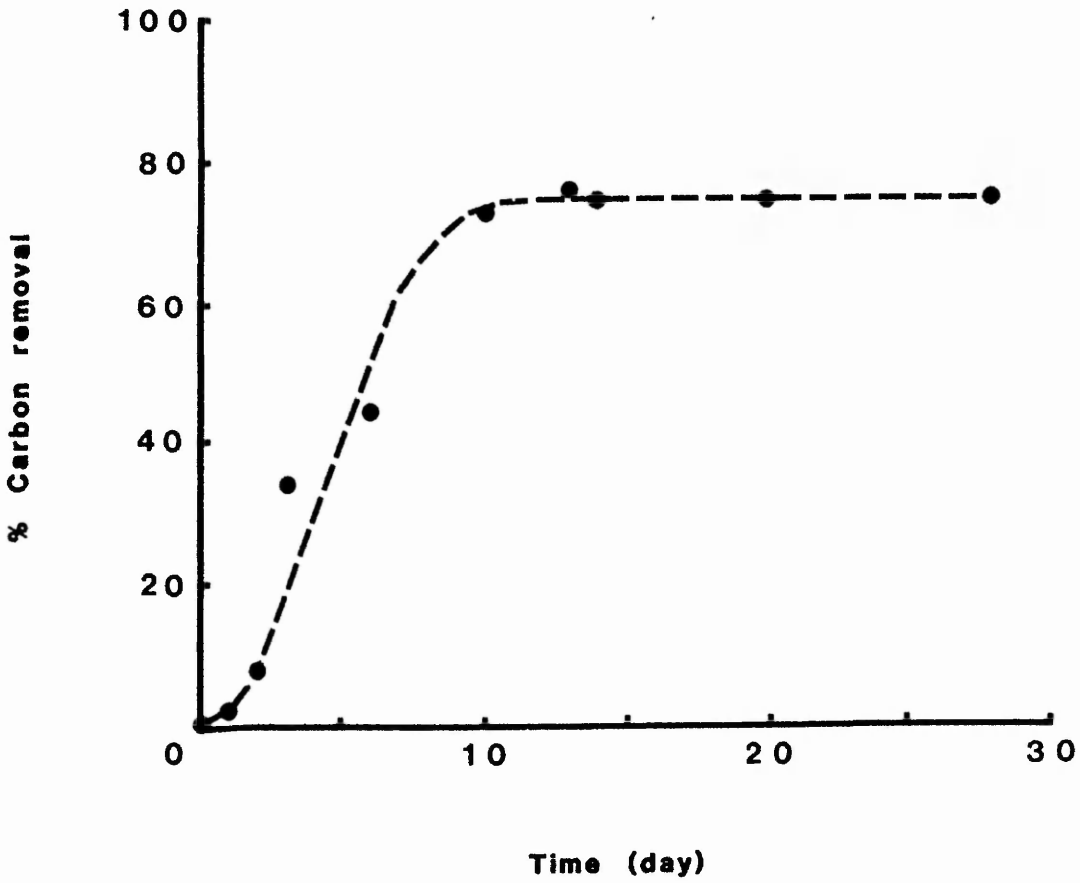


Table 45. Number of viable microorganisms ml^{-1} found in activated sludge after varying periods of exposure to hexa in the SCAS unit.

Day of SCAS test	Number of viable microorganisms ml^{-1} ($x10^7$)	
	Control unit	Hexa dosed unit
0	2.10	2.10
23	1.32	0.038
85	3.00	0.038
110	4.27	0.035
160	3.33	0.033
212	4.87	0.032

Table 46. Inhibition activated sludge growth by hexa. Values represent % reduction in O.D. (530 nm) relative to the control after 6 hours incubation.

	Concentration mg l^{-1}				
	1.0	3.2	10.0	32.0	100.0
% Inhibition	0	0	0	37	46

density between the control and test flasks indicating that the effect of hexa was to retard the growth rate rather than reduce the final cell population. The EC50 for hexa is greater than 100mg l^{-1}.

3.8.6 Isolation of hexa-degrading microorganisms.

Two microorganisms were isolated by enrichment of activated sludge with 0.5g l^{-1} hexa at 30°C and 200 rpm in batch culture using baffled flasks. These two isolates were designated KCH1 and KCH2 respectively.

3.8.7 Identification and characterization of KCH1 and KCH2.

3.8.7.1 Identification of KCH1 and KCH2 by NC1MB.

The isolates were found to be very similar and have been identified by NC1MB as being Methylobacterium species. These organisms are Gram-negative, non-spore forming, motile rods, catalase positive, oxidase negative by Kovac's reagent, capable of growing on methanol as their sole carbon source and can grow at 37°C. The colony morphology of KCH1 on Lab M nutrient agar at 30°C after 4 days was found to be round, regular, entire, smooth, semi-translucent, pink, low convex, punctiform colonies of less than 1mm in diameter. KCH2 was found to be very similar except that the colonies were much paler, almost white in colour.

3.8.7.2 API 20E-biochemical tests performed on KCH1 and KCH2 at 35°C.

Table 47 lists the results of biochemical tests performed (at 35°C) on the two isolates KCH1 and KCH2 using the API 20E test system. KCH1 was found in these tests to be motile, catalase-positive, used sodium pyruvate to produce acetoin and was able to utilize glucose, reduce nitrate to nitrogen gas and may be able to utilize tryptophane by production of tryptophane deaminase. KCH2 gave similar results to KCH1 except that it showed no indication of utilizing tryptophane by any method. Both isolates grew at 37°C on MacConkeys medium.

3.8.7.3 Carbohydrate utilization by KCH1 and KCH2.

Both isolates were tested for carbohydrate utilization in an attempt to differentiate further between them. Table 47 lists the results for carbohydrate utilization for both isolates, and it can be seen that both have identical utilization profiles. Of the 49 carbohydrates tested both isolates were found to be able to utilize 10. The carbohydrates which were utilized by KCH1 and KCH2 after 72 hours incubation were; L-arabinose, ribose, D-xylose, galactose, D-glucose, D-mannose, N-acetyl glucosamine, esculine, melibiose and D-fucose. The other 39 carbohydrates were not utilized by the isolates in this test.

Table 47. API 20E-biochemical tests performed on isolate KCH1 and KCH2.

Test	Test Substrates	Isolates KCH1	KCH2
ONPG	ortho-nitro-phenyl-galactoside	-	-
ADH	arginine	-	-
LDC	lysine	-	-
ODC	ornithine	-	-
CIT	sodium citrate	-	-
H_2S	sodium thiosulfate	-	-
URE	urea	-	-
TDA	tryptophane	+/-	-
IND	tryptophane	-	-
UP	sodium pyruvate	+	+
GEL	Kohn's gelatin	-	-
GLU	glucose	+	+
MAN	mannitol	-	-
INO	inositol	-	-
SOR	sorbitol	-	-
RHA	rhamnose	-	-
SUC	sucrose	-	-
MEL	melibiose	-	-
AMY	amygdalin	-	-
ARA	arabinose	-	-
OX	H_2S or ONPG tube	+	+
NO_3-NO_2	GLU tube	-	-
NO_3-N_2	GLU tube	+	+
Motility		+	+
MAC	MacConkey medium	-	-
CAT	in any negative sugar	+	+

Table 24. Utilization of carbohydrates by isolates KCH1 and KCH2.

	Isolates	
Carbohydrate	KCH1	KCH2
Glycerol	-	-
Erythritol	-	-
D-Arabinose	-	-
L-Arabinose	+	+
Ribose	+	+
D-Xylose	+	+
L-Xylose	-	-
Adonitol	-	-
β-methylxyloside	-	-
Galactose	+	+
D-Glucose	+	+
D-Fructose	-	-
D-Mannose	+	+
L-Sorbose	-	-
Rhamnase	-	-
Dulcitol	-	-
Inositol	-	-
Mannitol	-	-
Sorbitol	-	-
α Methyl-D-amoside	-	-
α Methyl-D-glucoside	-	-
N-Acetyl glucosamine	+	+
Amygdaline	-	-
Arbutine	-	-
Esculine	+	+
Salicine	-	-
Cellobiose	-	-
Maltose	-	-
Lactose	-	-

Carbohydrate	Isolate	
	KCH1	KCH2
Melibiose	+	–
Saccharose	–	+
Trehalose	–	–
Inuline	–	–
Melezitose	–	–
D-Raffinose	–	–
Amidon	–	–
Glycogene	–	–
Xylitol	–	–
β Gentibiose	–	–
D-Turanose	–	–
D-Lyxose	–	–
D-Tagotose	–	–
D-Fucose	+	+
L-Fucose	–	–
D-Arabitol	–	–
L-Arabitol	–	–
Gluconate	–	–
2 ceto-gluconate	–	–
5 ceto-gluconate	–	–

Table 49. Growth of isolates KCH1 and KCH2 in the presence of antibiotics.

	Isolates	
Antibiotic	KCH1	KCH2
Pencillin G 10iu	+	+
Ampicillin 25µg	+	+
Tetracycline 10µg	+	+
Kanomycin 20µg	+	+
Sulphonamide 300µg	-	-
Bacitracin 10iu	-	-/+
Sulphamethoxazole/ Trimethroprim 25µg	+	+
Gentamycin 30µg	+	+
Chloramphenicol 10µg	-	-
Streptomycin 25µg	+	+
Nitrofurantoin 300µg	+	+
Carbenicillin 100µg	+	+
Erythromycin 30µg	+	+
Mecillinam 25µg	+	+
Nalodixic acid 30µg	-	-
Neomycin 30µg	+	+

+ = Resistance (growth)

- = Inhibition (no growth)

3.8.7.4 Growth inhibition by antibiotics of KCH1 and KCH2.

Antibiotic resistance was investigated in order to provide further criteria for characterizing and distinguishing between KCH1 and KCH2. A washed cell suspension (0.5ml) containing approximately 10^{8} nutrient-broth grown-cells were spread and dried on to the surface of nutrient agar plates, on to which were placed antibiotic containing discs. The seeded plates were incubated at 30°C for 48 hours and resistance recorded if no zone of inhibition was observed. Table 49 represents the results obtained for growth inhibition by antibiotics of KCH1 and KCH2. Apart from bacitracin to which KCH1 was sensitive and the result for KCH2 which was inconclusive, the results for all of the different antibiotics were identical for both isolates. The two isolates were found to be sensitive to only three antibiotics sulphonamide (300μg), chloramphenicol (10μg) and nalodixic acid (30μg).

3.8.8 The effect of temperature on the growth characteristics of KCH1 and KCH2.

The isolates were grown in shake flask cultures at 25, 30 and 35°C in minimal medium containing 0.5g l^{-1} hexa in baffled flasks shaken at 200rpm. Growth was measured by following the change in the optical density of the cultures at 530nm against an uninoculated blank.

The change in temperature had no effect on the specific growth rate of KCH1 (μ=0.030h^{-1}), (Table 50). The temperature did affect the specific growth rate of KCH2, however, an increase in temperature bringing about an increase in the specific growth rate. The biggest increase was between 30°C, (μ=0.028h^{-1}) and 35°C (μ=0.040h^{-1}); the specific growth rate at 25°C was 0.025h^{-1} (Table 50).

3.8.9 Effect of storage of KCH1 and KCH2 on hexa-minimal medium agar slants at 4°C on growth rates.

As the length of time the isolates were stored increased so did their specific growth rates. The initial specific growth rate of KCH1 was 0.030h^{-1}, but on re-testing after 32 months maintenance and subculturing on hexa the specific growth rate had increased to 0.140h^{-1} (Table 51). A similar increase was observed with KCH2 although initially it had a lower specific growth rate than KCH1 at the time of isolation (0.027h^{-1}). After 32 months storage, KCH2 had a greater specific growth rate (0.150h^{-1}) than KCH1 (0.140h^{-1}).

3.8.10 Growth of KCH1 and KCH2 on other compounds as sole carbon source.

KCH1 and KCH2 were tested for growth in liquid culture with a variety of compounds as the sole source

Table 50. The effect of temperature on the specific growth rate of the hexa-utilizing isolates KCH1 and KCH2.

Isolate	Temperature	Specific growth rate (h^{-1})
KCH1	25	0.030
	30	0.030
	35	0.030
KCH2	25	0.025
	30	0.028
	35	0.040

Table 51. The effect of storage on hexa minimal media slants (4°C) on the specific growth rate at 30°C of the hexa-utilizing isolates KCH1 and KCH2.

Time (months) after isolation that specific growth rates were determined	Specific growth rate (h^{-1}) isolates	
	KCH1	KCH2
0	0.030	0.028
18	0.038	0.042
26	0.113	0.125
32	0.140	0.150

Table 52. Specific growth rates of KCH1 and KCH2 with various carbon sources.

	Specific growth rate (h^{-1})	
Compound	KCH1	KCH2
Hexa(1)	0.140	0.150
Hexa(2)	0.130	0.135
Formaldehyde(3)	0.080	0.090
Sodium formate	0	0
Methanol	0.135	0.130

(1) Hexa as sole source of carbon.

(2) Hexa as sole source of both carbon and nitrogen.

(3) Formaldehyde growth rate was determined by using sealed flasks containing formaldehyde and sacrificing flasks to measure O.D.

of carbon; Table 52 gives the calculated specific growth rates for KCH1 and KCH2 on these compounds. Both isolates could utilize hexa as both the sole source of carbon and nitrogen. Ammonia release has been demonstrated (by Nessler's reaction) when hexa was the sole source of carbon and nitrogen. The specific growth rates with no extra source of nitrogen present were only slightly lower than with an addtional nitrogen source. Growth with methanol as the sole source of carbon for KCH1 and KCH2 was almost as good as with hexa as the sole source of carbon. The growth rates of KCH1 and KCH2 were lower when grown on formaldehyde as the sole source of carbon, 0.080 and 0.090h^{-1} respectively, compared with 0.140h^{-1} for KCH1 and 0.150h^{-1} for KCH2 when grown on hexa. No growth was observed when formate was the sole source of carbon.

3.8.11 Respirometric studies with KCH1 and KCH2.

Respirometric studies were carried out with KCH1 and KCH2 in a Gilson respirometer in an attempt to indicate the possible mechanism of hexa degradation. All cells were initially grown on hexa minimal salts medium and harvested in mid-exponential phase, concentrated by centrifugation and washed in 0.1M phosphate buffer, pH 7.2. Table 53 shows the initial (maximum) oxygen uptake rates by KCH1 and KCH2 with a series of organic compounds. No oxygen uptake by either isolate was

observed in the presence of hexa and a similar observation was made with sodium formate. However, oxygen uptake rates were observed with methanol and formaldehyde for both isolates; the rates for methanol were less than those with formaldehyde. KCH1 had a lower oxygen uptake rate (0.98µl O_2 min^{-1}), than KCH2 (1.18µlO_2 min^{-1} mg^{-1}) when metabolizing formaldehyde. Table 54 gives the values for total oxygen uptake by experiment and the theoretical maximum values. Both sodium formate and hexa gave negligible values for total oxygen uptake with KCH1 and KCH2. With methanol both KCH1 and KCH2 gave the same percentage uptake of the theoretical maximum, 74%, and with formaldehyde KCH1 utilized 77% of the theoretical maximum oxygen uptake whilst KCH2 used 80%.

3.8.12 Utilization of hexa by KCH1 and KCH2.

The two isolates were inoculated in triplicate into minimal medium containing 0.1g l^{-1} of hexa and grown at 30°C and 200 rpm. Samples were taken at intervals and optical density, pH and residual hexa determined. The amount of residual hexa was determined as formaldehyde present when samples of supernatant were hydrolysed with acid and the formaldehyde present due to hydrolysis of hexa determined colormetrically by a modified Riker method, (see section 2.9.2.1). It is possible to

Table 53. Maximum oxygen uptake rates for KCH1 and KCH2.

Substrate	Max. oxygen uptake rate ($\mu l\ O_2\ min^{-1}\ mg^{-1}$ dry weight of cells)	
	KCH1	KCH2
Hexa	0	0
Formaldehyde	0.98	1.18
Sodium formate	0	0
Methanol	0.79	0.76

Table 54. Experimental and theoretical total oxygen uptake values for KCH1 and KCH2.

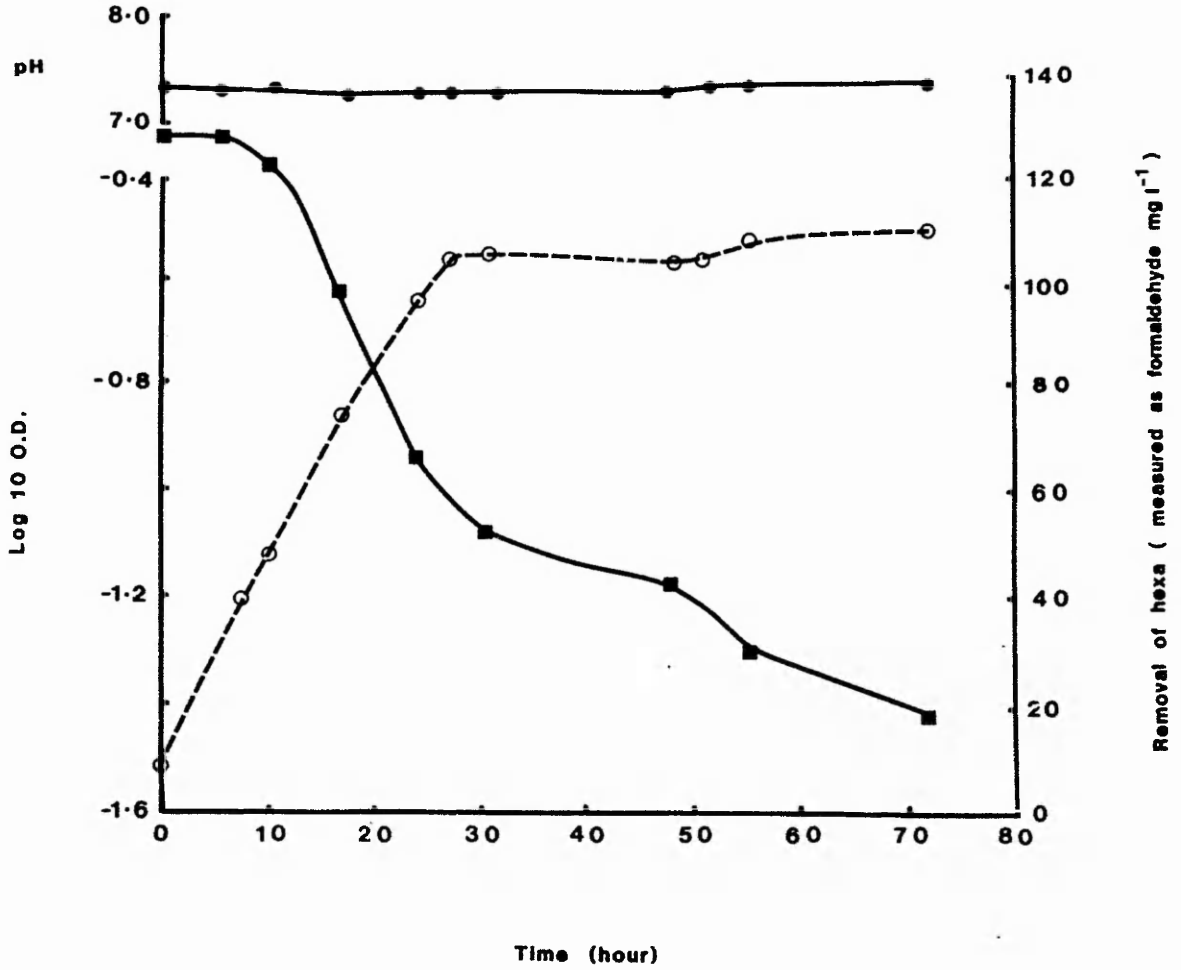
Substrate (μM)	Theoretical O_2 uptake (μl)	Observed O_2 uptake			
		KCH1		KCH2	
		μl	% Theoretical	μl	% Theoretical
2μM Hexa	268,8	1	0	3	1
10μM Formaldehyde	224	174	77	179	80
10μM Sodium formate	224	15	7	6	3
15μM Methanol	336	249	74	248	74

Table 55. Removal of hexa (mg l^{-1}) by KCH1 and KCH2 against time.

