Applikation von aeroben und anaeroben Bakterien in den Bereichen biologischer Schadstoffabbau und biologische Abwasserreinigung

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Abstrakt

In der hier vorgelegten Arbeit wurden vier distinkt verschiedene Probleme untersucht. Das erste Problem war die Untersuchung in den Abbau von Dichloroethylene (DCE) und 1,1-bis (p-chlorophenyl)-2-dichloroethylene (DDE) mithilfe von reinen bakteriellen Kulturen. Die zweite Untersuchung beschaeftigte sich mit dem Abbau von DDE und polychlorinierten Biphenylen (PCB's) mithilfe von anaeroben Sedimenten und Erden aus New Zealand. Die dritte Untersuchung behandelt die Granulation von anaeroben Fluss-sedimenten in Upflow Anaerobic Sludge Blanket (UASB) Reaktoren. Die letzte Untersuchung behandelt das Anfahren einer industriellen aeroben Abwasser-anlage und die Implementierung von biologischem Stickstoff- und Phosphat-abbau in dieser Abwasser-anlage.

Da die chemische Struktur von DCE und DDE gewisse Aehnlichkeiten besitzt, wurden hier wurden Bakterien untersucht, die in der Lage sind DCE abzubauen, ob diese DDE in einer cometabolischen Reaktion abbauen koennen. In den Experimenten wurden die aeroben Bakterien *Methylosinus trichosporium* und *Mycobacterium vaccae* und die anaeroben Bakterien *Acetobacterium woodii* und *Clostridium butyricum* benutzt. Ungefaehr 60% des hinzugefuegten DCE's wurde von *M. vaccae* abgebaut, whaerend *M. trichosporium* ca 50% abbaute. *A. woodii* und *C. butyricum* bauten jeweils 40% und 30% des zugefuegten DCE's ab. Weiterfuehrenden Experimente mit den obigen Kulturen und zugefuegtem DDE fuehrte zu einem mikrobiologischen Abbau von DDE in den Kulturen von 34.6% fuer *M. vaccae*, 14.1% fuer *C. butyricum*, 12.2% fuer *A. woodii* und 10.5% fuer *M. trichosporium*. Weitere Experimente, bei denen [14C]-DDE benutzt wurde, ergaben, dass das DDE nicht abgebaut worden war, sondern es stellte sich heraus, dass das DDE an die Bakterienzellen angelagert worden war.

Die zweite Untersuchung benutzte anaerobe Erden und Sedimente aus New Zealand um den anaeroben cometabolischen Abbau von DDE und PCB's zu studieren. Die Erden und Sedimente stammten von dem Fluss Waikato, aus Abwasser-Teichen in Kinleith, Meeresboden-Sedimenten aus Mapua, und verschiedene Erden die mit Pentachlorophenyl (PCP) kontaminiert waren. Die Kulturen aus diesen Erden und Sedimenten wurde mit verschiedenen Kohlenstoff- und Energie-Quellen aufgezogen. Neben DDE wurden Aroclor 1260 und ein Mix aus vier reinen PCB-Congeneren (ein Tetra-, ein Hexa, ein Hepta- und ein Deca-Chlorobiphenyl) fuer die reduktive Dechlorinierung benutzt. Die Aufzucht der Bakteria dauert sechs Monate, Proben wurden am Start der Kultivierung, nach drei und nach sechs Monaten genommen. Diese Proben wurden fuer die Veraenderung des Zellproteins, den Abbau der Kohlenstoof- und Energie-Quellen, und das Verschwinden der zugefuegten polychlorinierten Chemikalien ananlysiert. Die Organochlorine wurden mithilfe von reversed HPLC und dann FID-GC untersucht. Wenn eine Veraenderung in den Chromatogrammen auftrat wurden die entsprechenden Kulturen mithilfe von ECD-GC und GC-MS weitergehend untersucht. Die Resultate zeigten ein wachsen der Kulturen an, aber keinen Abbau von DDE und dem PCB-Mix, und nur geringe Veraenderungen der Komposition von Aroclor 1260.

Die dritte Untersuchung befasste sich mit der Granulierung von anaeroben Flusssedimenten in UASB Reaktoren. Dafuer wurden Sedimente von dem Waikato in New Zealand und der Saale in Deutschland benutzt. In beiden Faellen war die Granulation erfolgreich, was durch mikroskopische Vergleiche von den Sedimenten und den Granules festgestellt werden konnte. Die zwei hauptsaechlichen Bakterien Kulturen waren *Methanosarcina* und *Methanothrix* aehnliche Kulturen. Die Haupt-Kohlenstoff- und Energie-Quelle war Lactic Acid und wurde mit einer Konzentration von 21,8 g COD/L verwendet. Der Granulations-Prozess war eine Kombination von einer hohen COD-Konzentration verbunden mit einer niedrigen volumetrischen Ladungs-Rate. Vergleiche der spezifischen Abbauraten von verschiedenen Kohlenstoff- und Energie-Quellen zwischen den Sedimenten und den Granules, ergab keine erhoehten Abbauraten in Bezug auf die gleiche Zellmasse, aber die erhoehte Biomasse in den Granules sorgt fuer groessere Abbauraten in den UASB Reaktoren.