Time (h^{-1})	Calculated removal of hexa (mg l^{-1}) from culture fluids	
	KCH1	KCH2
0	0	0
6	0	0
7.5	-	0.8
10	2.3	-
17	23.4	35.9
24	50.0	60.9
30	69.4	71.9
48	73.4	75.0
55	76.6	75.0
72	85.9	84.4

Initial concentration of hexa = 100mg l^{-1}

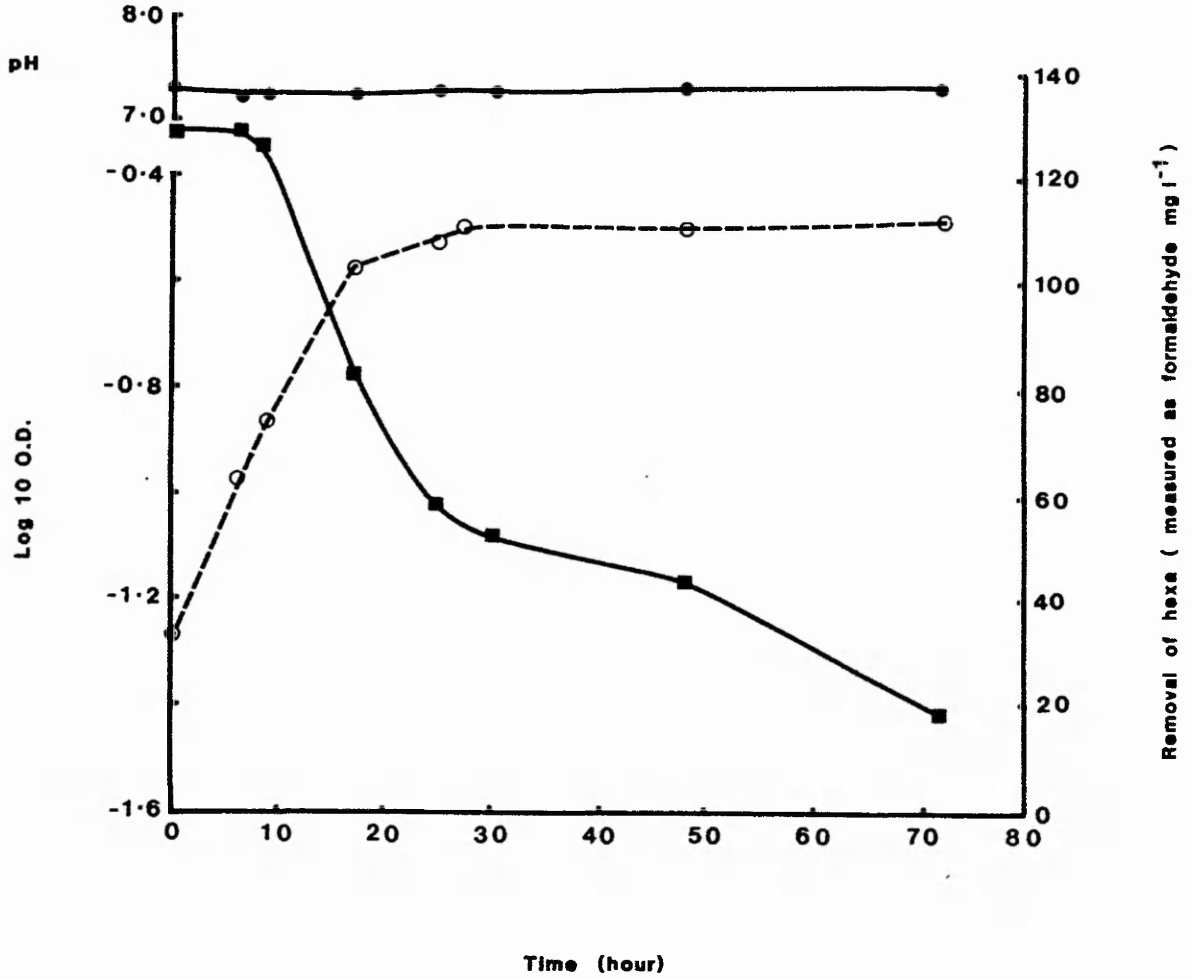
Figure 38. Removal of hexa (measured as formaldehyde) and change in pH of culture fluid during growth of KCH1.



relate directly the amount of residual hexa removed by the isolates to the amount of formaldehyde detected. This is because one molecule of hexa will breakdown under acid conditions into six molecules of formaldehyde. Table 55 shows the calculated values for the removal of hexa (mg l^{-1} hexa) during the growth of KCH1 and KCH2 on 0.1g l^{-1} hexa.

No removal of hexa was detected during the first 6 hours of incubation by either KCH1 (Figure 38) or KCH2 (Figure 39). A small decrease in the amount of hexa (measured as formaldehyde), in the cultures was recorded between 6 to 10 hours for KCH1 and 6 to 7.5 hours for KCH2. A rapid decrease in the level of detected hexa (measured as formaldehyde) was detected in the hydrolysed supernatant samples of both isolates until 30 hours of incubation. After 30 hours incubation 69.3mg l^{-1} of hexa had been removed by KCH1 and 71.9mg l^{-1} of hexa had been removed by KCH2 (when measured as formaldehyde) see Table 55. A slower removal of hexa (measured as formaldehyde) was observed after 30 hours of both KCH1 (Figure 38) and KCH2 (Figure 39). This slower removal of hexa almost corresponded to the onset of the stationary phase for KCH1 (26 hours), stationary phase for KCH2 having started at about 18 hours. From the growth curves for both KCH1 (Figure 38) and KCH2 (Figure 39) the specific growth rates can be calculated. It was found that KCH2 had a slightly higher specific

Figure 39. Removal of hexa (measured as formaldehyde) and change in pH of culture fluid during growth of KCH2.



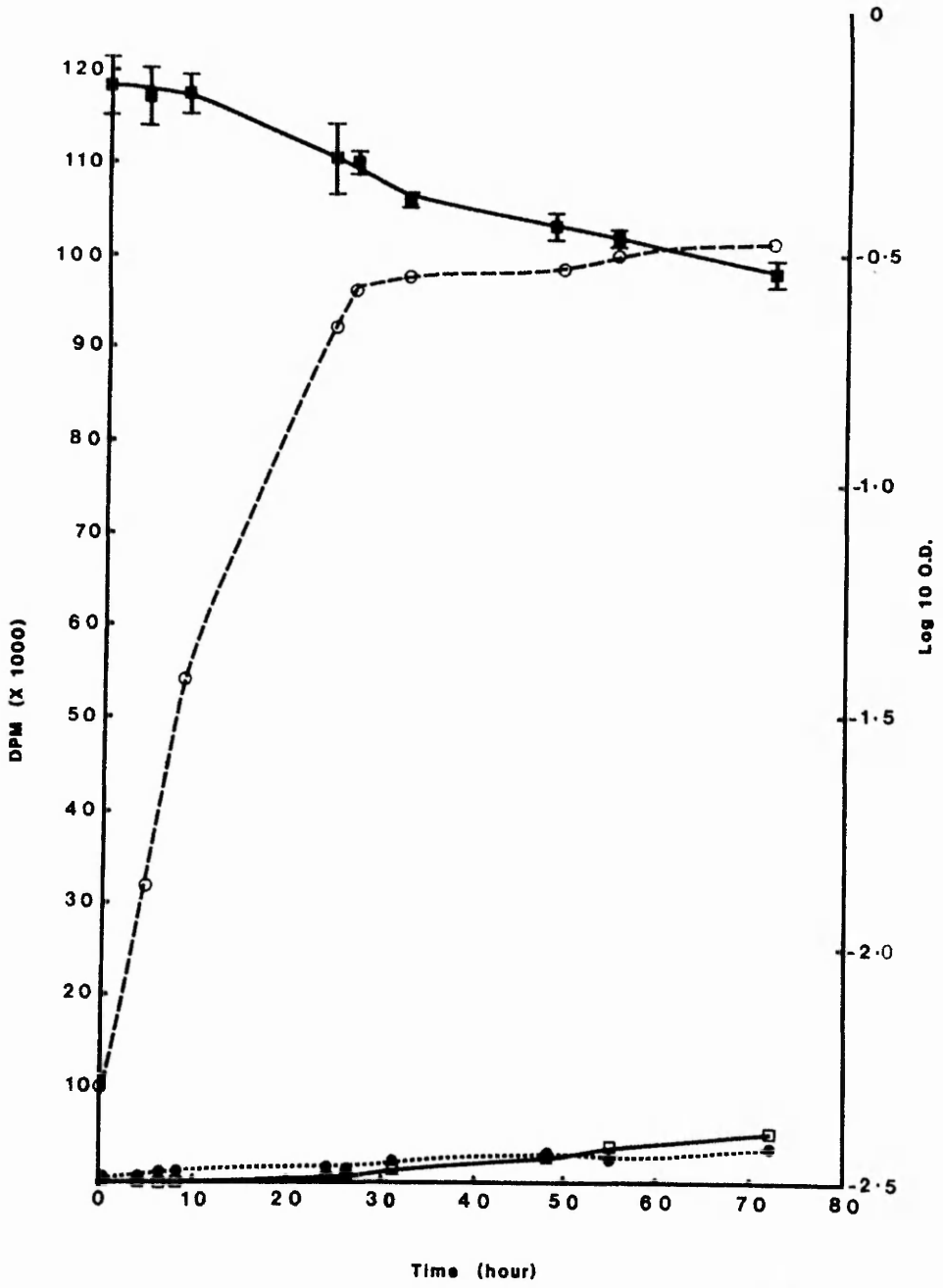
growth rate ($0.042h^{-1}$) than KCH1 ($0.038h^{-1}$). The percentage removals of hexa over a 72 hour incubation period for KCH1 and KCH2 were almost identical at 85.9 and 84.4% respectively as calculated from Table 55.

3.8.13 Determination of growth using ^{14}C radiolabelled hexa.

Hexa universally labelled with carbon 14 (^{14}C) was diluted with unlabelled hexa (1843:1) and KCH1 and KCH2 grown on this mixture at 30°C and 200 rpm in sealed baffled flasks. The removal of ^{14}C activity from supernatants was measured along with the increase in ^{14}C activity within the cell mass produced during growth. The release of radiolabelled carbon ($^{14}CO_2$) was also determined by collection in Carbo-sorb. In addition to monitoring radioactivity in each fraction, growth was determined by measuring optical density at 530nm.

As the optical density of KCH1 increased, the level of ^{14}C activity in the supernatant decreased slowly during the first 8 hours (Figure 40). This decrease in activity was equivalent to a decrease in hexa concentration of 4mg l^{-1} (Table 56). A faster removal of hexa was measured over the following 16 hours which gave a total removal after 24 hours of 38mg l^{-1}. This faster reduction of ^{14}C activity (and therefore hexa removal) continued until 32 hours of incubation at which

Figure 40. Removal of (U-^{14}C) hexa during growth of KCH1.



■—■ Supernatant activity (DPM X 1000)

⊡—⊟ Activity of $^{14}CO_2$ collected from exhaust gases (DPM X 1000)

●·●·● Cell mass activity DPM X 1000)

⊙—⊖ Optical density (Log_{10} O.D.)

point 53mg l^{-1} had been removed from the supernatant (Table 56). The rate of removal of ^{14}C activity decreased after 32 hours but removal continued slowly until the experiment was discontinued after 72 hours. The calculated amount of hexa removed ater 72 hours was 86mg l^{-1}. As ^{14}C activity was removed from the supernatant an increase in optical density was observed.

A gradual increase in the level of ^{14}C activity within the cell mass was measured over the 72 hours of the experiment (Figure 40). After 72 hours, 3.12% of the ^{14}C labelled hexa removed was measured as labelled carbon within the cell mass of KCH1. Only a small amount of labelled $^{14}CO_2$ was measured over the first 24 hours incubation, but subsequently the cumulative amount increased with time until the experiment was discontinued (Figure 40). KCH1 removes labelled hexa from the culture fluid almost from time 0; this removal corresponded with an increase in activity of the cell mass of KCH1. Growth of the isolate KCH1 corresponded to the increase in ^{14}C activity in the cell mass and decrease in the ^{14}C activity of the supernatant. Little labelled $^{14}CO_2$ was measured until 24 hours after incubation of KCH1.

When KCH2 was grown in the presence of radiolabelled hexa the optical density was observed to increase as the ^{14}C activity of the supernatant decreased over the first 8 hours (Figure 41). This decrease in activity was

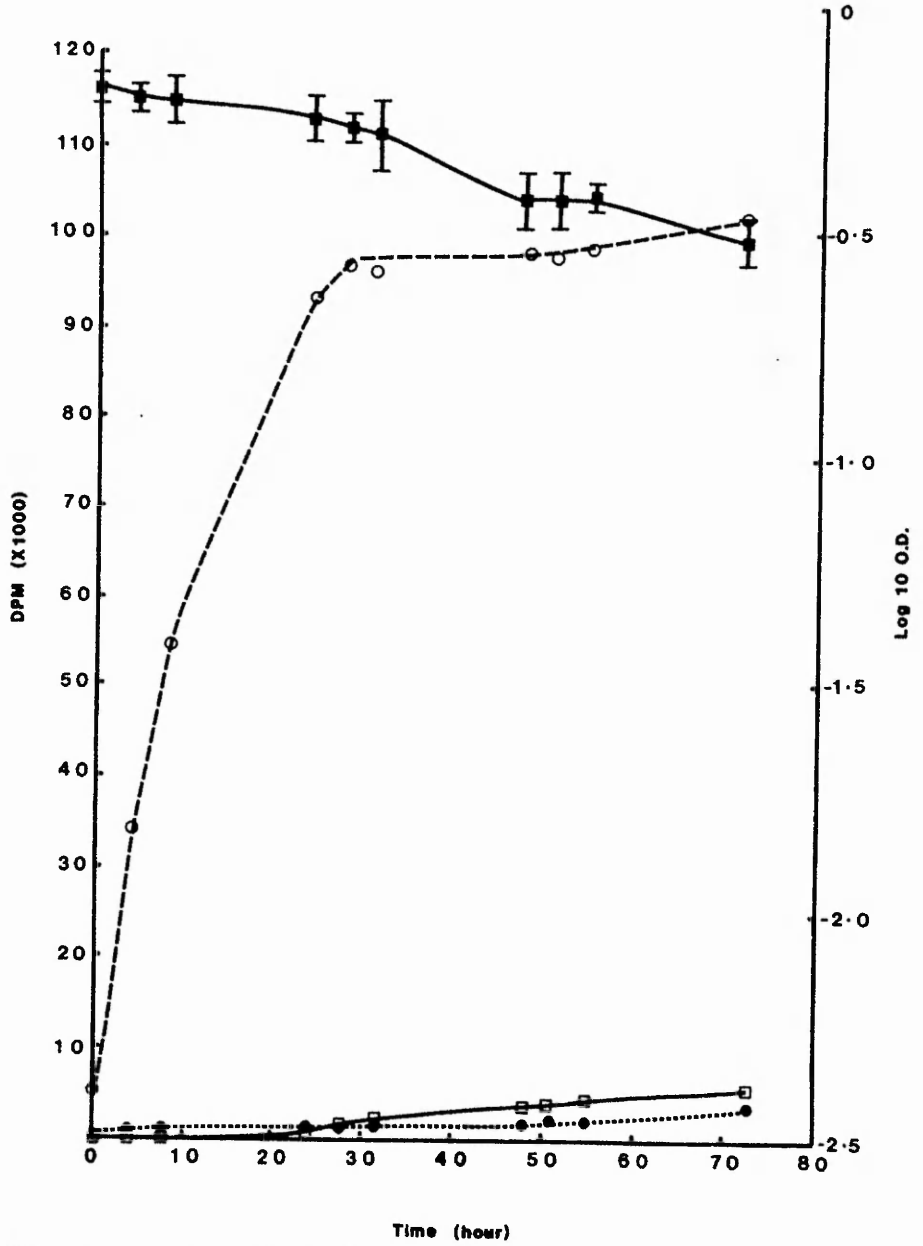
Table 56. Removal of hexa calculated by isotope dilution with KCH1 and KCH2.

	Removal of hexa (mg l^{-1})	
Time(h)	KCH1	KCH2
0	0	0
2	3.5	N.D.
4	7.5	6.5
8	4.0	8.0
24	38	15
26	40	28
32	53	42
48	66	51
55	70	50
72	86	75

N.D. = Not determined

Initial concentration of hexa was 500mg l^{-1}

Figure 41. Removal of (U-^{14}C) hexa during growth of KCH2.



■—■ Supernatant activity (DPM X 1000)

⊡—⊡ Activity of $^{14}CO_2$ collected from exhaust gases (DPM X 1000)

●-● Cell mass activity (DPM X 1000)

⊙—⊙ Optical density (Log_{10} O.D.)

equivalent to a removal of 8mg l^{-1} of hexa (Table 56). The level of activity decreased more rapidly between 8 and 24 hours with 15mg l^{-1} hexa having been removed, and after 32 hours 42mg l^{-1} hexa had been removed (Table 56). Figure 41 shows removal of activity in the supernatant continued until the experiment was discontinued after 72 hours. Table 56 shows that after 72 hours 75mg l^{-1} of hexa had been removed from the culture fluid by KCH2.

A gradual increase in the level of activity within the cell mass was measured over the 72 hours of the experiment (Figure 41). After 72 hours, 3.13% of the ^{14}C-labelled hexa removed was measured on ^{14}C within the cell mass of KCH2. Only a small amount of labelled $^{14}CO_2$ was measured over the first 24 hours incubation, after which time the cumulative amount increased with time until the experiment was discontinued (Figure 41). The efficiency of $^{14}CO_2$ collection method used in all radioisotope experiments has been determined by experimentation to be 38±4%.

With the efficiency of the $^{14}CO_2$ collection method determined it is possible to calculate mass balances for isolates KCH1 and KCH2 after 72 hours incubation in the presence of [U-^{14}C] hexa.

The mass balance equation is described below;

[$^{14}CO_2$ activity collected	Cell mass	Supernatant		Supernatant
	+	+	=	
x efficiency correction]	activity	activity time t		activity t=o.

The mass balance for KCH1 after 72 hours incubation with [U-^{14}C] hexa is:

(5275 x 2.66±0.28) + 3724 + 98783 = 115061 to 118016 dpm

CO_2 Cell Mass Supt

The actual activity of supernatant at t=0 was 118001 dpm.

It can be seen after correction for $^{14}CO_2$ collection efficiency, the calculated limits of the mass balance include the original supernatant activity.

The mass balance for KCH2 after 72 hours incubation with (U-^{14}C) hexa is:

(5529 x 2.66±0.28) + 3651 + 99000 = 115810 to 118906 dpm

The actual activity of supernatant at t=0 was 116500 dpm.

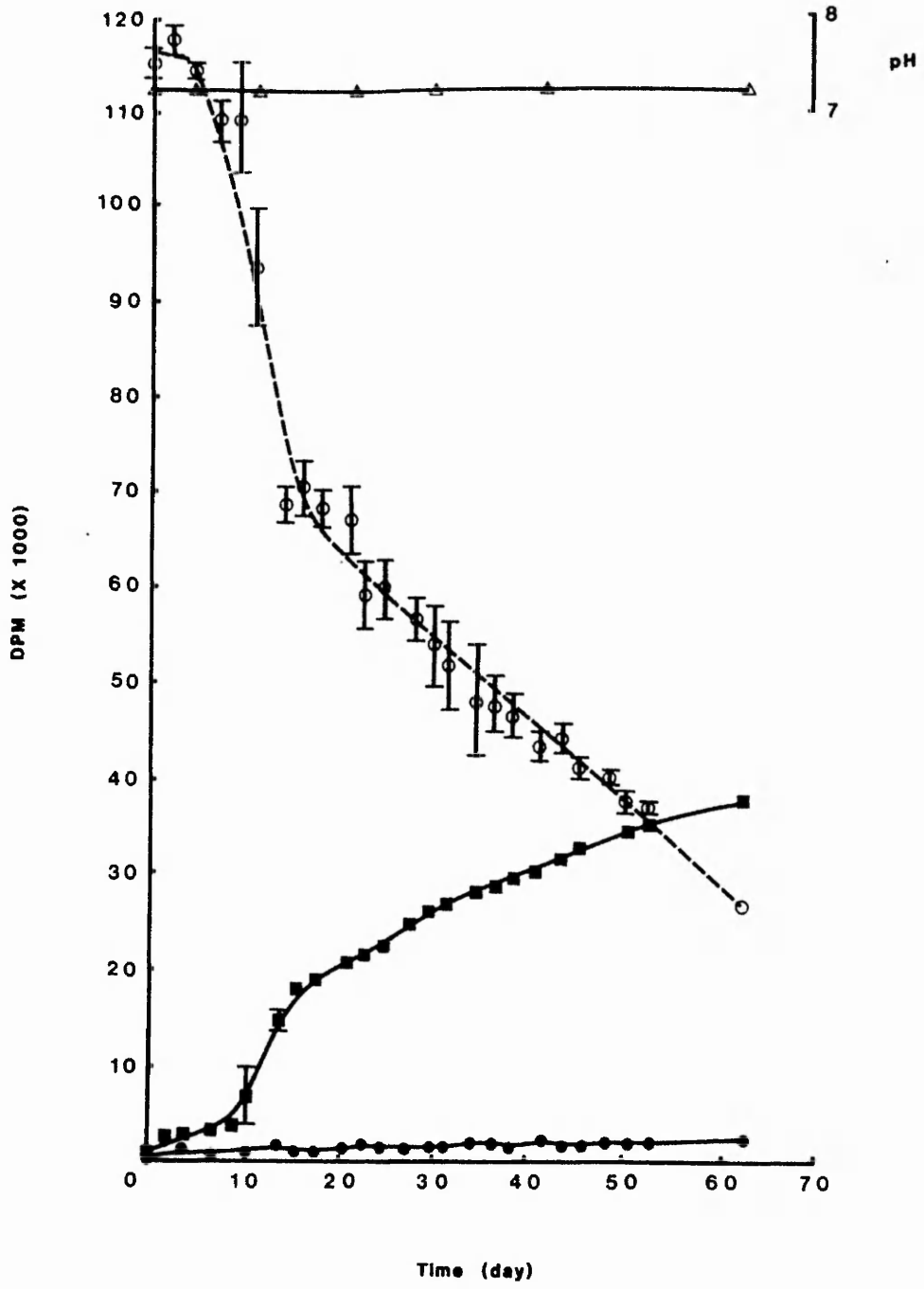
It can be seen that after correction for $^{14}CO_2$ collection efficiency the calculated limits of the mass balance include the original supernatant activity.

3.8.14 Removal of hexa at low concentrations.

3.8.14.1 Removal of hexa by activated sludge.

Previously unexposed activated sludge containing $5.5x10^{6}$ microorganisms ml^{-1} and at pH 7.2 was dosed with uniformly labelled ^{14}C hexa in sealed fermentation units, at levels of 530µg hexa l^{-1} and 54µg hexa l^{-1}. During these experiments the pH of the activated sludge was measured at intervals and no significant variation from pH 7.2 was observed. With 530µg l^{-1} of hexa present in the test units, a lag of 4 days was observed before removal of ^{14}C activity from the supernatant was detected. A rapid removal of ^{14}C activity was measured between days 4 and 14 (Figure 42), and after this time a steady although slower removal of activity was observed in the test units until the test was discontinued after 63 days. The total removal of ^{14}C activity was equivalent to 67% which corresponds to a removal of 407µg hexa l^{-1} (Table 57). From Table 57, it is possible to observe the initial lag in degradation; the rapid removal of hexa corresponded to 212µg l^{-1} in 10 days. Over the succeeding 49 days until the test was discontinued, 195µg l^{-1} was removed. This shows that over half of the removal of hexa occurred during the first 14 days. A small increase in the activity of the cell mass (corrected for adsorption) was measured as shown in Figure 42.

Figure 42. Removal of low levels of hexa (530 ug l^{-1}) by activated sludge.



When collection of radiolabelled $^{14}CO_2$ is considered it can be seen that after an initial lag of 9 days, a rapid increase in the accumulative amount of $^{14}CO_2$ collected occurred until day 18, after which a slower increase in the amount of $^{14}CO_2$ was measured until the test was discontinued. The efficiency of $^{14}CO_2$ collection for this apparatus was determined to be 23.75±6.25%. The mass balance equation for activated sludge exposed to [U-^{14}C] hexa (530µg l^{-1}) for 63 days is:-

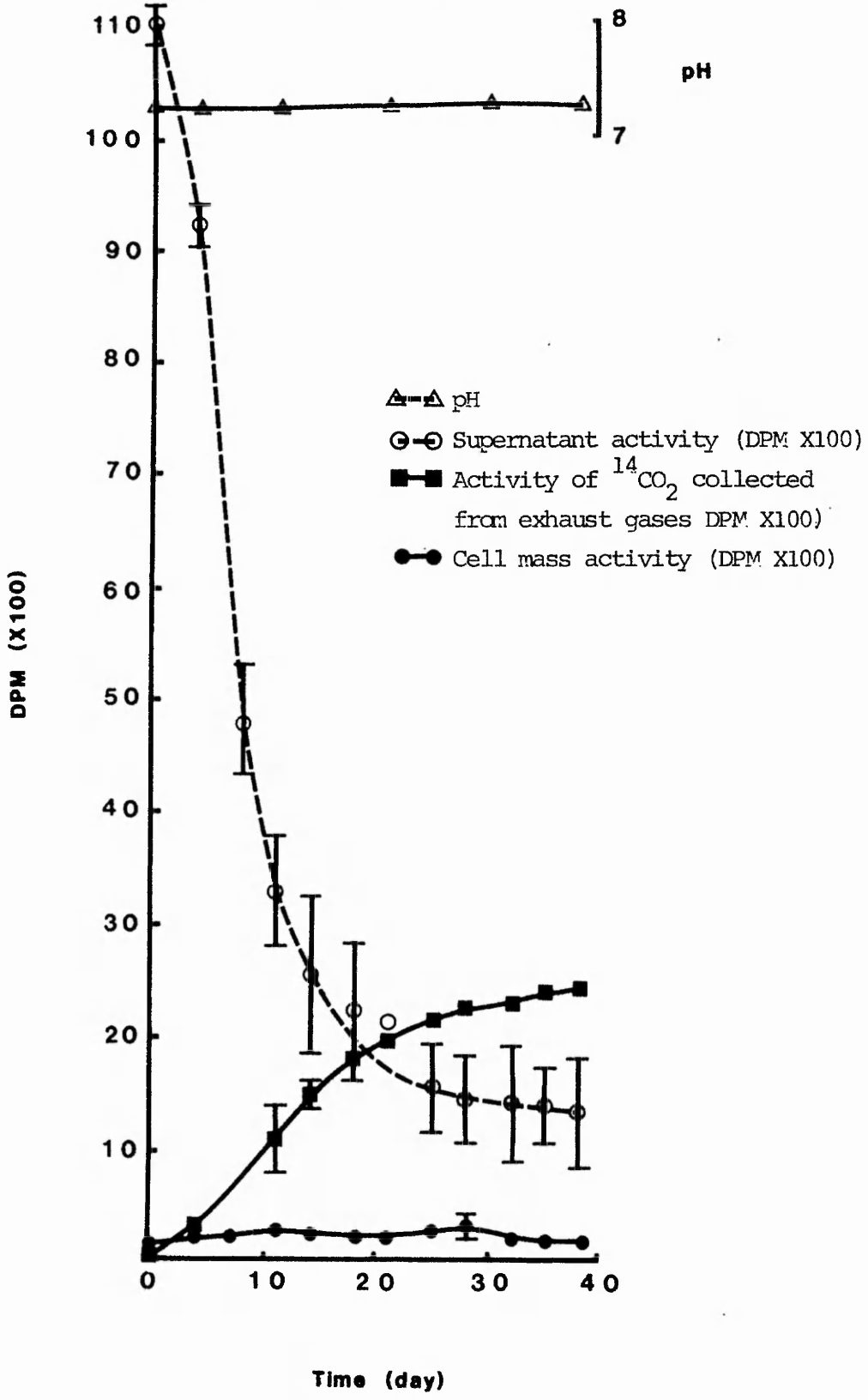
(36821 x 4.52±1.2) + 2514 + 26463 = 151590 to 238857 dpm

CO_2 Cell Mass Supt

The actual activity of the supernatant at t=0 was 114614 dpm. The original activity present in the supernatant was observed to be within the limits of the mass balance.

When the initial amount of hexa was 52µg l^{-1} no lag period was observed before removal of ^{14}C activity from the supernatant was detected. A rapid removal of activity occurred during the first 11 days which corresponded to 70% of the initial activity present at time 0 (Figure 43). Over the succeeding 28 days a further 19.6% of the activity was removed giving a total removal of activity of 89.6%. After day 28 only a very

Figure 43. Removal of low levels of hexa (53 ug l^{-1}) by activated sludge.



small amount of hexa was removed. The removal of hexa in $\mu g\ l^{-1}$ over the first 11 days was $36.8\mu g\ l^{-1}$ and $9.8\mu g\ l^{-1}$ over the following 28 days until the test was discontinued (Table 57). A total of $46.6\mu g\ l^{-1}$ of hexa was removed by the activated sludge over a 39 day period.

During the first 4 days little $^{14}CO_2$ was detected, but after this time until day 18 a rapid increase in the amount of $^{14}CO_2$ was measured. There then followed a gradual tailing off of $^{14}CO_2$ evolution. The mass balance equation of activated sludge exposed to [U-^{14}C] hexa ($53\mu g\ l^{-1}$) for 39 days is :-

$$(2384 \times 4.52 \pm 1.2) + 116 + 1164 = 9219 \text{ to } 14869 \text{ dpm}$$

CO_2 Cell Mass Supt

The actual activity of the supernatant at t=0 was 11149 dpm. The original activity present in the supernatant was observed to be within the limits of the calculated mass balance. The ^{14}C activity in the cell mass (corrected for adsorption) initially increased slightly as the ^{14}C activity of the supernatant decreased. After this initial increase the ^{14}C level remained almost static.

The activated sludge exposed to the higher level of hexa appeared to remove the compound more slowly than when exposed to the lower level of hexa.

3.8.14.2 Removal of hexa by a soil suspension.

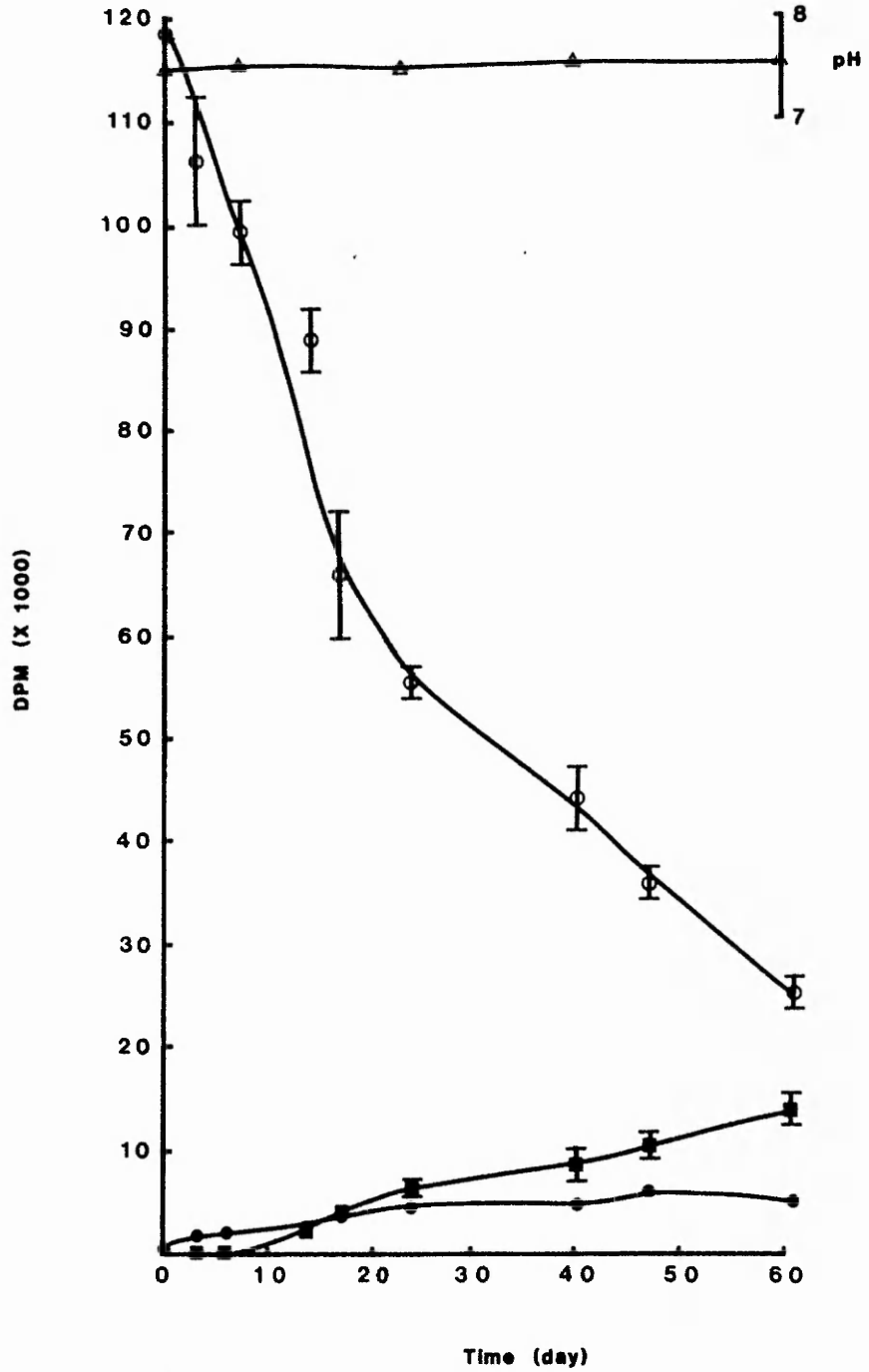
A soil suspension (10g l^{-1}) at pH 7.5 previously unexposed to hexa was dosed with labelled hexa in sealed fermentation units at levels of 553µg l^{-1} and 52.1µg l^{-1}. During these experiments the pH of the soil suspension was measured at intervals and no significant change was observed. The number of viable microorganisms per ml^{-1} of the final volume of the test suspension was $1.5x10^{8}$. With 553µg l^{-1} hexa present in the test units no lag period was observed before removal of ^{14}C activity was measured in the supernatant. A rapid removal of ^{14}C activity was measured in the supernatant. A rapid removal of ^{14}C activity was measured over the first 17 days of the test, but after this the rate slowed considerably (Figure 44) until the test was discontinued after 61 days. The observed total removal of ^{14}C activity corresponded to 441µg l^{-1} equivalent to 80% of that initially present (Table 58). It is possible to calculate from Table 58 that 249µg l^{-1} of hexa was removed during the first 17 days of the test which is more than half (56%) of the total hexa removed. Figure 44 shows that the level of ^{14}C activity in the cell mass (corrected for adsorption) increased initially

Table 57. Removal by activated sludge of hexa added at low concentrations.