Die vierte Untersuchung befasste sich mit dem Anfahren einer industriellen Abwasser-Anlage fuer eine Molkerei in Edendale, Southland, New Zealand. Diese Anlage besteht aus einer DAF-Unit (Dissolved Air Flotation), zwei Abwasser-Teichen mit aktiver Schlammbehandlung und zwei Klaerbecken, eines fuer die Aktiv-Schlamm-Beseitigung und das zweite fuer die Dosierung von Aluminiumsulphat und die Entfernung von Phosphat-Sulphat. Biologische Verfahren zum Abbau von Kohlenstoff-Verbindugen wurden optimiert und biologische Verfahren zur Verringerung von Stickstoff- und Phospaht-Konzentrationen im Abwasser wurden implementiert und optimiert. Biologische Abbau-Raten fuer COD von ueber 95%, fuer Stickstoff 85-92% und Phosphat 64-83% wurden erreicht.

Abstract

In the work here presented four distinctly different problems were investigated. The first problem was an investigation into the degradation of Dichloroethylene (DCE) and 1,1-bis (p-Chlorophenyl)-2-dichloroethylene (DDE) utilising pure bacterial cultures. The second investigation dealt with the degradation of DDE and polychlorinated Biphenyl's (PCB's) utilising anaerobic sediments and soils from New Zealand. The third investigation worked on the Granulation of anaerobic River-sediments in Upflow Anaerobic Sludge Blanket (UASB) Reactors. The last investigation describes the commissioning of an industrial aerobic Wastewater Treatment Plant and the Implementation of biological Nitrogen- and Phosphate removal in this Wastewater Treatment Plant.

Since the chemical Structure of DCE and DDE have certain similarities, Bacteria that were capable of degrading DCE, were tested here, whether they would also be able to degrade DDE utilising a co-metabolic pathway. In the experiments the aerobic bacteria *Methylosinus trichosporium* and *Mycobacterium vaccae* and the anaerobic bacteria *Acetobacterium woodii* and *Clostridium butyricum* were used. Approximately 60% of the added DCE was degraded by *M. vaccae*, while *M. trichosporium* degraded approximately 50%. *A. woodii* and *C. butyricum* degraded 40% and 30% respectively of the added DCE. Further experiments with these cultures and DDE lead to a microbial degradation of DDE to an extent of 34.6% for *M. vaccae*, 14.1% for *C. butyricum*, 2.2% for *A. woodii* and 10.5% for *M. trichosporium*. Additional experiments, utilising [14C]-DDE, showed that the DDE had not been degraded but were attached to the bacterial cells.

The second investigation utilised anaerobic soils and sediments from New Zealand to study the anaerobic co-metabolic degradation of DDE and PCB's. The soils and sediments originated from the River Waikato, from Wastewater Ponds in Kinleith, Marine-Sediments from Mapua, and a variety of soils comtaminated with Pentachlorophenyl (PCP). The cultures from these soils and sediments were raised on a variety of Carbon- and Energy-sources. Beside DDE, Aroclor 1260, and a mix of four pure PCB-Congeneres (one Tetra-, one Hexa, one Hepta- and one Deca-Chlorobiphenyl) were used to test for the reductive dechlorination. The cultivation process of the baceria lasted six months. Samples of the cultures were taken after zero, three and six months. These samples were tested for the increase of cell-protein, the degradation of carbon- and energy-sources, and the removal of the added polychlorinated chemicals. The organochlorines were analysed using reversed phase HPLC and FID-GC. When a change in the Chromatogram was detected the respective cultures were further analysed using ECD-GC and GC-MS. The results showed that the culutres grew under these conditions, but no degradation of DDE and the PCB-Mix could be detected, and only small changes in the composition/chromatograms of Aroclor 1260 were found.

The third investigation worked on the Granulation of River-Sediments in UASB-Reactors. Sediments from the River Waikato in New Zealand and the River Saale in Germany were used. In both cases the Granulation process was successful, which was demonstrated by microscopic comparisons of the Sediments and the resulting Granules.

The two main bacterial cultures detected were *Methanosarcina*- and *Methanothrix*-like cultures. The main carbon- and energy-source was Lactic Acid, which was used at a concentration of 21,8 g COD/L. The Granulation-Process was a combination of using high a COD-Concentration combined with a low Volumetric Loading-Rate. Comparisons of the specific degradation-rates of a variety of carbon- and energy-sources between the Sediments and the Granules, showed no increased degradation rates in regard to the same cell-mass, but the increased bio-mass in the Granules allowed for higher degradation-rates within the UASB-reactors.

The fourth investigation describes the commissioning of an industrial Wastewater Treatment Plant for a Dairy-Site in Edendale, Southland, New Zealand. This Plant consists of a DAF-Unit (Dissolved Air Flotation), two Extended Aeration Lagoons with Activated Sludge and two Clarifiers, one for the Activated Sludge and the second for the dosing of Aluminium-Sulphate and the removal of Phosphat-Sulphate. Biological processes for the removal of carbon- and energy-sources were optimised and biological processes for the reduction of Nitrogen- and Phosphate-Concentrations within the wastewater were implemented and optimised. Bilogical removal rates for COD of 95% and above, for Nitrogen of 85-92% and Phosphate of 64-83% were achieved.

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Table of Contents

List of Figures

List of tables

List of Abbreviations

The following abbreviations have been used in this thesis:

atm	atmosphere
GC	gas chromatography
ppb	parts per billion
ppm	parts per million
UV	ultra violet
DSM	Deutsche Sammlung für Microorganism
NCIMB	National Collection of Industrial and Marine Bacteria
ATCC	American Type Culture Collection
rpm	revolution per minute
l	wavelength lambda
VFAs	volatile fatty acids
TCD-GC	thermal conductivity detector-gas chromatography
FID-GC	flame ionisation detector-gas chromatography
ECD-GC	electron capture detector-gas chromatography
HPLC	high performance liquid chromatography
GC-MS	gas chromatography-mass spectroscopy
ESR	environmental science and research institute
UASB	upflow anaerobic sludge blanket
COD	chemical oxygen demand
HRT	hydraulic retention time
ER-photo	electron microscopy photo
VSS	volatile suspended solids
TSS	total suspended solids
SRB	sulphate reducing bacteria
PCBs	polychlorinated biphenyls
DDT	1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane
DDE	1,1-bis (p-chlorophenyl)-2-dichloroethylene
DDD	1,1-dichloro-2,2-bis (p-chlorophenyl)ethane
DDMU	1-chloro-2,2- (p-chlorophenyl)ethylene
DDMS	1-chloro-2,2-bis (p-chlorophenyl)ethane
DDNU	2,2-bis (p-chlorophenyl)ethylene
DDNS	2,2-bis (p-chlorophenyl)ethane
DDOH	2,2-bis (p-chlorophenyl)ethanol
DDA	bis (p-chlorophenyl)acetic acid
DDM	bis (p-chlorophenyl)methane
DBH	p,p-dichlorobenzhydrol
DBP	p,p-dichlorobenzophenone
PCE	tetrachloroethylene
TCE	trichloroethylene
DCE	dichloroethylene
VC	vinylchloride
PCP	pentachlorophenyl

1. Introduction

1.1 General Introduction

The work presented in this thesis will mainly deal with anaerobic bacteria/consortia, and the impact of successive anaerobic/aerobic environments and bacterial activity within these environments.

Microorganisms of aerobic and anaerobic environments are essentially capable of utilising any organic molecule that structurally resembles a natural substrate (Bhatnagar and Fathepure) and have been reported to utilise certain xenobiotic chemicals. In some cases sequential steps of degradation might be necessary and anaerobic consortia provide one example of how nature has solved the problem of sequential degradation by a variety of bacteria together. Complete mineralisation usually requires one or more trophic groups to convert even non-toxic chemicals by a multi step reaction to mineralised end products. It has been shown by a variety of researchers, that synthetic, halogenated compounds can be reductively dechlorinated by biologically mediated reactions and thus act as electron acceptors (Zehnder and Stumm). This is also the case in wastewater treatment in regard to nutrient removal.

1.2 Introduction on Polychlorinated Biphenyls (PCBs)

PCBs are a family of chemicals produced commercially by direct chlorination of biphenyl using ferric chloride and/or iodine as the catalyst. Chlorine can be placed at any or all of the ten available sites, with 209 theoretically possible variations, producing different congeners (Alford-Stevens, 1986). Of these, only about half are produced in the synthesis due to steric hindrances. PCBs are a family of stable, water insoluble industrial chemicals that were widely used for nearly 50 years (1929 - 1978). By 1975, it was estimated that some 57 x 107 kg had been produced in the United States, and about 8 x 107 kg had passed into its soils, sediments and waters (Durfee et al., 1976). PCBs were manufactured and sold as complex mixtures with different average chlorination levels under trade names like: Aroclor (Monsanto, USA), Phenclor and Pyralene (Prodelec S. A., France), Clophen (Bayer AG, Germany) and Kanechlor (Kaneguchi Chemical Industrial Co. Ltd, Japan). The manufacturers also assigned product numbers that usually reflected the degree of chlorination by either the average number of chlorine's/biphenyl or the weight percentage chlorine in the mixture. For example, Aroclor 1260 (12 carbon atoms and 60 % chlorine) Clophen A 60 (average of 6 chlorine's per biphenyl; Abramowicz, 1990).

Although the synthesis of PCBs was first described in 1881 by Schmidt and Schultz (Schmidt and Schultz, 1881) the potential industrial applications were not fully realised until about 1930. PCBs were first introduced to solve a public safety hazard - electrical equipment fires, because of their non-flammable and heat-resistant properties they were utilised for a variety of industrial purposes, including fluid-filled capacitors and transformers, hydraulic fluids, heat transfer fluids, plasticisers and carbonless copy paper, mainly because of their chemical and thermal stability, low or non flammability and good electrical insulating properties (Lerman et al., 1982).

The detailed characterisation of chemical composition changes in environmental PCB extracts is made possible by a unique composition of PCB commercial mixtures, like fingerprints. All of the commercial PCB products, like Aroclor, consisted of complex mixtures of chlorinated biphenyl homologous and congeners that were originally produced in a fixed proportion, because a single manufacturing process, iron-catalysed chlorination to a fixed weight gain, was used during the entire period of PCB production. However, chemical, physical or biological PCB transformation processes might show

selectivity pattern (eg. set of relative transformation rates) for attacking the various individual congeners present in an Aroclor. Thus, an environmentally altered Aroclor has been shown to produce a new congener distribution (and GC pattern) characteristic of the transformation process; and detailed analysis of that degradation pattern may elucidate the chemical nature of the transformation.

PCBs are frequently encountered as contaminants in soil environments, usually originating from electrical transformer leaks or improper disposal of PCB-containing wastes. Remediation of PCB-contaminated soils has typically involved excavation followed by impoundment in landfills or destruction by incineration. Issues of long-term liability, high cost, and facility access stand to limit the continuing implementation of these strategies and have provided the impetus needed for the examination of alternative technologies, bioremediation being among these.