Time (days)	Amount of hexa $\mu g\ l^{-1}$ remaining	
0	530	52
2	547	N.D.
4	531	43
7	509	22.2
9	510	N.D.
11	434	15.2
14	318	12.1
16	330	N.D.
18	318	10.2
21	312	9.8
23	276	N.D.
25	279	7.0
28	266	6.6
30	252	N.D.
32	243	6.4
35	224	6.0
37	221	N.D.
39	217	5.4
42	203	
44	207	
46	192	
49	187	
51	176	
53	173	
63	123	

N.D. = Not determined

Figure 44. Removal of low levels of hexa (530ug l^{-1}) by soil.



(24 days) and then remained steady until the end of the test.

It can be seen that a lag of 7 days occurred before any $^{14}CO_2$ was detected (Figure 44). After this time the accumulative level of $^{14}CO_2$ increased until the end of the test at day 61.

The mass balance equation for soil exposed to [U-^{14}C] hexa (530µg l^{-1}) for 61 days is :-

(14950 x 4.52±1.2) + 5021 + 25111 = 79916 to 115347 dpm

CO_2 Cell Mass Supt

The actual activity of the supernatant at t=0 was 118026 dpm. The original activity present in the supernatant was observed to be just outside the upper limit of the calculated mass balance.

When the initial amount of hexa was 52.1µg l^{-1} a slight lag of 3 days was observed before any significant removal of ^{14}C activity was detected (Figure 45). After this time a rapid removal of activity was measured until day 17 equivalent to 58.5% removal of ^{14}C activity added. The removal of hexa over the first 17 days was 21.6µg l^{-1} and 15.9µg l^{-1} was removed over the following 44 days (Table 58). A total of 37.5µg l^{-1} of hexa was removed by the soil over a 61 day period (Table 58).

Figure 45. Removal of low levels of hexa (5.21 ug l^{-1}) by soil

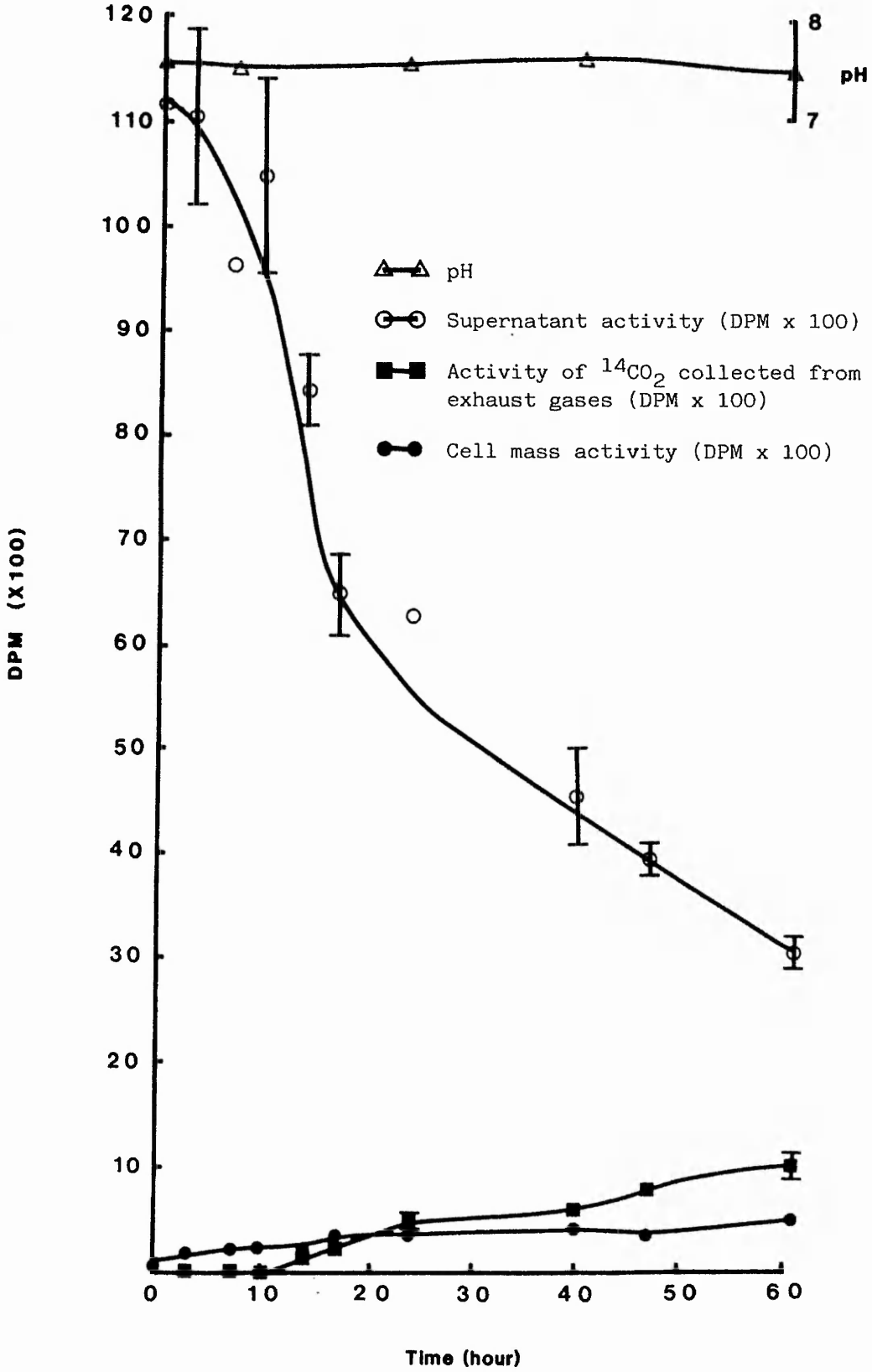


Table 58. Removal by soil of hexa added at two concentrations.

Time (days)	Amount of hexa ($\mu g\ l^{-1}$) remaining	
0	553	52.1
3	495	50.6
7	464	44.5
10	N.D.	49.0
14	414	39.4
17	304	30.5
24	259	29.3
40	207	21.9
47	169	18.7
61	112	14.6

During the first 10 days of the experiment no $^{14}CO_2$ was detected, but after this time an increase in the accumulative $^{14}CO_2$ level was measured until the discontinuation of the experiment (Figure 45). The mass balance equation of soil exposed to [U-^{14}C] hexa (53μg l^{-1}) for 61 days is :-

(1155 x 4.52±1.2) + 558 + 3251 = 7655 to 10393. dpm

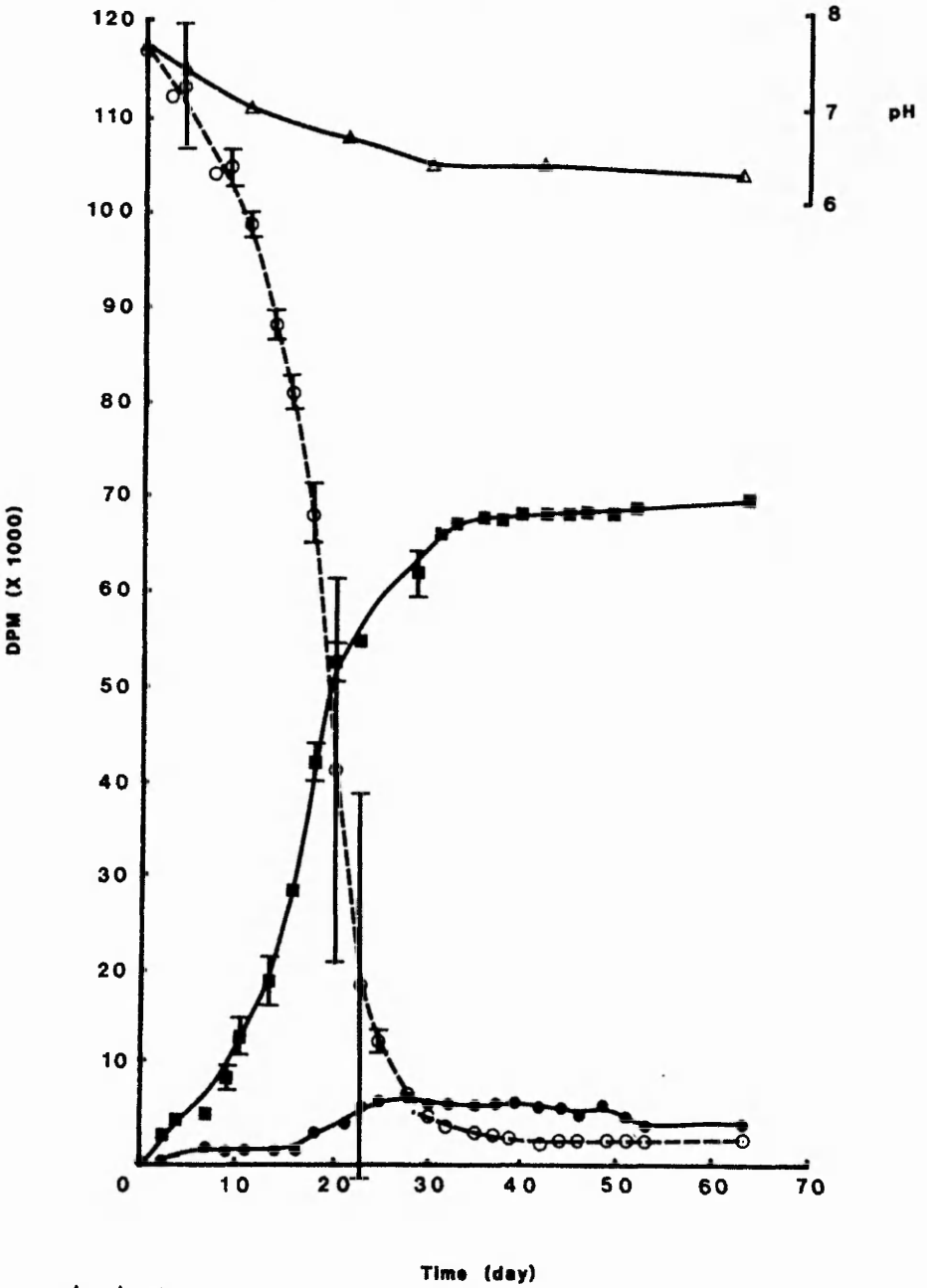
CO_2 Cell mass Supt

The actual activity of the supernatant at t=0 was 11025 dpm. The original activity present in the supernatant was observed to be just outside the upper limit of the calculated mass balance. An increase in the cell mass activity (corrected for adsorption) showed an increase from time 0 until the test was the end of the experiment at day 61. Both soil experiments gave very similar results in terms of the trends observed.

3.8.14.3 Removal of hexa by river water.

River water was dosed with 530μg l^{-1} hexa. The number of viable microorganisms in the river water was $2x10^4$ microorganisms ml^{-1}. The pH of the river water fell from pH 7.6 down to pH 6.3 over a 63 day period with fastest drop over the first 23 days (Figure 46). This corresponded with a rapid drop in supernatant ^{14}C

Figure 46. Removal of low levels of hexa 530 ug l^{-1}) by river water.



△—△ pH

⊖—⊖ Supernatant activity (DPM x 1000)

■—■ Activity of $^{14}CO_2$ collected from exhaust gases (DPM x 1000)

●—● Cell mass activity (DPM x 1000)

activity during which 84% of the original activity disappeared from the supernatant (Figure 46). After slow initial evolution the level of accumulative $^{14}CO_2$ measured increased rapidly from day 10 until day 21 at which time the rate of increase slowed considerably. Very little increase in the accumulative $^{14}CO_2$ level occurred after day 32 (Figure 46). The mass balance equation for river water exposed to [U-^{14}C] hexa (530µg l^{-1}) for 63 days is:-

(69527 x 4.52±1.2) + 3740 + 2155 = 237402 to 402199 dpm

CO_2 Cell mass Supt

The actual activity of the supernatant at t=0 was 116158 dpm. The original activity present in the supernatant was not observed to be within this limit. The amount of activity measured in the cell mass (corrected for adsorption) increased slowly at first then more rapidly until reaching a plateau at day 28. After day 42 a gentle decrease in activity was measured until the end of the test (Figure 46).

3.8.14.4 Biodegradation rates at low levels of hexa.

Table 59 gives the calculated first order biodegradation rates for hexa with different environmental samples determined from the decrease in

Figure 47. Effect if pH value on the degree of hydrolysis over 6 hours.

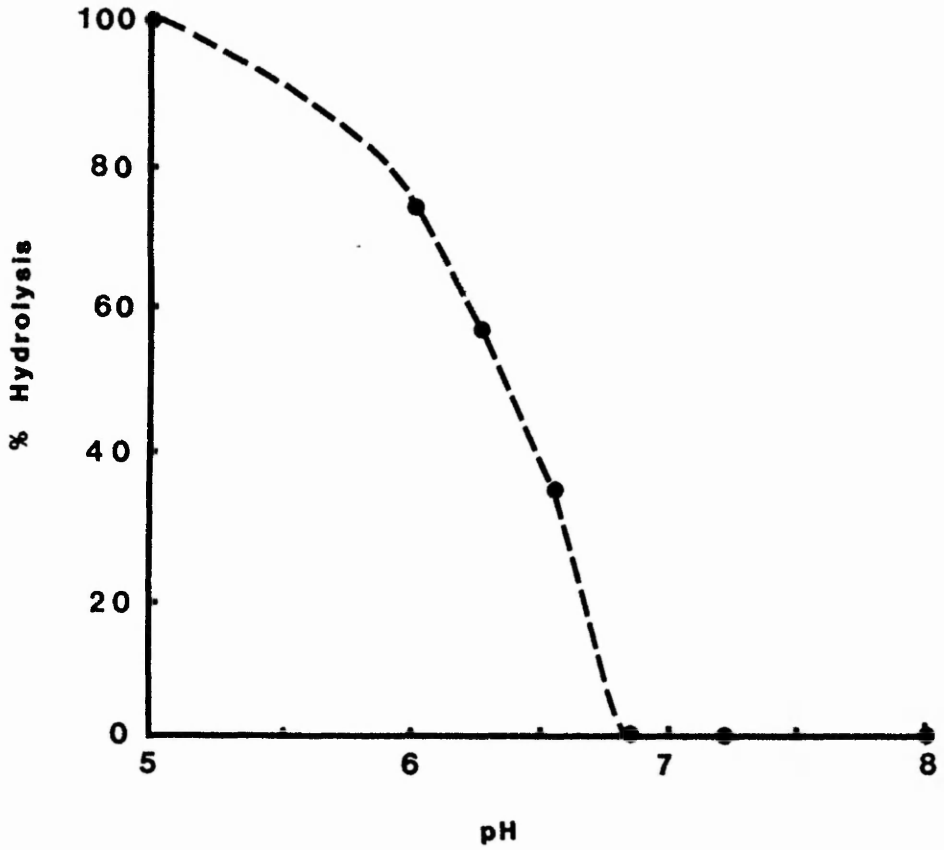


Table 59. First order biodegradation rates (d^{-1}) of activated sludge, soil and river water with hexa.

	Level of hexa	
	10^{-4}g l^{-1}	10^{-5}g l^{-1}
Activated sludge	0.367	0.450
Soil	0.271	0.276
River water	0.728	N.D.

N.D. = Not determined

the activity of the supernatant (Figures 42-46). Soil behaved almost identically at both levels of hexa, whereas activated sludge had a much higher biodegradation rate at the lower level of hexa. Both values for activated sludge were faster than those obtained with soil. River water had a "biodegradation rate" much greater than any of the other samples at 0.728 d^{-1}.

3.8.15 Acid hydrolysis of hexa.

To duplicate flasks of varying pH was added 0.1g l^{-1} of hexa and these flasks were incubated at 25°C and200rpm for 6 hours. It can be seen from Figure 47 that after 6 hours incubation no detectable hydrolysis was measured at pH 6.8 and above. As the pH fell the amount of hydrolysis increased; at pH 6.5 ≈ 33% hydrolysis occurred after 6 hours incubation rising to 100% at pH 5.0.

3.8.16 Plasmid detection in KCH1 and KCH2.

Detection of plasmids was attempted by the method of Kado and Liu (1981) but none were found in either KCH1 or KCH2 by this method of isolation.

3.8.17 Cell-free extract activity of KCH1 and KCH2 towards hexa.

Cell-free extracts prepared from KCH1 and KCH2 showed no activity towards hexa when measured as a function of oxygen uptake in a Gilson respirometer over a period of 5 hours.

3.8.18 Vertical gel electrophoresis of cell free extracts of KCH1 and KCH2.

Electrophoresis of cell-free extracts was performed as described in section 2.9.18. After staining and destaining no significant differences in protein banding between the two isolates KCH1 and KCH2 were found showing that the two isolates were very similar.

4.0 DISCUSSION.

4.1 Introduction.

The eight compounds tested during this study have previously been shown in degradation tests to exhibit aberrant behaviour and/or have given conflicting data about their degradability. It was this inconsistent behaviour which has made these chemicals of particular interest and therefore the subject of this study. It is intended to discuss the results obtained for each chemical separately relating them to previously reported work.

4.2 Degradation of BDSA.

BDSA was not degraded in the SCAS test during the 85 days of its operation. This is in agreement with Gerike and Fischer (1979) and Painter et al., (1983) and also with results they obtained for the degradation of BDSA in screening tests. Painter et al., (1983) also reported that in a SCAS test they performed for 5 months (150 days) no degradation of BDSA was observed. Again, this agreed with the SCAS test results obtained in this study (Figures 18a and 18b).

In die-away tests performed with BDSA-exposed activated sludge as the inoculum, positive values (25%) for degradation were observed only with inoculum which

had not previously been exposed to BDSA. After exposure to BDSA the activated sludge used as inoculum for die-away tests gave negative values. This increase in DOC may be explained by lysis of the inoculum cells during the test. As the period of exposure of activated sludge to BDSA increased (acclimatization) no increase in degradation of BDSA was observed. On the contrary as already mentioned effluent DOC values increased giving negative values of removal. This disagrees with Gerike and Fischer (1981) who observed that activated sludge acclimatized in a Husmann unit dosed with BDSA (4 weeks), could degrade this compound in the closed bottle and modified OECD tests. Gerike and Fischer (1981) go on to say that this proves that degradation of BDSA is "just a matter of acclimatization". In the Husmann simulation test these authors obtained 84% DOC removal after a lag of 4 weeks, while Painter (1985) reported only 25% after 8 weeks. Pitter (1976) after acclimatizing activated sludge for 21 days with BDSA also reported that it was degraded but at a very low rate (3.4mg COD g^{-1} h^{-1}). It is possible that the difference in the results for degradation of BDSA (i.e. Gerike and Fischer, Pitter, Painter and the present study) may be due to the basic difference in the activated sludge used as an inoculum. The activated sludge used by Gerike and Fischer (1981) needed to be acclimatized to BDSA before it would degrade it and the

same need for acclimatization was observed with Pitter (1976).

However the activated sludges used both in this study and that of Painter et al., (1983) did not acclimatize to degrade BDSA and the assertion made by Gerike and Fischer (1981), that degradation of BDSA is "just a matter of acclimatization" needs to be qualified for the inoculum and the conditions of the tests used in their determinations. No mention is made by either Gerike and Fischer (1981) or Pitter (1976) about the source of activated sludge they used. The activated sludge used in both the two British studies was obtained from water reclamation plants treating predominantly domestic sewage. If Gerike and Fisher's activated sludge had come from water reclamation plants treating greater proportions of industrial effluent, the predominant organisms may have been more susceptible to acclimatization to degrade BDSA. In this connection, it is of interest to note that in a ring test of the manometric method (King and Painter, 1983) only one laboratory reported biodegradation of BDSA with 95% ThOD and 99% DOC removal. This laboratory had used as inoculum activated sludge known to be constantly receiving aromatic sulphonic acids of various types.

During the operation of the SCAS unit it was observed that the number of viable microorganisms present fell dramatically when exposed to BDSA over the first 28

days. Then followed a slower decrease over the next 57 days (Table 15). The reduction of viable cells in the activated sludge is thought to be only marginally due to inhibition of microorganisms present by BDSA because the maximum calculated inhibition of bacterial growth was 28% at 100mg l^{-1} BDSA. This level of inhibition when applied to the viable microorganisms in the SCAS unit would not give the reduction in viable microorganisms observed. No plausible reason for the reduction in viable cells can be put forward.

From this investigation it has been concluded that BDSA is not readly biodegradable. It is not suprising that no microorganism capable of degrading BDSA was isolated. No information about the environmental fate of BDSA is available and so environmental hazard assessment is not possible.

4.3 Degradation of NMA.

Whether NMA can be classed as readily biodegradable in the strict OECD sense is doubtful. Gerike and Fischer (1979, 1981) found it to degrade in screening tests only when acclimatized inocula were used and King et al., (1985) achieved positive degradation in the ISO test on only 3 out of 13 occasions. NMA was, however, observed to be degraded in the SCAS unit after a lag of 77 days (Figures 20a and 20b) thus confirming the results of King et al., (1984, 1985) who used the SCAS

test and Gerike and Fischer (1979) who used the Husmann simulation method. Thus, NMA is inherently degradable and treatable. Figures 20a and 20b demonstrated graphically the lag with little degradation followed by a dramatic increase in degradation between day 60 and day 77. By contrast degradation was observed in die-away tests performed with activated sludge taken from SCAS unit dosed with NMA, even after the activated sludge used as the inoculum had only been exposed to NMA for 2 days. The initial level of degradation of NMA after 28 days in the die-away test using 2-day exposed sludge was 86% this increased to 100% after the sludge used as the inoculum had been exposed to NMA for 85 days.

The abiotic die-away test result showed a 55% removal of NMA by a non-biological mechanism. It is obvious that not all of the removal of NMA in the die-away tests is due to degradation although almost half of it is.

After the SCAS unit has been in operation for 115 days the addition of NMA was discontinued to observe if the activated sludge became deacclimatized to NMA. It was found that in die-away tests, performed during the following 83 days that 100% removal of NMA was still observed during the 28 days of each test performed as shown in figure 21.

This indicates that once the activated sludge has been acclimatized to NMA it does not lose its

degradative ability during an 83 days period. King and Painter (1985) demonstrated that NMA acclimatized activated sludge was not deacclimatized during a 202 day period of time. It is suggested that NMA will be degraded in water reclamation works becuase it is degraded in SCAS units during a 24 hour period after acclimatization.

The viability of microorganisms in activated sludge treated with NMA remained similar to the number found in untreated activated sludge. This is consistent with the data for inhibition of growth of activated sludge microorganisms by NMA which showed little or no inhibition upto 100mg l^{-1} of NMA.

It has been possible to isolate a microorganism capable of utilizing NMA as its sole carbon source. This microorganism, a Gram negative bacterium, has been identified with an 86.6% reliability of identification as being an Acaligenes species. The isolated bacteria KCNM1 was found to be very sensitive to antibiotics, being resistent to only 3 of those tested namely sulphamethoxazole/trimethroprim, nitrofurantoin and mecillinam. The growth rate of KCNM1 varied only slightly at the temperatures tested, 25, 30 and 35°C. It is believed that with a doubling time of 2 days that the isolate would be retained in the activated sludge process. The pH of the growth medium remained constant during growth of KCNM1. It was observed that a purple

colour formed if the growth flasks were left longer than 72 hours. It is thought that the catabolic function associated with the degradation of NMA is not plasmid borne because no plasmids were isolated from KCNM1.

NMA is synthesised from aniline by the addition of methylchloride. Several bacteria that can utilize aniline as a sole source of carbon and energy have been isolated (Aoki et al., 1983, 1984; Helm and Reber 1979; and Zeyer et al., 1985). The degradative pathways involved have not all been fully elucidated, but all those studied so far have involved oxidative deamimation to catechol, followed by either ortho- or meta-cleavage. Few reports have been published concerning bacterial metabolism of toluidines. (ring methyl-anilines). Pseudomonas strain JLI is capable of growth on o-toluidine via meta-cleavage pathway (Latorre et al., 1984). Growth has also been observed on m- and p-toluidine. Other reports of toluidine metabolism also involved meta-cleavage pathways. Rhodoccus rhodococcus Sb4 has been shown to degrade o-toluidine via 3 methyl catechol (Appel et al., 1984) and Pseudomonas testosteroni has been shown to degrade p-toluidine via 4-methylcatechol (Raabe et al., 1984).

NMA is a secondary amine with the methyl group on the nitrogen (Figure 10) unlike the other methylanilines where the methyl group is on the benzene ring, thus making NMA less susceptible to microbial attack. No

microorganism has been reported in the literature able to degrade NMA. Although during this study, no investigation of the metabolic pathway of degradation of NMA was undertaken, it is possible to speculate about the pathway of NMA degradaton.

It is very unlikely that degradation is through methyl catechol as proposed for the degradation of m-toluate and p-toluate by Acaligenes eutrophus (Hughes et al., 1984) who proposed that the first degradation step was deamination. This is because demethylation of NMA by KCNM1 must occur before deamination can occur therefore methyl catechol cannot be formed since the methyl group would already have been removed. What is more likely is that NMA is demethylated resulting in the formation of aniline which is then degraded via catechol and β-ketoadipate as shown in Figure 48. It has been observed that ammonia is released during growth of KCNM1 on NMA although no quantitative data was obtained.

The speculated model of NMA degradation (Figure 48) utilizes a known pathway for aniline degradation after the initial new step of deamination. Microorganisms very rarely create new pathways of assimilation/ dissimilation but develop existing pathways. The speculated pathway in Figure 48 is put forward based on this assumption.

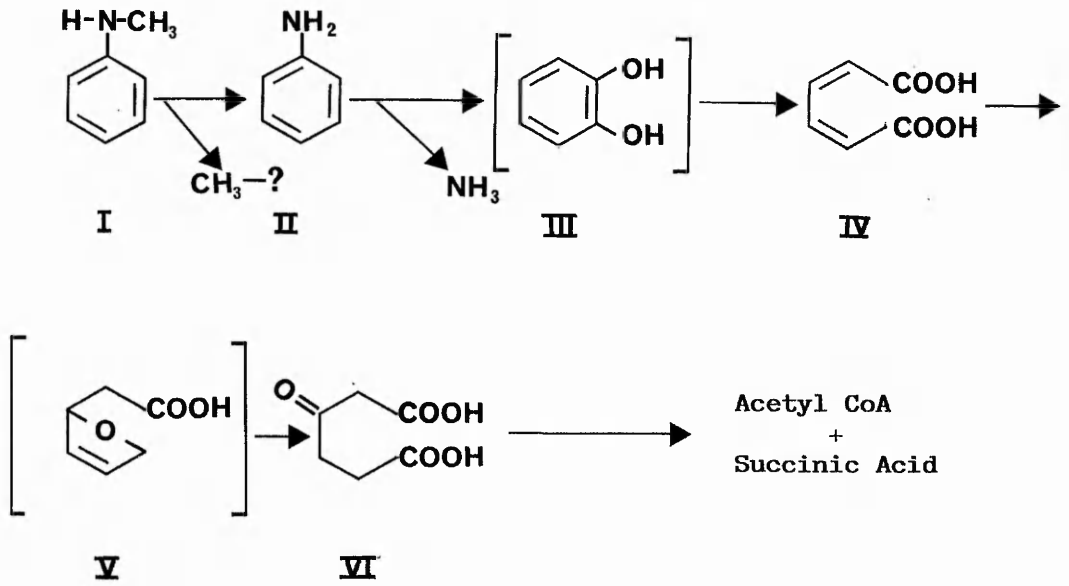


Figure 48. Speculated pathway for the biodegradation of NMA by KCNM1. (I) NMA; (II) Aniline; (III) Catechol; (IV) cis, cis-Muconic acid; (V) Muconolactone and (VI) β-Ketoadipate.

Future work with KCNM1 might include an investigation into the degradative pathway of NMA utilized by KCNM1 using the speculated pathway (Figure 48) as a starting point. This would be of academic value because no work has been published about the degradation of NMA. In addition to determining the pathway of NMA degradation by KCNM1 it would be of interest to determine degradation rates for environmentally realistic concentrations of NMA using ^{14}C labelling. It would also be worth investigating degradation of NMA by activated sludge and KCNM1 at realistic environmental temperatures to assess the true likelihood of NMA being degraded in the environment.

4.4 Degradation of 2 and 3CBA.

4.4.1 Degradation of 2CBA.

It is generally accepted that 2CBA is not readily biodegradable even when ready tests were performed with acclimatized inocula (Gerike and Fischer, 1981). In this study 2CBA was not degraded in a SCAS test during the whole 275 days of the test (Figures 19a and 19b), but King *et al.*, (1985) using only 2mg 2CBA C l^{-1} instead of 20mg l^{-1}, found that the compound was degraded. Inocula taken from the SCAS units degraded 2CBA by 90% in 14 days in DOC die-away tests. Lund and Rodriguez (1984) reported that they found no adaptation

of activated sludge to 2CBA (1g l^{-1}) in a modified SCAS procedure over more than 30 days. However, Haller (1978) observed degradation of 2CBA (16mg l^{-1}) after a lag of only 25 days in waste water at 30°C.

Gerike and Fischer (1979) found 2CBA to be degraded (97% removal of DOC) in a simulation test (Husmann) using synthetic sewage, but King et al., (1984) under the same conditions found only 30-40% DOC removal, although when the experiment was performed with domestic sewage 100% degradation was observed after a lag of 4 weeks. Incidentally, King et al., (1984) found virtually no difference in either the percentage removal, or standard deviations of the percentage removal when using the Husmann units in "single" or in "coupled" mode, as used by Gerike and Fischer (1979).

No acclimatization of activated sludge to 2CBA was observed in the present study over the 245 days of the SCAS test and no degradation was observed in any of the die-away tests performed with inoculum taken from the SCAS test.

2CBA had little effect on the total number of viable microorganisms present in the SCAS unit. Neither did it have any affect on growth of sludge derived microorganisms below 32mg l^{-1} 2CBA. It did show 30% inhibition at 100mg l^{-1} 2CBA indicating a weak inhibitory effect; the EC50 value for 2CBA was calculated to be greater than 100mg l^{-1}. No further

investigation into the inhibitory properties of 2CBA were made as it did not appear to be degradable so did not have any further interest to this project. It is thought that 2CBA would have little effect on activated sludge if it entered water reclamation works at levels below 100mg l^{-1}. At higher concentrations 2CBA is known not to be easily soluble and so may not be soluble at a level equivalent to its EC50 value.

From the information about the biodegradability of 2CBA it is clear that it is not readily biodegradable but under certain conditions it exhibits inherent biodegradability. When simulation tests were performed on the 2CBA it was found to be degraded by two separate groups of workers, Gerike and Fischer (1979), and King et al., (1984). These data indicate that 2CBA should not present an environmental problem as it will be degraded under environmental conditions.

There is uncertainty about how degradable 2CBA is, which may be due to the different sources and types of inoculum used in the test methods employed by various workers. This study used activated sludge from a water reclamation plant treating predominantly domestic effluent and did not degrade 2CBA under the test conditions employed. Other workers may have used activated sludge or sewage from water reclamation plants treating industrial effluents which would have a greater likelihood of having previously been exposed to 2CBA or

similar compounds. This would increase the chances of the activated sludge containing microorganisms with the ability to degrade 2CBA.

It has been demonstrated by Shamat and Maier (1980) that enriched activated sludge in continuous culture can be adapted to produce a population of predominantly Pseudomonas species capable of degrading 2CBA. Cometabolism in continuous culture has been demonstrated by Veerkamp et al., (1983). This information helps to substantiate the idea about the effect the source and type of inoculum may have on degradation of 2CBA.

Under normal conditions of use it is not believed that 2CBA would enter the environment directly, but rather it would appear in the environment because of the breakdown of the products of which it is an intermediate or key component. These products include glues and paints where it is used as a preservative, it is also used as an intermediate in the manufacture of fungicides and dyes.

From this study it would appear that 2CBA is not degradable at 20mg C l^{-1} (36.3mg l^{-1}), but it is degradable at lower levels, Painter (1985) 2mg C l^{-1} (3.7mg l^{-1}) and Haller (1978) 16mg l^{-1}. This observation would warrant further investigation and could perhaps be coupled with investigation of the behaviour of environmental levels of 2CBA (µg l^{-1}) using ^{14}C labelled 2CBA with assorted environmental samples.

4.4.2 Degradation of 3CBA.

It took 54 days for the convergence of the SCAS unit being dosed with 3CBA and the control SCAS unit, indicating complete degradation of 3CBA during each 24 hour cycle of the SCAS test (Figure 25a). This result indicates that activated sludge requires a finite length of time to acquire the ability to degrade 3CBA in the SCAS test (acclimatization). With the die-away tests performed with activated sludge from the SCAS unit being dosed with 3CBA, very high removals of 3CBA were observed; however, even with previously unexposed activated sludge as the inoculum high removals were obtained. This indicates that the ability to degrade 3CBA is already present in activated sludge before exposure to the compound.

Zahn and Wellens (1980) found 3CBA to be readily biodegradable using river water as the inoculum source. It was also found to be readily biodegradable by Painter et al., (1983) using the ISO die-away test. King et al., (1984) reported that 3CBA was removed extensively in simulation tests using both synthetic and domestic sewage. Not all workers have found 3CBA to be as easily degradable as already described. Two groups of workers found that 3CBA did not degrade unless the inoculum had previously been acclimatized to the compound, namely Gerike and Fischer (1979 and 1981) and also Lund and Rodriguez (1984). It would appear that these workers

are in the minority with the majority of workers finding 3CBA easily degradable.

In the die-away tests it was observed that 3CBA was still degraded even when the inoculum had not been exposed to 3CBA for up to 178 days (Figure 26). This indicates that the ability to degrade 3CBA is not lost even when the activated sludge has not been exposed to the compound for long periods, that is, it is not deacclimatized. It is clear that the 3CBA degradative mechanism is very stable. It is also clear from the die-away tests that it is a biological mechanism which removes the 3CBA because only about 20% removal was observed in the abiotic control.

The ability to degrade 3CBA would appear always to be present and does not need time for the activated sludge to acclimatize. Time is required for the activated sludge to select for organisms which can degrade 3CBA fastest. This is why there was a 54 day lag before 3CBA was completely removed during each 24 hour cycle of the SCAS test.

The viability of the microorganisms found in activated sludge was not affected by 3CBA during the operation of the SCAS unit. Neither was inhibition due to 3CBA observed during inhibition tests performed on activated sludge, the EC50 value for 3CBA on activated sludge being greater than 100mg l^{-1}.

4.4.2.1 Competent Isolates.

It was possible to isolate four microorganisms capable of utilizing 3CBA as their sole source of carbon at 0.5g 3CBA l^{-1}. The four isolates KC13, KC16, KC20 and KC21 were found to be very similar and were identifed by using the API 20E test system; all four were found to be Pseudomonas species. Isolate KC13 was identified as Pseudomonas putrefacens with a 92.9% reliability of identification, isolates KC16 and KC21 were identified as Pseudomonas fluorescens/putida with 87 and 91% reliability of identification respectively. Isolated KC20 was identified only to species level and was found to be a Pseudomonas species.