Although the use of PCBs was prohibited in many countries, their release into the environment continues and they are regarded as global contaminants (Kipli et al., 1988). Like PCBs, DDT and its derivatives were regarded as highly water insoluble, with a high molecular weight and low vapor pressure, the evaporation should therefore have been low. Both compounds had high activity coefficients in water, which caused high equilibrium vapor partial pressure and thus high rates of evaporation, which was the major transport through the atmosphere (Mackay and Wolkoff, 1973) . PCBs and DDT have been found in the Arctic and Antarctic regions where they could only have got to by atmospheric deposition. Recent work on DDT in tropical soils has shown, that in all cases the half lives for DDT and DDE were between 2 and 9 month due to volatilisation-dissipation, whereas in temperate zones the half lives for these chemicals are between 2 and 10 years. Ballschmitter and Wittlinger (Ballschmitter and Wittlinger, 1991) reported on the inter hemisphere exchange of PCBs, DDT, DDE and other organochlorines. They suggested a south/north ratio between the two hemispheres for DDT of 7 - 8 and for DDE of about 3. The inter hemisphere exchange only distributed the problem worldwide and did not solve it, because the chemicals were not degraded, but volatilised. Samuel and Pillai (Samuel and Pillai, 1989) showed that the bulk of DDT loss occurred because of volatilisation, which rose with increasing temperature. The degradation of DDT to DDE was also faster with higher temperatures as were the binding of DDT to soil. DDT and DDE are found in higher concentrations in tropical regions in plants than in the northern hemisphere (Calamari et al., 1991). They describe plants as good indicators of tropospheric contamination levels by chlorinated hydrocarbons.

Another question arises regarding the threshold of organochlorines like PCBs, DDT and DDE. It has to be noted that experiments with pressure bottles aiming for the anaerobic reductive dehalogenation have utilised high concentrations of organochlorines, eg. Tiedje (Tiedje et al., 1993) utilised 200 - 500 ppm of PCBs before detecting dechlorination. If such a high concentration is a prerequisite for microbial dechlorination this might be one explanation why these chemicals have spread more or less worldwide in relatively low concentrations.

The conclusion of these findings would be that although PCBs, DDT and DDE show very little direct toxicity their accumulation throughout the food chain is a strong concern. A term often used in the literature is the "sink", which is supposed to highlight the fact that these organochlorines are accumulating in biota and are found in higher concentrations in the steps/grades of the food chain (Mosser et al., 1974; Bates et al., 1990; Solly and Shanks, 1974; Fuller and Hobson, 1986; Pal et al., 1980).

No single pure bacterial strain has been found which is able to carry out the reductive dehalogenation of highly chlorinated organochlorines like PCBs or DDT. Consortia of anaerobic bacteria were identified to be responsible (Dolfing and Tiedje, 1991; Linkfield et al., 1989; Tiedje et al., 1987; Tiedje et al., 1993; Ye et al., 1995

Other researchers (Kohler et al., 1988; Alvarez-Cohen and McCarty, 1991; Pfaender and Alexander, 1973; McCarty and Smith, 1986; Hendriksen et al., 1992) were convinced that the reductive dechlorination was a cometabolic process for which an enrichment would not have been possible.

There are other groups who propose an abiotic process mediated by biological factors like extracellular enzymes, or coenzymes either from lysed cells, decomposed plant material, or released by microorganisms. This is similar to the white rot fungus lignin peroxidase system. Assaf-Anid (Assaf-Anid et al., 1992) showed the ability of Vitamin B12 to catalyse dehalogenation of PCB congeners under anaerobic conditions.

The sediments used for the reductive dechlorination of complex commercial PCB mixtures, in most cases Aroclor mixtures, came from rivers and contaminated sites, which had been exposed to the contamination over a long time period. These adaptation times would have to be defined in years (Quensen III et al., 1990) and several months

rather than weeks and days. Linkfield (Linkfield et al., 1989) found acclimatisation periods of three weeks to six months before dehalogenation of halogenated benzoates occurred in anaerobic lake sediments.

There are several different means to degrade PCBs abiotically, these are:

1. Photolysis by solar near-ultraviolet light (Bopp et al., 1984).
2. Decomposition of Aroclor 1260 using Fenton's Reagent Sato (Sato et al., 1991; Sedlak and Andren, 1991)
3. Reductive dechlorination of one pentachloro-biphenyl to two tetrachlorobiphenyls with vitamin B12 as the catalyst (Assaf-Anid et al., 1992).
4. Reductive dechlorination of chlorophenols utilising vitamin B12 reduced by titanium (III) citrate from vitamin B12 to catalyses the reaction (Smith and Woods, 1994).
5. Reductive dehalogenation of organochlorines with zero-valent iron or other metals as catalyst (Gillham and O'Hannesin, 1994; Matheson and Tratynek, 1994).

To be economically viable the concentration of PCBs has to be relatively high, or the contaminated soils and sediments have to be excavated. As an alternative, research into the biodegradation or biotransformation of PCBs has been conducted. A question addressed was whether the different catalysts for reductive dehalogenation like iron porphyrins and corrinoids might be supplied by biological systems (eg. cell lysis or extracellular enzymes). The common denominator here are the transition metals or transition metal complexes. If these are introduced by biota one could talk of an abiotic but biologically induced system. In general dechlorination of PCBs occur more readily in meta- and para- positions on the benzene-rings than in ortho-positions. This fact is relevant for abiotic as well as for biotic mechanisms. Nies and Vogel (Nies and Vogel, 1990) talk about a general relationship between reduction potential, chlorine substituent number, chlorine substitution pattern and dechlorination rate.

Bioremediation, especially in situ bio clean up, has the advantage of cleaning up a contaminated site without excavation and therefore one is still able to use this particular site for commercial use. This is not necessarily a big problem in countries with vast land resources, but in highly populated and highly developed countries it is a major benefit. There are two major goals for any biodegradation technology aiming for highly toxic chemicals. One is to remove the bulk of toxic compounds and their toxic metabolites as

fast as possible. The other is to eventually reduce their levels to below the critical risk level.