Although KC16 and KC21 were identified as the same type of organism they were found to be different from one another in several respects. KC21 utilized melibiose and trehalose, and in addition was found to be resistant to kanomycin, while KC16 was not able to utilize the two sugars and was sensitive to kanomycin. This information shows that all four isolates are not the same and are independent of each other.

Isolate KC21 had the highest specific growth rate followed by KC16, KC13 and KC20. While specific growth rates were unaffected by temperature between 25 and 30°C, they were lower at 35°C.

Storage of the isolates on 3CBA minimal salts medium over 20 months with continuous subculturing, resulted in

selection of mutants with higher specific growth rates because of continuous selection pressure. After 20 months isolates KC13, KC16 and KC20 all had the same specific growth rate as one another and higher than originally determined. KC21 had in the same time almost doubled its original specific growth rate and grew much faster than the other three isolates.

All four isolates were found to contain plasmids, indicating that the type of catabolic function could be either plasmid borne or chromosomal. Isolates KC13, KC16 and KC20 contained one plasmid which appeared to be common for all three, but KC21 had 3 plasmids all different to those observed in the other isolates.

Other workers have isolated 3CBA-degrading organisms with possibly the most documented being Pseudomonas B13 isolated by Dorn et al., (1974). Other 3CBA-utilizing pseudomonads have been isolated by Johnstone et al., (1972) and Hartman et al., (1979). In addition to microorganisms which can utilize 3CBA as their sole source of carbon, organisms which can degrade 3CBA by cometabolism with benzoate have been isolated. Examples of these organisms are an Arthrobacter species isolated by Horvath and Alexander (1970) and a Pseudomonas species isolated by Veerkamp et al., (1983).

It has been established in the present work that all four 3CBA degrading isolates degrade 3CBA by causing cleavage of the benzene ring before chloride is released

(Figures 27 to 30). Each isolate released the maximum theoretical available chloride present, although at different rates. KC21 released chloride the most rapidly after ring cleavage, followed by decreasing rate of chloride release by KC16, KC13 and KC20. This difference is presumably due to differences in the activities of the enzymes which cause dehalogenation. The dehalogenation of 3CBA may not be due to specific dehalogenases but rather to enzymes which metabolize halogenated intermediates formed by ring cleavage to unstable compounds which in turn spontaneously liberate halide ions. This idea was proposed by Knackmuss (1979) after he demonstrated that benzoate-grown cells of Pseudomonas B13 possessed enzymes with only low levels of activity towards chlorocatechol, but did observe release of chloride ions.

During this study it was shown that all four 3CBA-utilizing isolates could grow on 3CBA upto 1.0g l^{-1} releasing the theoretical maximum available chloride. It was observed that the amount of 3CBA present affected chloride release differently in each isolate. With KC16 an increase in 3CBA present during growth increased the rate of chloride release, whereas with KC13 and KC20 an increase in 3CBA produced a lag before chloride release was observed. Overall for isolates KC13 and KC20 a reduction in the rate of chloride release was observed compared to KC16 indicating an initial temporary

inhibition of isolates KC13 and KC20 only with increased levels of 3CBA. With isolate KC21 chloride release was faster at 0.75g l^{-1} 3CBA than at 0.5g l^{-1} but it was slower at 1.0g l^{-1} than at 0.75g l^{-1} though faster than at 0.5g l^{-1}. This would indicate that between 0.75g l^{-1} and 1.0g l^{-1} the optimum concentration for growth of 3CBA has been passed and is now beginning to inhibit the rate of chloride release by KC21.

It was found that none of the isolates could grow on 2 or 4CBA, indicating that the mechanisms to degrade these chlorobenzoates were not present in the 3CBA-degrading isolates. Growth of the four isolates on other chlorinated compounds was investigated and only chlorosuccinate gave growth of all four isolates. All four isolates grew weakly on chloroacetate and chloropropionate with the exception of KC13 which could grow well on chloropropionate. None of the isolates could grew on chloroacetaldehyde.

Three degradative ring fission pathways for benzoid rings are possible, the *ortho* or β-ketoadipiate pathway, the *meta* and *gentisate*-pathways. Each pathway has specific intermediates associated with them, the *ortho*-pathway having acetate and fumarate, the *meta*-pathway, acetaldehyde and pyruvate and the *gentisate*-pathway gentisate, pyruvate, fumarate and maleate. Each isolate was tested for growth on all of these compounds except

maleate. The four isolates grew on pyruvate, fumarate and acetate, but none grew on acetaldehyde. These data would appear to discount the *meta*-pathway because acetaldehyde is an intermediate of this pathway. Further evidence to discount the *meta*-pathway comes from the fact that all the isolates exhibit ring cleavage followed by dehalogenation, while in the *meta*-pathway ring cleavage and dehalogenation appear to occur simultaneously rather than sequentialy. This would strongly indicate that the *meta*-pathway is not used by the four isolated microorganisms. In addition it has been reported that the *meta*-pathway produces toxic and inhibitory metabolites (Knackmuss, 1979; Murray et al., 1972) causing self inhibition.

With respect to the *gentisate*-pathway the starting compound, gentisate, is not utilized by any of the isolates, which almost certainly indicates that this pathway is not responsible for the degradation of 3CBA by the four isolates. Some contradictory evidence arises from the fact that both fumarate and pyruvate, intermediates in this pathway, are both utilized. These are commonly utilized compounds and it is proposed that the isolates utilize the compounds by some other pathway such as the *tricarboxylic*-pathway in the case of fumarate and pyruvate. Glucose was tested to show that the isolates were viable at the time of the experiment.

As yet no microorganisms have been isolated by any workers which can utilize the *gentisate*-pathway. This is most probably due to the production of toxic and inhibitory metabolites.

The final pathway not yet discounted is the *ortho*-pathway; the intermediates studied which are part of this pathway were acetate and fumarate. Both these compounds were utilized by all four isolates, which is a good indication that this is the pathway utilized to degrade 3CBA by the four isolates.

This hypothesis was further tested by respirometry of the four isolates with acetate, acetaldehyde, fumarate gentisate and β-ketoadipate. In addition to the compound previously tested, β-ketoadipate was also tested as this is the key intermediate in the *ortho*-pathway. Gentisate was not utilized by any of the isolates, which is further evidence for this not being the pathway for 3CBA degradation. All isolates were shown to utilize oxygen in the presence of acetaldehyde (all less than 47% of the maximum theoretical uptake). It is true that pyruvate the other important metabolite associated with the *meta*-pathway was utilized by all the isolates which is evidence for this pathway. However, when the maximum oxygen uptake rates were calculated it was found that the rate was much lower for acetaldehyde than for any of the other intermediates. This appears to indicate that the acetaldehyde is being transformed into

toxic or inhibitory metabolites resulting in the oxygen uptake observed. Dagley and Chapman (1971) suggested that low oxygen uptake such as obtained with acetaldehyde indicates that it does not lie on the pathway of oxidation. β-Ketoadipate was utilized by all the isolates consuming between 44 and 60% of the theoretical oxygen uptake. In addition it was calculated that the maximum oxygen uptake rates were very high indicating the compound was being actively metabolized. All the isolates utilized both acetate and fumarate the two important intermediates of the *ortho*-pathway. Between 51 and 66% of the theoretical maximum oxygen demand possible from acetate was observed, this together with the high calculated oxygen uptake rates indicates that the isolates are actively metabolising acetate. For fumarate, 65-83% of the maximum theoretical oxygen uptake was observed for the all isolates, and was coupled with very high maximum oxygen uptake rates. The combination of all these data leads to the conclusion that the pathway by which the isolates utilize 3CBA is the *ortho*-pathway (Figure 49). One possible reason for intermediates associated with the other two pathways being oxidised readily e.g. pyruvate is that the enzymes for its metabolism may be constitutive (Stanier, 1947).

Figure 49. Compound Index

(1) 3-chlorobenzoic acid

(2) 3-chlorol-dihydroxy benzoate

(3) 5-chloro-dihydroxy benzoate

(4) 3-chloro-catechol

(5) 4-chloro-catechol

(6) α-chloro-muconic acid

(7) β-chloro-muconic acid

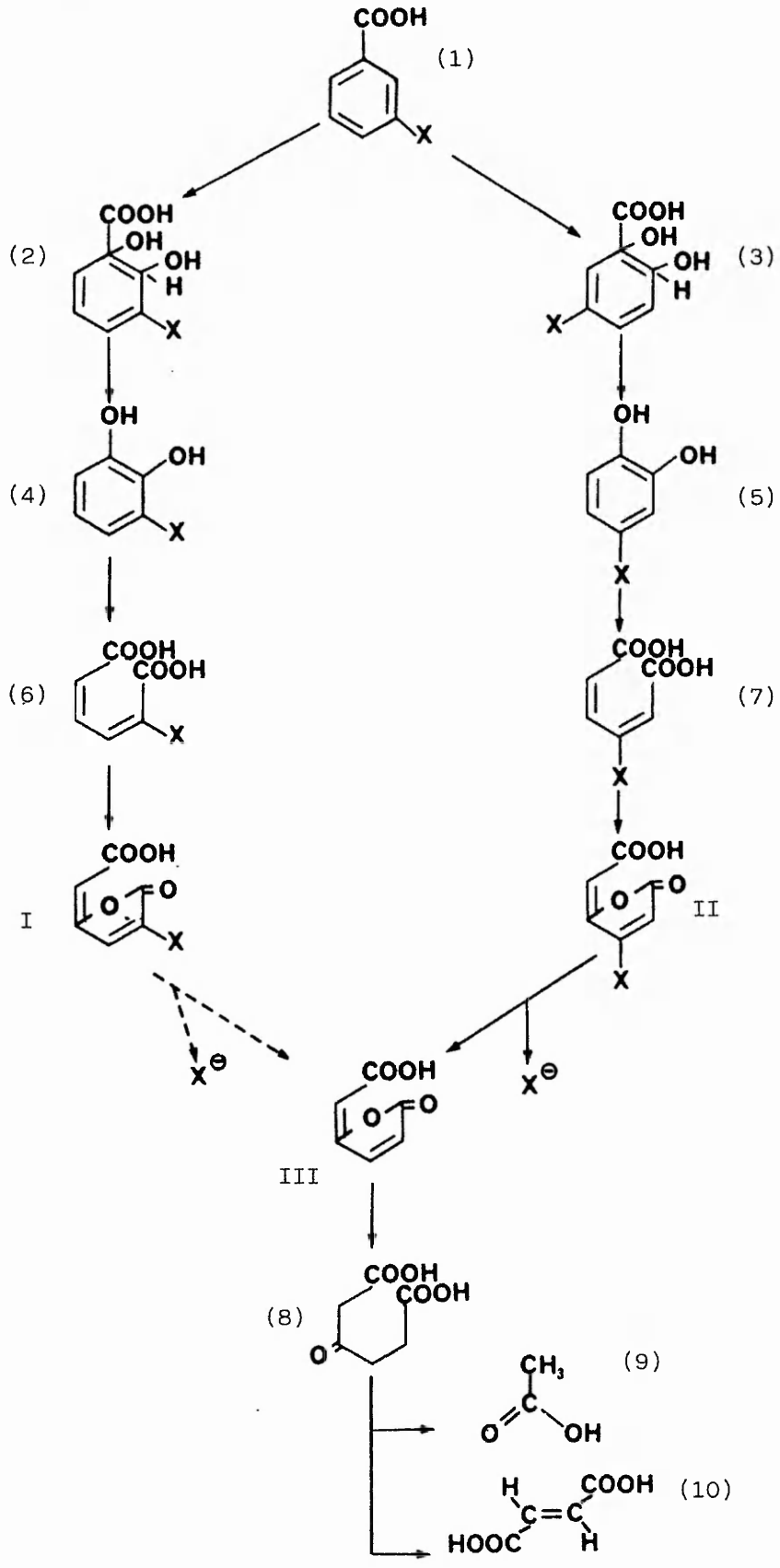
(8) β-ketoadipate

(9) acetate

(10) fumarate

I, II and III are hypothetical unstable intermediates.

Figure 49. Ortho (β-ketoadipate) pathway



The maximum theoretical oxygen uptake was not observed except on a few occasions namely for sodium benzoate and 3CBA. The short-fall was most probably due to only a proportion of the substrate being completely oxidized, the remainder being assimilated as intracellular metabolites (Barker, 1936; Clifton and Logan, 1939; Clifton et al., (1936)). Dagley and Nicholson (1970) proposed entry into the tricarboxylic acid cycle for the biosynthesis of amino acids. Fewson, (1980) proposed that only two-thirds of an aromatic compound is mineralized, whilst the remaining third is converted into new cell material under aerobic conditions.

It is not a surprising conclusion that the *ortho* or β-ketoadipate-pathway is involved in the degradation of 3CBA, both on consideration of the data and the fact that it is one of the major microbial pathways for the dissimilation of aromatic and hydroaromatic compounds, (Stanier and Ornston, 1973).

It might be appropriate to discuss here why only the 3-isomer was found to be degraded. The literature has other examples of organisms capable of degrading 3CBA which have already been discussed, but examples of 2 and 4CBA-degrading organisms are much less common. Hartman et al., (1979) and Marks et al., (1984) have detected growth on 4CBA in some species of Pseudomonas, while the work of Hartman et al., (1979) showed isolates capable of growing on either 3 or 4CBA, but with a lower growth

rate on 3CBA. Marks et al., (1984) isolated a strain of Arthrobacter species capable of utilizing 4CBA as the sole carbon source but not 2 or 3 CBA. This organism dehalogenated 4CBA as the initial step in the degradative pathway in a similar manner to the strain isolated by Johnston et al., (1972).

It is significant that 2CBA was not degraded by activated sludge neither was it utilized by the 3CBA degrading isolates. These same isolates were found to be unable to utilize 4CBA. The most probable reason for the lack of 2CBA utilization is steric hindrance at the 2 or ortho-ring position making dehalogenation very difficult. When the chlorine is in the 4 or para ring position a negative inductive effect reduces the reaction rate to a negligible value. Consequently only the 3 or meta substituted chlorobenzoic acid is readily biodegradable

4.4.2.2 Future work.

3CBA is clearly degradable both on the evidence of this study and that of other workers. Further work on this compound therefore might include an investigation into the dehalogenation mechanism of the isolates, to determine if chloride release is spontaneous or due to a specific or non-specific dehalogenase.

4.5 Degradation of CPTCA and THFTCA.

4.5.1 Degradation of CPTCA.

The cis, cis, cis, cis isomer of CPTCA was not found to be degraded in the SCAS test during the 175 days of its operation. This result is in agreement with those of Painter (1986) who also performed an activated sludge simulation. Gerike (1978) did not find any evidence for either ready or inherent biodegradability of this compound, and further work (Gerike and Fisher, 1979) showed that in seven tests in which they investigated CPTCA, no degradation was observed. Gilbert and Lee (1980) found that the cis, trans, cis, trans isomer of CPTCA was degraded by 85% in the SCAS test after an eight week lag. They also reported 0-30% removal in a ready test depending on the sterioisomer but the isomers investigated were not recorded in their paper.

The activated sludge in the SCAS unit in the present work was found not to have acclimatized to CPTCA and did not develop the ability to degrade CPTCA as shown by only negative DOC values for degradation after 28 days incubation in die-away tests (Table 7).

CPTCA had little effect on the total number of viable microorganisms present in the SCAS test unit compared to the control (Table 8). The compound was found to be non-inhibitory to microorganisms and had an EC50 value of greater than 100mg l^{-1}. Painter (personal

communication) has found that the inherent error of the test for inhibition of microorganisms maybe as much as ± 20%. It is not surprising that microorganisms capable of degrading CPTCA were not isolated since the compound was not found to be inherently biodegradable by this investigation.

The intended use of CPTCA was as a phosphate substitute in detergent powders to combat hardness of water. It is because detergents are widely used that the potential problem is very large. Substitution of phosphate is on first consideration a good idea because phosphate can be a problem in water courses causing algal blooms which on decomposition may release toxic or noxious substances.

However, the available data indicates that the cis, cis, cis, cis isomer of CPTCA is not biodegradable and the cis, trans, cis, trans isomer is biodegrable but only after an 8 week lag period. Would the replacement of phosphates by CPTCA in detergents create a different but equally important problem namely the accumulation of CPTCA ?. Some CPTCA is at best degraded only after a lag period of 8 weeks, or not at all, depending on the isomer. It may prove to be a greater environmental problem than the phosphates it is supposed to replace, even if sufficient quantities of the degradable isomer could commercially be produced. It is true that it has an EC50 value of greater than 100mg l^{-1} but nothing is

known of its behaviour in the environment or its accumulation in the environment. More work must be performed to determine what environmental impact CPTCA would have if it were to be used as a phosphate substitute in detergents..

It would appear from the data available for this compound that it may not be a suitable replacement for phosphates in detergents. This is because CPTCA appears only to degradable in the cis, trans, cis, trans form after an 8 week acclimatization period and other available information is very limited. It is not known if the degradable isomer would be removed during effluent treatment in water reclamation works. Further work is required including simulation tests and investigation of the environmental toxicity of this compound.

4.5.2 Degradation of THFTCA.

THFTCA did not initially degrade in the SCAS test during the first 57 days, but a gradual increase in degradation from negligible to approximately 100% degradation was observed between day 57 and day 90 (Figures 22a and 22b). This shows that the 20mg C l^{-1} of THFTCA present in the SCAS unit at the start of each aeration could be totally degraded during a 24 hour aeration period. Complete acclimatization of activated sludge to THFTCA took 90 days; a similar observation was

made by Painter, (1986) in SCAS tests. This is strong evidence to suggest that adaptation of the activated sludge population requires an extended period of exposure to THFTCA (11 to 13 weeks).

No removal of THFTCA in 28 day die-away tests was observed with activated sludge exposed to THFTCA for only 2 days as inoculum, although 89% removal was observed with inoculum which had been exposed to THFTCA fo 30 days. All further die-away tests showed 100% removal of THFTCA. Figure 23 graphically shows the lag in degradation in die-away tests, and also that it was not possible to deacclimatize the activated sludge over a 3 month period. Painter (1986) reported an 11 week acclimatization period of the activated sludge inoculum for 100% removal to occur. The degradation of THFTCA has been demonstrated to be due to a biological function as no degradation was observed in the abiotic control. It is believed that the ability of activated sludge once acclimatized to degrade THFTCA is stable as it was not lost over a three month attempt to deacclimatize it.