For the aerobic microbial attack at least two adjoining carbons of at least one of the benzene rings have to be chlorine free. An aerobic microbial PCB degradation can go up to a maximum of six chlorines per biphenyl while mono and di-chlorobiphenyls are preferred, higher chlorinated PCBs are not accessible for this kind of attack (Field et al., 1995).

Abramowicz (Abramowicz, 1990) summed up the aerobic and anaerobic biodegradation of PCBs in his review as follows: "A large number of naturally occurring, aerobic microorganisms have been isolated from many different locations and studied for their ability to degrade PCBs. The organisms range from common soil bacteria to more complex fungi. Some of the major findings follow:

1. Most soils contaminated with PCBs contain organisms with some level of PCB-degrading ability.
2. These microorganisms display congener specificity and therefore degrade individual congeners at different rates.
3. Most aerobic bacteria that have been isolated degrade only the lightly chlorinated congeners, although some bacteria have been isolated that are capable of attacking congeners containing as many as seven chlorines.
4. For the known cases, the 2,3-dioxygenase pathway is common and quite similar in otherwise unrelated organisms.
5. Similarities in the genes are being transferred between bacteria in the environment.
6. In general, the effect of aerobic bacterial PCB biodegradation is to remove the less chlorinated congeners.
7. No aerobic microorganisms have been reported that degrade the more highly chlorinated commercial mixtures Aroclor 1260 or Clophen A 60."

Thus a two-step process, consisting of dechlorination followed by oxidative biodegradation, may be required for complete PCB degradation and ultimately mineralisation.

The aerobic biodegradation of PCBs is generally limited to congeners with 5 or fewer chlorines that must contain two adjacent unsubstituted carbon atoms (Bedard et al., 1987a; Bedard et al., 1987b; Furukawa et al., 1979). Fava (Fava et al., 1993; Fava and

Marchetti, 1991) found that Fenclor 42 congeners with 4 or fewer chlorines per biphenyl could be degraded by mixed aerobic cultures. The aerobic degradation of most components of Aroclor 1242 (Bedard et al., 1987a; Bedard et al., 1987b; Brunner et al., 1985; Dolfing and Tiedje, 1987) and some of Aroclor 1254 (Bedard et al., 1987a; Bedard et al., 1987b) has been reported. There is no convincing evidence for the aerobic degradation of Aroclor 1260. Therefore, the biologically mediated reductive dechlorination of PCBs (Brown Jr. et al., 1987a; Brown Jr. et al., 1987b; Quensen III et al., 1988) is of great interest for the degradation of Aroclor 1260 or similar highly chlorinated PCB mixtures. It would allow removal of chlorines of the higher chlorinated compounds and thus make them more susceptible for an aerobic biodegradation.

It was previously noted that greater dechlorination activity was associated with PCB-contaminated sediments than with un-contaminated sediments (Quensen III et al., 1988). This inferred that the responsible selective pressures might have arisen from the ability to use PCBs as terminal-electron acceptors and/or from the ability to use the energy that is potentially available from dechlorination Therefore, any microorganisms that could use PCBs as terminal-electron acceptors would be at a selective advantage over microorganisms lacking this ability (Brown Jr. et al., 1987a; Brown Jr. et al., 1987b).

Brown (Brown et al., 1984) reported in 1984 that PCBs in the sediments of the upper Hudson River were undergoing a previously unreported type of compositional transformation, which was confirmed by Bopp (Bopp et al., 1984) and later by Brown (Brown Jr. et al., 1987a; Brown Jr. et al., 1987b). These PCB extracts from upper Hudson river sediments all showed smaller proportions of most higher chlorinated PCB congeners and increased proportions of certain less chlorinated congeners, including some that were virtually absent from the Aroclor composition originally discharged. The GC pattern of different sites could be grouped into certain dechlorination patterns, because of the differently changed GC-chromatograms. Brown (Brown Jr. et al., 1987a; Brown Jr. et al., 1987b) observed that the higher more heavily chlorinated PCB congeners were preferentially targeted by the observed dechlorination process including all those that were either pharmacological active or persistent in higher animals. All the lower (less heavily chlorinated) PCB congeners formed by the dechlorination were chemical species known to be biodegradable by bacteria of aerobic environments. Brown (Brown et al., 1984) showed that the extent of composition change was only minimal near the surface and maximal within and below the sub-surface strata where the total PCB levels were highest. They concluded that the observed transformation resulted from

in situ dechlorination rather than differential migration of PCB congeners. A microbial reductive dechlorination of PCBs was proposed (Brown Jr. et al., 1987a; Brown Jr. et al., 1987b; Brown et al., 1984).
The environmental dechlorination of PCBs has now been observed in a large number of contaminated (in most cases Aroclor mixtures) anaerobic sediments. The wide spread occurrence of this natural process indicated that it could be a general phenomenon.

For a microbial cell of any kind to be able to attack chemicals like PCBs and DDE several conditions have to be met:

1. Uptake of the organochlorine into the cell.
2. For the reaction to be favourable it has to have a negative free energy balance.
3. Need for an enzyme to catalyse the reaction.

4a. If organism benefits, it is a metabolic reaction.

4b. If organism does not benefit, it is a cometabolic reaction.

Superimposed over 1, is the 'threshold concept', which means, that below a certain concentration the microorganisms are not attacking the respective organochlorines. There are some indications in the literature that this concept is realistic, but unfortunately it is so far poorly understood. The second superimposition over 1, is the possibility of adaptation.