Painter (1986) indicated that THFTCA may be inhibitory, at least initially, to activated sludge in a Porous Pot simulation test; no degradation of the compound was observed during the 9 week period of the test. No difference in the growth of activated sludge on sodium acetate/nutrient broth was observed between dosed and undosed activated sludge with THFTCA during

this study. The inhibition test on activated sludge showed that THFTCA was not inhibitory.

A microorganism capable of utilizing THFTCA as its sole source of carbon has been isolated and designated KCTI. This microorganism has been identified as a non-pathogenic, gram positive, coryneform rod, exhibiting a rod/coccus life cycle by the NCIMB. There is no evidence in the literature of any other microorganism(s) capable of utilizing THFTCA.

KCT1 was shown to be temperature sensitive; as the temperature increased the specific growth rate decreased. It was also observed that over an 82 hour period the pH of the culture fluid increased from pH 6.4 to pH 7.1. This increase in pH was presumably due to decarboxylation of THFTCA. Indeed, Smith and Tricket (personal communication) have recently reported high levels of decarboxylase in KCT1, although no pathway for THFTCA has yet been proposed. The catabolic function for THFTCA degradation is not thought to be plasmid borne as no plasmids were isolated from KCT1.

It is not envisaged that THFTCA would provide an accumulative environmental hazard in sewage treatment works at levels upto 100mg l^{-1}. The reasons for this are i) it is broken down in SCAS tests within 24 hours once the activated sludge has acclimatized at 20mg C l^{-1} and ii) it is not inhibitory up to this concentration.

From the environmental point of view, THFTCA would be a better substitute for phosphate in detergent formulations than CPTCA, which is not readily degradable in the cis, cis, cis, cis form.

Future work with THFTCA would involve the use of ^{14}C radioisotope to investigate degradation of THFTCA at environmental levels ($\mu g\ l^{-1}$) and at realistic environmental temperatures. Attempts to elucidate the metabolic pathway of THFTCA degradation should also be attempted.

4.6 Degradation of t-butanol.

Reports of the biodegradation of this compound are confused because of its volatility, which most authors appear to have ignored. From its structure ie tertiary carbon atom, it would be expected to present difficulties. The majority of workers report that t-butanol is not readily biodegradable, since for example, in a ring test using a respirometric method only 2 of the 11 participants reported more than a 60% theoretical oxygen demand (King et al., 1985). The oxygen demand in these two cases indicated that t-butanol was being degraded within the enclosed test vessels. In the present study t-butanol was removed from the SCAS unit (23 hours aeration period) from the start of the SCAS test without the need for an acclimatization period (Figure 15a and 15b) and continued to be removed until

the end of the test on day 118. This is in contrast to results of simulation tests (3-hour retention period) with activated sludge; neither Gerike and Fischer (1981) nor King et al., (1984) found much removal of t-butanol in the 9 weeks duration of the test, although Gerike and Fischer (1981) did observe removal of t-butanol when using the more lenient "square wave feed" version of the Husmann test. In the study by Gerike and Fischer (1981) acclimatized sludge failed to degrade t-butanol in a "ready test" but Painter et al., (1983) found SCAS acclimatized sludge did not degrade t-butanol after 18 days.

Volatilization of t-butanol was demonstrated in a SCAS unit containing only 20mg C l^{-1} of t-butanol in aqueous solution and aerated at the same rate as in a normal test (0.5 l min^{-1}). During a 24-hour period 92% of the t-butanol present at the start of aeration had been removed. The only possible mechanism for this occurrence was volatilization. This idea of volatilization is in direct contradiction to the results reported by Horn et al., (1970), who reported that only 1% of the t-butanol removed was lost by evaporation (air stripping) and the remaining 99% by biodegradation in an activated sludge plant. King and Painter (1983) reported that in aerated Husmann units receiving t-butanol in the presence of no biological agent, only 8% of the t-butanol originally present disappeared due to

volatilization in the normal 3 hour aeration period. Gerike and Fischer (1981) measured 0% CO_2 production with t-butanol in the 28 day-Sturm (ready) test, but observed 32% DOC removal which would indicate removal by volatilization.

Removal of t-butanol was observed in all the die-away tests from the first one set up with inoculum taken on day 2 of the test (Figure 16). The removal of t-butanol continued even when the inoculum was activated sludge which had not been exposed to t-butanol. It was interesting that the abiotic control exhibited 63% removal of t-butanol during the 28 days of the die-away test. Obviously the removal of t-butanol was not due to any biological agent.

It was proposed that t-butanol may have been more volatile in aqueous solution than previously thought. This idea was tested by conducting a series of further die-away tests (Table 11). This data shows that shaken flasks closed with porous bungs have slightly more t-butanol removed than static flasks that is, 89 and 81%. With similar flasks sealed with silicone bungs the proportion removed was much less, namely 35% in shaken flasks and 29% in static flasks. In abiotic controls removal of t-butanol was observed in all flasks although less than in the flasks with viable activated sludge. Thus the majority of t-butanol removal was by volatilization and less than 20% of the removal observed

could be related to biodegradation. A further set of abiotic control flasks containing no activated sludge, gave very similar results to flasks containing sterile activated sludge showing that loss of t-butanol was not due to adsorption.

As a direct result of this study, Painter (1986) performed further die-away tests using the method developed during this investigation and described in section 2.4.4 and found that up to 63% of t-butanol was lost by volatilization in unsealed flasks normally used. This method of using sealed flasks could perhaps be used to help determine the biodegradability of volatile chemicals which is difficult to do using current methods. It is a simple, cheap and effective method which are important factors when testing large numbers of volatile compounds.

During the operation of the SCAS unit slightly lower viable counts were obtained for the unit exposed to t-butanol than for the control unit. The reason for this is unclear but it is not thought to be due to inhibition of microorganisms in the activated sludge as the counts are within acceptable experimental error.

No microorganism capable of utilizing t-butanol was isolated even though there was evidence to suggest t-butanol is degraded to some extent by activated sludge. It has however been reported by Romadina et al., (1984) (English abstract) that two Pseudomonas species have

been isolated which could degrade t-butanol and they continued to do so when added to activated sludge units.

The data from this study indicates that major mechanism of t-butanol removal is volatilization. However, the fact that other workers (Romadina et al., 1984) have isolated microorganisms which can degrade t-butanol must not be discounted. It is suggested that discrepancies between the data of different workers may be arise from uneven distribution of competent microorganisms globally. This may be due to the fact that the reservoirs of microorganisms used by workers have been exposed to t-butanol prior to use in experimentation, perhaps allowing acclimatization to the test compound.

In conclusion t-butanol has been demonstrated to be removed from tests although the mechanism is unclear. In this study removal has been attributed to volatilization although other workers have demonstrated a biological mechanism.

4.7 Degradation of hexa.

In a SCAS unit being dosed with 20mg C l^{-1} of hexa daily, it was not possible to detect any significant daily removal of the compound; with only approximately 17% removal on average over the 24 hours aeration period. The SCAS unit was operated for 205 days without the removal of hexa altering significantly. This agrees

with the results (15-20% DOC removal) of Painter and King (1986) for simulation tests performed on hexa. These results suggest that hexa is neither inherently degradable nor treatable. However when the daily addition of hexa and synthetic sewage to the SCAS unit was discontinued and removal of hexa followed for a number of days, it was found that 75% DOC was removed over a subsequent 10 day period indicating at least inherent biodegradability.

Further, Painter and King (1986) reported that 100% removal of hexa was obtained in die-away tests when acclimatized activated sludge was used as the source of inoculum. Similarly, high levels of hexa, up to 87%, after 28 days were obtained in die-away tests during this investigation, though when the activated sludge used as inoculum had been exposed to hexa for as long as 205 days in a SCAS unit, 100% removal was not observed as reported by Painter and King (1986). The ability to degrade hexa in die-awy tests was not lost even after 3 months deacclimatization.

The viability of microorganisms in the SCAS unit being dosed with hexa, fell from 2.1×10^{7} microorganisms ml^{-1} to 3.8×10^{5} microorganisms ml^{-1} over the first 28 days after which it remained constant, whilst the control SCAS unit viable count remained unaltered in the order of 10^{7} microorganisms ml^{-1}. No satisfactory explanation for this reduction in the viable count has

been found during this study. Inhibition of activated sludge growth due to hexa has been measured; 37% at 32mg l^{-1} hexa after 6 hours incubation although after 24 hours no apparent inhibition could be detected. In light of this explanation it is difficult to point to inhibition as the sole reason for the reduction in viable microorganisms. Further work in this area might include an investigation into the inhibitory effect on the component species of activated sludge by hexa, in conjunction with a study of the growth kinetics of these component species. There may well be a link between inhibition of activated sludge microorganisms and their growth rates to account for the reduction in the viable count of activated sludge exposed to hexa.

It is known that hexa can be hydrolysed under acid conditions to ammonia and formaldehyde (Strom and Won Jun, 1980; Tada, 1960) and this study has shown that little hydrolysis can be measured colorimetrically above pH 6.9. The lowest pH value measured over the 28 day period of any die-away tests performed on hexa was pH 6.95. Thus it may confidently be proposed that significant removal of hexa in die-away test is not due to acid hydrolysis. This idea can be further substantiated by the fact that no abiotic removal of hexa was observed, and that Painter and King (1986) calculated from the data of Strom and Won Jun (1980) and

those of Tada (1960) that the half life of hexa at 30°C and pH 7.0 is as high as 160 days.

4.7.1 Utilization of hexa by competent isolates.

Two microorganisms KCH1 and KCH2 have been isolated from activated sludge which had been exposed to hexa. These two isolates were found to be very similar and have been identified as being Methylobacterium species. The isolates were shown to be different species by the use of specific biochemical, carbohydrate utilization and antibiotic resistance tests. The two isolates can conveniently be described as pink pigmented facultatively methylotrophic bacteria (PPFM's).

No significant differences in protein banding was observed by vertical gel electrophoresis of cell-free extracts from the two isolates. No plasmids were detected in either isolate by the method of Kado and Liu (1981). The growth rates of isolate KCH1 at 25; 30 and 35°C were at 0.03 h^{-1}; with KCH2, however, the specific growth rate increased with increasing temperature having the highest growth rate at 35°C (0.04 h^{-1}).

Storage of the isolates over a period of 32 months on minimal medium agar slants plus 0.5g l^{-1} hexa resulted in higher specific growth rates. This increase in the specific growth rates of KCH1 and KCH2 (to 0.14 and 0.15

h^{-1} respectively) was probably due to natural strain selection of isolates during storage.

There are four possible assimilatory pathways found in methylotrophs (Zatman, 1980), ribulose monophosphate pathway (RuMP), xylulose monophosphate pathway (XuMP), ribulosebiphosphate carboxylase pathway (RuBPC) and serine pathway (Figure 50). If methane is the carbon source to be utilized it is metabolized via methanol to formaldehyde. Methanol is metabolized directly to formaldehyde which is the common starting point for the four metabolic pathways (Attwood and Quayle, 1984) shown in Figure 50. It is proposed that the initial breakdown of hexa to formaldehyde and ammonia is enzymatic because the pH observed during growth never drops low enough to allow acid hydrolysis as discussed earlier.

The XuMP cycle can be discounted as the probable pathway because it is found only in methylotrophic yeasts (Zatman, 1980) and so will not be found in either KCH1 or KCH2. Of the remaining three potential pathways for hexa degradation, the RuBPC cycle can be discounted because it requires conversion of formaldehyde to formate. It has been demonstrated that neither isolate grew on or consumed oxygen when sodium formate was the sole source of carbon.

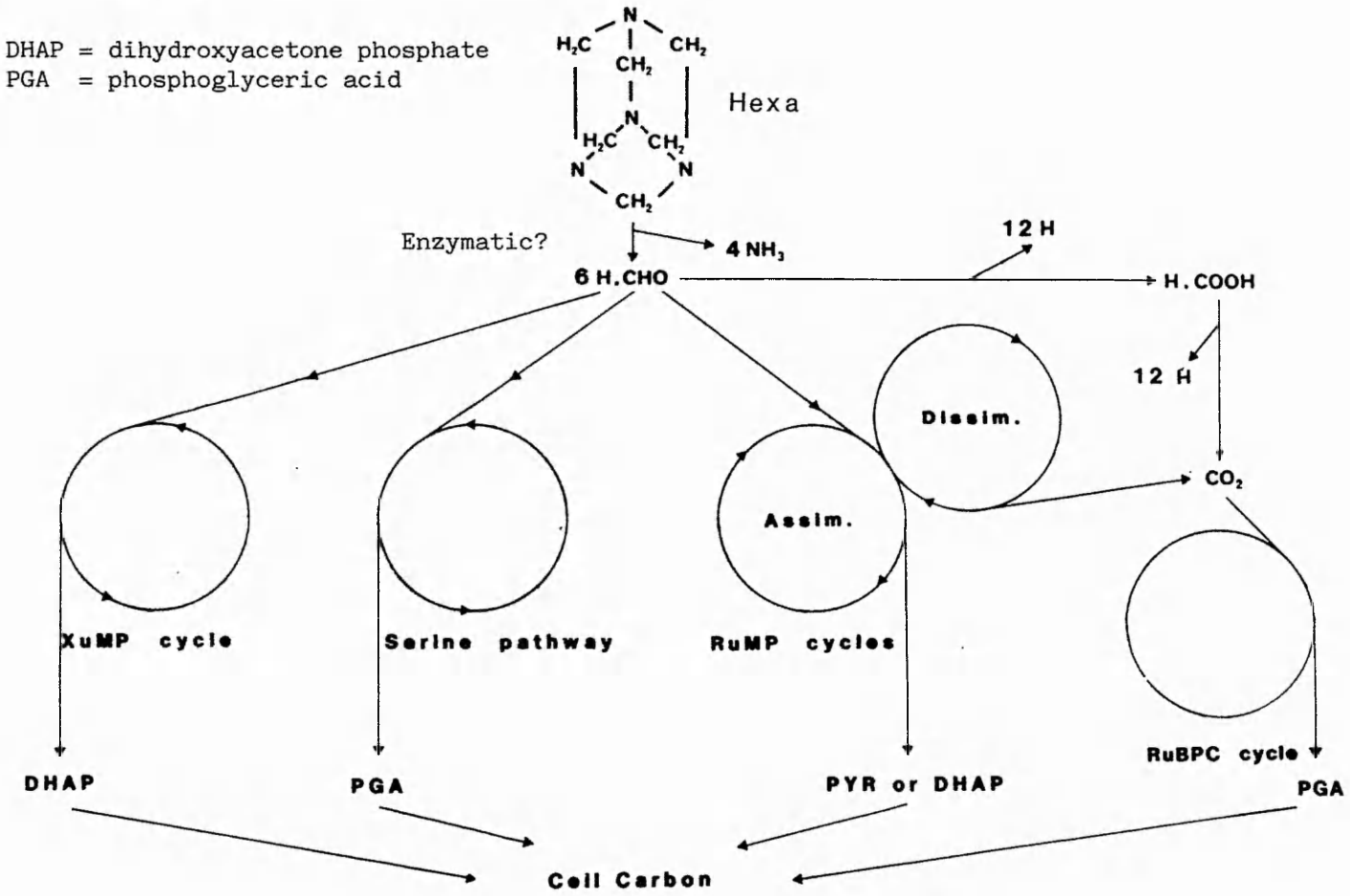
When hexa was the sole source of both carbon and nitrogen, the specific growth rates of each isolate was slightly less than with supplemented nitrogen (Table

52). Methanol was used by the two isolates as a carbon source with similar rates as those observed when hexa was both the sole carbon and nitrogen source. This is strong evidence that isolates KCH1 and KCH2 are facultative methanol users, one of the properties used by Byrom (1980) to classify methylotrophs. As would be expected both isolates grew on formaldehyde as their sole carbon source, but slower than when hexa was the sole carbon source.

Although no specific enzymatic studies were performed with either isolate during this study it has been demonstrated by Zatman (1981) that lack of the enzyme formate dehydrogenase in methylotrophs has been linked with the utilization of the dissimilatory cycle of the RuMP pathway. Thus of the two remaining possible pathways, the RuMP pathway is presumably the more likely pathway by which the two isolates utilize the formaldehyde produced by the breakdown of hexa. Although no enzyme activity was found in cell-free extracts towards hexa, the existence of such activity cannot be discounted. Further investigation of this hypothesis will be discussed later.

When the respirometric oxygen uptake data are compared to the growth rates of KCH1 and KCH2 on methanol and formaldehyde the following observation can be made. The two isolates had higher growth rates on

Figure 50. Dissimilatory and assimilatory pathways in methylotrophs.



methanol than on formaldehyde but during respirometry, formaldehyde produced a higher oxygen uptake rate by KCH1 and KCH2 than did methanol. This would indicate that during growth rate determinations of the two isolates, the formaldehyde present in the test flasks may have been inhibitory to the isolates. It is known that formaldehyde has an antimicrobial property, which was not observed during respirometry as the number of cells present was high and the concentration of formaldehyde was much lower than in growth rate determinations.

Hexa itself was not utilized during the 6 hours of the respirometric test (Table 53) which is not as surprising as it may at first appear. This is because no removal of hexa was measured during the growth of KCH1 and KCH2 when detected colorimetrically as free formaldehyde (Table 55). Either no hexa was utilized in this time or the method of detection was not sensitive enough. To investigate this [U-^{14}C] hexa was used to follow the removal of hexa by isolates KCH1 and KCH2. It was observed that over an initial 8 hour period 4-8mg l^{-1} of hexa was utilized by KCH1 and KCH2 respectively. Therefore it can be deduced that the colorimetric detection of hexa as formaldehyde was not sensitive enough to detect the initial removal of hexa over the first 6 hours although both methods gave comparable values for hexa utilization after 72 hours.

Respirometric studies of hexa with the two isolates was found in light of this data to be too insensitive over the initial period of investigation and the apparatus unreliable over an extended time period.

Battersby and Bealing (1988) have indicated that nitrification is required for hexa degradation and so they have tentatively suggested that the initial microbial attack on hexa may be for hexa as a source of nitrogen rather than a carbon or energy source. This would appear to be another starting point for further work on hexa.