The process of anaerobic reductive dehalogenation mediated by bacteria has been confirmed in a number of laboratories with sediments from many distinct aquatic systems. Some of the most significant findings from current anaerobic dechlorination experiments follow.

1. Microbial reductive dechlorination has been observed in a number of sediments and the process seems to be widespread in the environment.
2. Although congener preferences are demonstrated, in general the organisms present in Hudson River sediments exhibit broad dechlorination activity on the more highly chlorinated PCBs.
3. These anaerobic microbial consortia are capable of dechlorinating even recalcitrant, highly chlorinated PCB congeners contained in Aroclor 1260.
4. All results to date involve consortia cultured from sediments and pure PCB reductively dechlorinating strains have not yet been isolated.
5. Dechlorination preferentially removed meta and para chlorines, significantly reducing toxicity associated with PCBs.

6. Most of the less chlorinated congeners that are produced are known substrates for aerobic bacterial systems.

1.3 Introduction on 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (DDT) and 1,1-bis (p-chlorophenyl)-2-dichloroethylene (DDE)

Prior to World War II agricultural pesticides and fertilisers were derived from naturally occurring minerals, plant products and faeces, which were neither very toxic nor persistent in nature. After the war, the total world production of synthetic organics used as insecticides, plastics, flame retardants, electrical transformers, ink, or lubricants increased dramatically. These halogenated aromatics were relatively rare in nature. Their recalcitrant nature is determined by the type, position and amount of halogenation. The electronegative halogens are able to form stable bonds to ring carbons because they help to delocalise the electrons. The large halogens also tend to interfere with the degradative activity of ring-cleaving enzymes, which adds to their persistence (Slayer and al., 1984).

During World War II, DDT was introduced as a pesticide especially targeting insect-borne diseases like typhus and malaria, it was extremely beneficial. The immense biological potency of these chemicals not only as pesticides but also to persist in the environment soon became apparent (Hoffman et al., 1990). Early work by Stickel (Stickel, 1946) warned users of potential risks of DDT to the wildlife. In the early 1950s decline in the population of American robins (Turdus migratorius) was linked to DDT spraying for the Dutch Elm disease. It was also found that the bald eagle (Haliaeetus leucocophalus) the national symbol of the USA, osprey (Pandion haliateus) and certain fish-eating mammals were at risk (Carson, 1962). Eggshell thinning related to DDT and especially DDE caused reproductive failure in European raptors (Ratcliffe, 1967) and North American raptors (Blus et al., 1974) and fish-eating birds (Hickey and Anderson, 1968; Wiemeyer and Porter, 1970). Bioaccumulation of contaminants in food chains with resulting biomagnification became evident when mortality of American robins was linked to DDT in earthworms (Carson, 1962). The work of Blus (Blus et al., 1974) showed that eggs from various colonies of the brown pelican in the Southern States of the USA had eggshell thinning. All analysed eggs contained measurable quantities of DDE, most also contained measurable quantities of DDD, DDT, dieldrin or PCBs. They concluded that DDE had the strongest influence on the eggshell thinning and in the lowering of reproductive success. Sericano (Sericano et al., 1990) reported significant levels of DDT, DDE and DDD in oysters in the Gulf of Mexico even 15 years after the

ban of DDT. Fries (Fries et al., 1969a; Fries et al., 1969b) fed dairy cows pure DDT, DDE and DDD and were able to recover 25.8% of the DDE, 7.6% of the DDD and 5.1% of the DDT in the milk. The increasing knowledge of the health and environmental hazards led to the ban of DDT in the industrialised countries in the early 1970s (from 1972 onwards).

Hileman (Hileman, 1994) reviewed the effect of "environmental oestrogens" in regard to reproductive abnormalities and cancer. Oestrogens have important roles in female vertebrates but a specific ratio of oestrogens to androgens (male hormones) is necessary for sexual differentiation and proper formation of reproductive organs. “Oestrogen mimics” are a diverse group of chemicals that have no obvious structural similarity, but attach themselves to oestrogen receptor cells. Examples would be DDT, DDE and PCBs. It was reported to be most likely that DDE has a devastating effect on Alligators in Lake Apoka, Florida (Hileman, 1994). This Lake had been contaminated in the 1980s by Dicofol (at that stage containing 15% DDT). The effect of DDT on the ratio and fertility of Seabirds was reported by others. Clear evidence exists for the detrimental effects of DDE on animal reproduction (Kelce et al., 1995). Another potential health risk associated with DDE is the role as a suspected human carcinogen. The p,p'-DDE molecule has been shown to bind tightly to androgen hormone receptors in experimental animals inducing reproductive disorders such as deformation of the sexual organs during pubescence (Kelce et al., 1995).

DDT has been used in New Zealand until the early 1970s for the control of grass grub (Costelytra Zealandica) in pastures (Bates et al., 1990).

A survey by Copplestone (Copplestone et al., 1973) reported that the proportion of DDE in the total DDT residue was virtually constant at any given time throughout the New Zealand population but increased from 68.7% in 1965 to 79.5% in 1969 and then to 88.7% in 1974. It has to be expected that this development will continue, which makes DDE the most persistent organochlorine contaminant in New Zealand.