Using [U-^{14}C] hexa it was demonstrated that as hexa was utilized by isolates KCH1 and KCH2 some of ^{14}C accumulated within the cell mass and some passed out of the cells as $^{14}CO_2$. This demonstrated that the isolates were actually utilizing the compound. The method of $^{14}CO_2$ collection was found not to be very efficient and satisfactory mass balance calculations could not be made by using the equation in section 3.8.12. The reasons for using this method of $^{14}CO_2$ collection and for its inefficiency will be discussed later.

When previously unexposed activated sludge (pH 7.2) was dosed with [U-^{14}C] hexa at levels of 52 and 530µg l^{-1} the hexa was removed. The lower dose of hexa was removed more quickly than the higher dose with relative first order kinetic rates of 0.45 and 0.367 d^{-1} respectively for the removal of ^{14}C in solution. If

Alexander's threshold theory is correct (Alexander, 1985) then the threshold level for hexa is below 52$\mu g\ l^{-1}$. The pH of the activated sludge remained constant at pH 7.2 during these experiments so that acid hydrolysis can be discounted.

When a similar experiment to that performed with activated sludge was performed using an alkaline soil (pH 7.5), removal of [U-^{14}C] hexa was observed. Both the higher and lower doses had similar first order kinetics of removal by soil, 0.271 and 0.275 d^{-1} respectively. Again as for activated sludge if Alexander's threshold theory is correct (Alexander, 1985) then the threshold for degradation of hexa by soil must be below 52.1 $\mu g\ l^{-1}$.

Attempts were made to investigate how low levels of hexa are degraded by river water, but it was found that the river water became more acidic during the experiment (Figure 46) and so hydrolysed the hexa to formaldehyde and ammonia masking any direct microbial attack on hexa. The formaldehyde was utilized by the bacteria in the river water hence the observed degradation of hexa, ^{14}C accumulation in the cell mass and eventual $^{14}CO_2$ release.

Returning to the point made earlier about the poor efficiency of $^{14}CO_2$ collection. Because the reaction vessel containing the activated sludge/soil or river water was to be operated continuously for an extended

period (up to 60 days), the only practical way to collect $^{14}CO_2$ was by passing CO_2-free air through the reaction vessels (Rubin and Alexander, 1983). This served two functions, firstly to drive out any $^{14}CO_2$ and secondly to replace the oxygen content of the reaction liquid and head space of the reaction vessel. In addition it allowed continuous monitoring of the experiment without too much disruption of the experimental set-up associated with other methods. The least satisfactory part of this experimental set-up was the collection of $^{14}CO_2$ in the exhaust gases of each vessel.

Although it has been demonstrated that the $^{14}CO_2$ collection was inefficient and did not allow accurate mass balances to be calculated, production of $^{14}CO_2$ has been demonstrated as has the removal of hexa and build up of ^{14}C in microbial cells. The combination of these and other factors demonstrated the biological nature of hexa degradation.

In concluding this discussion on hexa the following points can be made:-

1) Hexa is inherently biodegradable but requires longer to degrade than is allowed during the 24 hour aeration period of the SCAS test. This does not explain why other workers obtained aberrant results in a ring test of the enclosed manometric method for ready biodegradability, where only 5 out of 24 participating

laboratories reported >70% DOC removal after 28 days (Painter and King, 1985a).

2) The two isolated bacteria probably breakdown hexa to formaldehyde and ammonia by some as yet unknown enyzmatic reaction. The formaldehyde is then utilized by either the RuMP cycles or serine cycle with the former being the more probable. Further investigation into the pathway of hexa degradation is required, perhaps starting with the initial break up of the hexa molecule followed by an enzymatic investigation of the two possible pathways.

3) Hexa is degraded by naturally occuring microbial samples e.g. activated sludge and soil at levels as low as 10^{-5}g l^{-1}. This level of hexa more closely represents levels likely to be found in the natural environment and so will give far more relevant data than normal degradation tests performed at concentrations as much as 1000 times greater than this. It is proposed that further work at environmentally realistic temperatures be carried out. This would allow a reliable model of the environmental fate of hexa to be produced and evaluated.

4.8. Further comments.

4.8.1. Test Results.

Of the eight chemicals tested, BDSA, 2CBA and CPTCA were found not to be inherently biodegradable and were not degraded in any of the tests performed during this study. No other workers have reported BDSA to be degradable with 3 exceptions, King and Painter (1983), Gerike and Fischer (1981) and Pitter (1976) all of whom observed some degradation with inoculum which had been acclimatized to BDSA.

It is agreed that 2CBA is not readily degradable and reports on its inherent biodegradability are inconsistent. 2CBA has been reported as not being inherently biodegradable by Painter et al., (1983), Lund and Rodriguez and in this study in which no degradation was observed even after 245 days in the SCAS test. However, degradation has been reported by Gerike and Fischer (1979) in the Zahn-Wellens tests (inherent) and in an activated sludge simulation test after acclimatization, but not with synthetic sewage. This would indicate that 2CBA is treatable and therefore inherently biodegradable.

In this study cis, cis, cis, cis CPTCA was found not to be inherently biodegradable, which is in agreement with Gerike and Fischer (1979) and Painter (1986). However, the cis, trans, cis, trans isomer was found to

be inherently biodegradable after 8 weeks acclimatization in the SCAS test (Gilbert and Lee, 1980).

3CBA was found to be readily biodegradable in this study and also by Zahn and Wellens (1980) and Painter et al., (1983). Two other groups of workers reported that 3CBA required an acclimatized inoculum for degradation and so was only inherently biodegradable (Gerike and Fischer, 1979, 1981; Lund and Rodriguez, 1984). Nearly all of the studies indicate that 3CBA is degradable and King et al., (1984) demonstrated that it was degraded in a simulation test. Gerike and Fischer (1979, 1981) surprisingly found that 3CBA degraded in the Zahn-Wellens test (therefore inherently biodegradable) but did not degrade in a simulation test.

NMA was found to be degraded by activated sludge after a period of acclimatization (70 days) and the sludge had not lost this ability 3 months after exposure was discontinued. Gerike and Fischer (1979), and King and Painter (1985) also found that the inoculum had to be acclimatized to NMA before being able to degrade it; both groups of workers observed it to be treatable in simulation tests.

In the only reported study of THFTCA Painter (1986), showed that activated sludge required a period of acclimatization (11 weeks) for degradation in the SCAS test. This was also the case in the present study

although the period was slightly longer (13 weeks). In the die-away test THFTCA was not degraded by activated sludge exposed to the THFTCA for only 2 days, but was by activated sludge exposed for 30 days. Painter (1986) reported no degradation during a 9 week simulation test.

Removal of t-butanol was observed in both the SCAS and die-away tests. Volatilization has been shown to be the main route of removal of t-butanol, not biodegradation, by this study. Most workers found it not to be readily biodegradable although Horn et al., (1970) reported degradation after 8 weeks acclimatization in activated sludge units. Bacteria which can degrade t-butanol have been isolated by Romidina et al., (1984), showing that it can be degraded. It is the conclusion of this study that t-butanol is lost mainly by volatilization although there is evidence that it can be degraded.

Hexa has shown aberrant behaviour. In the EEC respirometry ring test Painter and King, (1985a) reported only 8 out of 24 participants found hexa to degrade, determined as oxygen uptake over 60% of theoretical oxygen demand (ThOD). In the Husmann simulation test less than 25% hexa was removed during the 9 weeks of the test. In this study hexa was found not to degrade in the SCAS unit except over an extended period (10 days) of exposure. It was however degraded in die-away tests without acclimatization. In addition

hexa was found to be degraded by unacclimatized activated sludge and soil at realistic environmental levels as low as 10^{-5}g l^{-1}.

Disparity of results with other chemicals have been observed by Painter and King (1985a) in the EEC respirometry ring test. Apart from aniline, only 2-phenyl phenol was degraded in the tests carried out in all laboratories in the ring test. For 1 methyl-propylamine, sodium benzene sulphinate, 1-naphthol, 1-naphthoic acid and pentaerythritol over 50% of the participants reported greater than 60% ThOD while for N-methylpyrrole, diphenic acid and thioglycollic acid the proporition finding the compounds readily biodegradable was only 40-50%. In the remaining cases (tetrahydrofuran, t-butanol, hexa, 3-aminophenol and sulphanilic acid,) as little as 20-34% of the laboratories reported ready biodegradability.

There was clear evidence in the case of suphanilic acid that the only laboratory reporting definite ready biodegradability had used inoculum taken from an activated sludge plant constantly receiving aromatic sulphonic acids. This was also the case with BDSA as reported in an earlier ring test, King and Painter (1983).

Bacteria capable of utilizing 3CBA, hexa, NMA and THFTCA as their sole carbon source have been isolated and no previous reports of such bacteria able to degrade

the latter three chemicals have been seen. Both CPTCA and THFTCA have been proposed as phosphate substitutes in detergents, and from this and other investigations it would appear that THFTCA is a better proposition than CPTCA with respect to biodegradability. However, much more work is required before any definite conclusions can be drawn.

4.8.2 Biodegradation - general.

For a xenobiotic compound to be degraded microorganisms which possess enzymes with the capability to breakdown that xenobiotic compound must exist. If a single microbial species does not contain all the enzymatic mechanisms required to degrade a xenobiotic compound on its own, then it is common for communities of microorganisms to degrade the xenobiotic collectively, i.e. mutualism. An example of mutualism is that between Alcaligenes eutrophus and Pseudomonas B13 which individually cannot breakdown 3CBA. In a mutualistic relationship A.eutrophus can convert 3CBA to chlorocatechols and accumulation and autooxidation of these toxic chlorocatechols is prevented by the metabolic activity of Pseudomonas B13, (Knackmuss, 1983). Individual species e.g. Arthrobacter sp have been demonstrated to degrade 3CBA while utilizing benzoate as their sole source of carbon and energy (Horvath and Alexander, 1970). In this study all the

bacteria isolated in the absence of other substrates have been able to utilize specific xenobiotics as their sole carbon and energy source. This is very advantageous as it reduces the complexity of the conditions needed for degradation to occur.

It is unlikely that new pathways would evolve to degrade xenbiotic compounds as this would require many millions of generations to occur naturally. What is most probable is the adaptation of existing pathways which facilitate the initial degradation of xenobiotics into intermediates which can feed into existing pathways; some may call these modified pathways "new pathways".

It is of interest to speculate on the spatial or geographical distribution of the newly acquired ability by microbial populations to degrade xenobiotic compounds. (Such distributions have been demonstrated in the "ring tests" described earlier). Whereas competent bacteria for degrading "common" chemicals are fairly evenly distributed, the distribution of those species degrading xenobiotics is very uneven. Presumably the "new" or modified pathways necessary for degrading the xenobiotic chemical are induced at disparate rates depending on the use pattern and distribution of the chemical itself. Until a chemical has been in use for many years and unless it has

widespread use, uneven distributions of the competent bacteria are inevitable.

4.8.3 Biodegradability testing.

There are many factors that affect microbial activity and therefore, affect biodegradability in a given sample. These may vary from test to test and from laboratory test conditions to those in nature. Some of the important variables include : type and numbers of microorganisms, mineral salts composition, test chemical concentration, supplementary organic nutrients, oxygen concentration, temperature and pH. It is these variations together with the arbitrary time scale of the various tests which contribute to the disparate results already discussed.

Variation in test results from different laboratories using identical test methods such as in the ring test Painter and King (1985a), have been observed. For example, in this test only 8 out of 24 participating laboratories found oxygen uptake greater than 60% ThOD when hexa was the test compound. Essentially the only difference in the test method at each laboratory was the inoculum. No data were given about the source of the inocula used or of the type of effluent to which they were predominantly exposed. This is important as some of the inocula may have been "pre-acclimatized" to the test compound before the tests by virtue of the fact that the

sewage works from which they were taken regularly received that compound. Other inocula by contrast may only have been exposed to domestic effluent and therefore were not "pre-acclimatized" to the test compound and so did not degrade it.

Perhaps when testing "new" or xenobiotic chemicals it would be prudent to pre-acclimatize inocula taken from a number of sources before applying a die-away test. In this way nature is "hurried along" and if ring tests were done with such inocula better agreement should be obtained. This scheme would be of more use to the EEC and to national competent authorities in their deliberations on estimating the hazardous nature of new chemicals.

In many tests a high ratio of concentration of test compound to numbers of microorganisms is required to allow detection of the test compound, which may in turn lead to inhibition of the microbial seed. Many tests do not allow pre-adaptation (acclimatization) of the microbial seed to the test compound which would allow a lesser chance of inhibition and a greater chance of degradation occurring during the test.

4.8.4 Biodegradability testing and this study.

During this investigation it has become clear that the SCAS test on its own, is not very suitable as a method of monitoring biodegradation of test compounds.

It is true that biodegradation of 3CBA, NMA and THFTCA was detected, but no degradation of BDSA, t-butanol 2CBA, CPTCA and hexa. This would appear to be very straight forward until the results of the die-away tests are also considered. Hexa is now found to be degradable and t-butanol was found to be removed by volatilization. With the case of hexa, if the SCAS test results were taken on their own then hexa would appear not to be degradable, but it was found to be degradable in die-away tests. This is a false-negative, and would be misleading to manufacturers and to competent authorities if hexa was a new compound to be marketed. On the other hand the data from the SCAS test performed with t-butanol would indicate that it is degradable, but the data from the die-away tests show that t-butanol is largely removed by volatilization. This is a false positive, although t-butanol is removed it is not principally by a biological process.

This study has found that the SCAS test is a very good method of studying acclimatization/ deacclimatization and biodegradation when in conjunction with die-away tests which are used to monitor biodegradation. This study has shown that two of the eight chemicals tested (25%) gave false results when the amount of carbon remaining in the SCAS unit after each 24 hour aeration period was taken on its own as a measure of biodegradation.

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Appendix

Microbial degradation of hexamethylenetratramine.

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Semi-continuous activated sludge (SCAS) units were used to study degradation hexamethylenetetramine (hexa). When SCAS units were supplied daily with substrate, degradation was not observed over the succeeding 24h period. Die-away tests (DAT) using inocula from SCAS units previously fed with hexa showed 60% degradation within 28 days indicating that the microbial population had become acclimatized to slowly breakdown the substrate.

After 208 days no further hexa was added to the SCAS unit and a study made of deacclimatization. The microbial population retained the ability to degrade hexa in DAT for 96 days after cessation of hexa addition to the SCAS units. When addition of hexa to the SCAS was discontinuned, the residual hexa decreased by 73% over the succeeding 13 days, supporting the DAT evidence that hexa was slowly degraded.

Hexa can be hydrolysed to ammonia and formaldehyde under acidic conditions. Our data shows that little hydrolysis occurs above pH 6.9 and this value is lower than the pH measured during the die-away tests on this compound. No degradation of hexa was observed when sterilized inocula were used in the DAT. Two pink pigmented facultatively methylotrophic bacteria capable of utilizing hexa as the sole source of both carbon and nitrogen have been isolated from activated sludge and growth rates of both organisms have been determined.

Microbial populations were acclimatized to breakdown hexa, but the rate of degradation was slow and only observed in the prolonged DAT. The ability to degrade hexa was retained after deacclimatization for 96 days. Significant degradation of hexa may not occur in sewage works activated sludge, but accumulation of hexa in the environment is unlikely as degradation will occur after prolonged exposure.

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Biodegradability of Hexamethylenetetramine

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In the paper by Painter and King[3] (Chemosphere, Vol 15, No 4, pp 471-478, 1986), it was reported that 100% removal of the compound hexamethylenetetramine (Hex) was obtained in ISO Die-Away Tests when activated sludge was used as inoculum. We too obtained a high removal of Hex, up to 87%, after 28 days incubation when activated sludge was used as inoculum, but not 100% as reported by Painter and King[3]. Even when the activated sludge used as inoculum had been exposed to Hex for up to 205 days, in a semi continuous activated sludge (SCAS) unit, 100% removal was still not detected. From our results the length of exposure time to this compound does not enhance the ability of activated sludge inoculum to degrade it in an ISO die away test of 28 days duration.

In a SCAS unit dosed with 20mgC/l of Hex daily, it was not possible to detect any significant daily removal of the compound, only 13% on average over a 24 hour aeration period. This agrees with the results of Painter and King for their activated sludge simultation tests on Hex. However, when daily addition of Hex and synthetic sewage to the SCAS unit was stopped, it was found that over a subsequent 13 day period, 73% of the Hex present when addition was discontinued had disappeared. This observation supports Painter's assumption that in simultation or SCAS tests, Hex will break down over an extended period, though it was noted that no further degradation occurred after 13 days in our test.

Is the removal of Hex due to physicochemical factors in the tests observed? It is known that Hex can be hydrolysed under strong acid conditions to ammonia and formaldehyde[5]. It is unlikely that any significant removal of Hex in die away tests is due to hydrolysis for two reasons. i) 100% removal of Hex was not always detected by Painter and King[3] and it was never detected in our laboratory on any occasion.
ii) The lowest pH value measured over the 28 days of the die away tests performed on Hex was 6.95%. In the absence of organisms no hydrolysis was

observed at pH values above pH 6.9 over a six hour period using a modified Riker[1] method to detect production of formaldehyde. This value is higher than the lowest pH value observed in the die away tests. This would strongly indicate that acid hydrolysis is not responsible for Hex removal in the tests.

In the SCAS units the initial microbial population of the activated sludge was found to be in the order of 10^7 micro-organisms /ml but in the Hex unit this fell to 10^6 micro-organisms /ml during the first 28 days of the SCAS test. The population remained at this level until the test was stopped at day 212. The number of micro-organisms present in the control unit remained at 10^7 micro-organisms /ml throughout the test.

Using the draft ISO Standard Method[6] we have observed inhibition by Hex on both sewage and activated sludge and an LD50 of 15mg l^{-1} has been calculated for this compound. In view of the observed inhibition it is possible that the reduction in the numbers of viable cells in the SCAS unit was in fact due to inhibition of the organisms by Hex. Painter[2] found no evidence of inhibition by Hex on either sewage or activated sludge organisms.

We have recently isolated two organisms which can utilize Hex as the sole carbon source. In the light of this fact it is possible that Hex can be metabolised by a fraction of the population and is inhibitory to several other species.

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