There are several mechanisms for the degradation of DDT residues by non-metabolic processes (Crosby, 1969), like incineration, which is not only fairly expensive but could also cause secondary pollution (Rappe, 1984), chemical reductive dehalogenation, photochemical breakdown, pH, free radicals and reduced iron porphyrins. Photochemical breakdown of DDE to DDMU and other derivates has been described by Zepp (Zepp et

al., 1977) for dissolved DDE in the top 10 cm of water. Castro (Castro, 1964) showed that DDT was transformed to DDD in anhydrous and anaerobic solution of ferrous deuteroporphyrin. Miskus (Miskus et al., 1965) achieved the same result under anaerobic conditions with haemoglobin or haematin in presence of excess sodium dithionite. Zoro (Zoro et al., 1974) increased the solubility of the haematin and rapid DDT degradation was achieved with addition of ferrous sulphate under anaerobic conditions. Wedemeyer (Wedemeyer, 1966) used cell free extracts from Aerobacter aerogenes and found that under anaerobic conditions reduced Fe(II) cytochrome was responsible for the dechlorination of DDT.

This means, that there are possible routes for the abiotic degradation of DDT and its residues, but one major problem is the availability of the organochlorines for the different types of degradation, as discussed previously. Another problem is, that no major abiotic mechanism for the degradation of DDE exists, which results in the accumulation in the environment.

The disappearance and mineralisation of [^{14}C]-DDT in nutrient-deficient cultures of the white rot fungus Phanerochaete chrysosporium was shown by Bumpus and Aust under aerobic conditions (Bumpus and Aust, 1987). DDD nor DDE accumulated in the DDT metabolism by Phanerochaete chrysosporium but some water-soluble metabolites were detected, indicating ring cleavage. In 1993, Bumpus (Bumpus et al., 1993), were the first who reported a biodegradation of DDE through the fungus Phanerochaete chrysosporium, at a rate of about 6% in 60 days. The breakdown of DDE by Phanerochaete chrysosporium is significant, because it shows the first reliably reported microbial degradation of this chemical.

Typical degradation pathways of DDT have been summed up:

1. DDT -> DDD via reductive dechlorination by bacteria, anaerobic conditions are required. DDD can be further degraded.
2. DDT -> DDE via dehydrochlorination by bacteria, greatest conversion under aerobic or highly alkaline conditions, dead end.
3. DDT -> Dicofol via oxidation, more in higher organisms and fungi probably because of a defined mixed function oxidase system

Bacterial degradation:

4. DDT aerobe to DDE and then not further

5. DDT -> DDD -> DDMS (anaerobe) DDMS -> DDNS -> DDOH -> DDA -> DDM -> DBH -> DBP (aerobe) reductive dechlorination of DBH or DBP would also be anaerobically.

These reports emphasise that a degradation of DDT is possible, but no major microbial and even abiotic degradation of DDE has been reported. In the contrary accumulation and biomagnification of DDE in soil/sediments and biotic fat tissue has been reported. The result is that DDE is one of the most persistent xenobiotic chemicals in the environment and research into the transformation of this chemical is highly desirable.

Working hypothesis for the reductive dehalogenation of DDE and PCBs by sediments and soils from New Zealand

By using microorganisms present in anaerobic sediments, from a variety of New Zealand environments, the aim was to determine whether the dechlorination of PCBs and DDE under strict anaerobic conditions was possible.

If the reductive dechlorination of PCBs is a cometabolic process, then these sediments should be able to dehalogenate these chemicals under methanogenic conditions. This is because of the common eco-physiological pathways in anaerobic sediments and environments (Thiele, 1988). Conversely, if the process of dehalogenation needs a long term adaptation or exposure of the microorganisms to the chemicals, the sediments used here should be comparable to work already published. This is because these New Zealand sediments have had negligible exposure to PCBs.

DDT is aerobically transformed to DDE and accumulates in environments around the world. Due to the previous widespread use of DDT in New Zealand, one would expect a dechlorination to DDE. If vitamin B12 is a necessary catalyst to dehalogenate PCBs, then in its' absence there should not be any dehalogenation. In this work the bottles were wrapped to exclude any photochemical degradation and no vitamin B12 or any other catalyst (like ferredoxin, thioredoxin, etc.) was added. Therefore, all known abiotic degradation/dechlorination possibilities were excluded. Methanogens and other organisms produce vitamin B12 and release this compound at cell lysis. The production of vitamin B12 is a biotic process and thus depends on the microorganisms. In contrast, the dehalogenation of organochlorines catalysed by vitamin B12 is an abiotic process, therefore the whole sequence can be regarded as abiotic, but biologically induced.

The hypothesis addressed in this work, regarding PCBs, were whether bacteria from sediments are involved in reductive dehalogenation of PCBs, as extensively described in work based on the heavily polluted methanogenic sediments in the USA and Europe. These polluted sediments also occur in New Zealand, but without extensive PCB pollution, could these bacteria carry out PCB dehalogenation. With long term usage of DDT in New Zealand and contamination of marine sediments (Mapua) and pasture soils (Winchmore) for over 30 years, questions arise regarding new microbial adaptation. Adaptation and effective reductive dehalogenation of DDT by bacteria have been shown overseas, but did this occur in New Zealand?

1.4 Introduction on Upflow Anaerobic Sludge Blanket (UASB) reactors

One of the major problems of anaerobic wastewater treatment is the washout of active biomass. To overcome this problem a variety of designs have been developed and tested to hold back the anaerobic biomass in wastewater treatments (Andrews; Guiot and Berg van den, 1985; Merrath et al., 1993; McCarty and Smith, 1986). A common feature offered by all the high rate processes was the ability of effectively separate hydraulic and solids retention times. This allowed accumulation of high biomass concentration and use of relatively low hydraulic retention times (HRT). One example of the anaerobic systems to treat wastewater is the concept of the Upflow Anaerobic Sludge Blanket (UASB) reactor. It has been used successfully around the world for different types of wastewaters, at a variety of COD levels, with a number of different temperature and pH conditions and with different process regimes. UASB's were first designed and operated at the Agricultural University of Wageningen in 1987 (Zeeuw de, 1987). The concept was aimed at treating low and medium strength wastewater at high volumetric loading rates. The core of the high performance of these type of wastewater treatment is always the granule or pellet, which allows a high loading rate and good settling abilities, because of its dense bacterial biomass. Weimin (Weimin et al., 1987) reviewed the different seed sources utilised by a variety of researchers so far. Most conveniently granules were used to start up new UASB reactors, up to 10 - 30% of the reactor volume is required as inoculum (Hickey et al., 1991). The advantage of utilising granules as inoculum is, that the start up time for a new wastewater composition is reduced. Fully grown granules are not always readily available and instead digested sewage sludge, cow manure and sewer slurry have been used. But all seed sources have the same problem, that they are not necessarily readily available all around the world. Weimin (Weimin et al., 1987) tested therefore the applicability of aerobic activated sludge as seed. In this work anaerobic river sediments were utilised as inoculum source. These originated from the river Saale in Germany and the river Waikato in New Zealand.

The process to obtain granules is called granulation. So far a number of scientists have worked on the problem to determine what the causes for granulation are. A variety of feeding regimes have been proposed and empirical descriptions have been given, but the underlying concept is not yet fully understood.

Hulshof Pol (Hulshof Pol et al., 1987) identified the sum of hydraulic loading rate and gas loading rate as the parameters of selection pressure. Under low rates, they were not able to granulate the seed sludge, and found that a selective washout of dispersed sludge during the start-up period was important. For their work with higher COD influent levels (10,000 mg/L) the volumetric loading rate played an important role in the selection process. Washout became effective at a volumetric loading rate of 0.02 m/h (at a HRT of 2.2 days) and a gas loading rate of 1.6 m/day. The expansion washout is defined as a sludge bed expansion above the top of the settling compartment within the reactor, which results in a washout of the surplus granules or flocs from the top (Zeeuw de, 1987). For granulation with lower COD-influent levels (500 mg/L) this effect was less important. The substrate concentration determined directly the hydraulic loading rate at a given space loading rate. A low concentration of wastewater resulted in a high selection pressure. But too low concentrations would have resulted in too low growth rates of the "most important organism in the methanogenic granule (Methanothrix soehngenii)". Also potential granule precursors might have been washed out. They recommended a selection for Methanothrix-granules because Methanosarcina-granules were too small and could not be obtained in the reactor. Wiegant and de Man (Wiegant and Man de, 1986) found that Methanosarcina-granules performed less well than Methanothrix-granules. The conclusion of Hulshof Pol (Hulshof Pol et al., 1987) was, that granulation occurred, because of attachment of bacteria to growth nuclei. These nuclei might consist of inert organic, or inorganic material or of small bacterial aggregates. After a time of growth these pregranules might break up and together with detached biofilms are being utilised as secondary nuclei to produce the granules. This will go on and over time the granules will develop from voluminous filamentous aggregates into dense granules. De Zeeuw (Zeeuw de, 1987) stated that expansion washout will occur with all types of seed sludge when too much seed is used, reducing the seed sludge hold-up in the reactor to its maximum value under the prevailing conditions. Only very coarse particles are retained selectively, which will be used as carrier material for granules. The erosion washout on the other hand is described as very selective, it is defined as a washout of sludge particles from the sludge bed, provided that the top of the sludge bed is still well below the settling compartment. It only removes the lightest particles from the system. Therefore he recommended a bed erosion washout with a concentrated digested sewage sludge type and a medium strength wastewater to obtain granules.

Weimin (Weimin et al., 1987) pointed out that the use of other seed material than granules might be a major option for countries where access to granules is difficult. Here

we took this idea even further by using a different loading scheme to the one proposed by Lettinga and gained granules from anaerobic river sediments. Published studies have shown that UASB reactors inoculated with a variety of material containing anaerobic microorganisms and operated under conditions combining a low strength feed (little carbon and energy sources) with a high volumetric loading rate (how much feed goes in and out of the reactor) to form bacterial granules. In the here described experiments a high strength feed was combined with a low volumetric loading rate, but the reactor content was pumped in a recycle loop. This has been described in the literature before and has the same effect as the high volumetric loading rate, which is a selection for bacterial granules with good settling abilities. The sediments utilised in this work originated from the river Saale in Germany and the river Waikato in New Zealand. By using river sediments as seed source one might be able to combine two advantages. First anaerobic river sediments are readily available if a slow flowing river or backwater of a river can be located. Secondly, if there is a pollutant influx into the river (eg. from pulp and paper industries, sugar refineries, communal wastewater discharges) bacteria in an anaerobic river sediment downstream of this pollution input might be adapted to this particular contamination. In the literature the biological breakdown of certain chemical pollutants has only been described for anaerobic bacteria. One example would be the anaerobic reductive dehalogenation of PCBs by bacteria from Hudson river sediments under methanogenic conditions. It is not fully understood whether this process is a catabolic pathway, for which a selection should be possible, or if it is a cometabolic pathway, for which selection is not possible. If such a river sediment would be granulated, a higher cell density of these bacteria would be obtained, and either of the above mentioned possibilities would gain from a higher cell density.

1.5 Introduction on Biological Nutrient Removal from Wastewater in Treatment Plants

Wastewater Treatment Plants have first been introduced to reduce the Carbon- and Energy-Loadings from wastewater to be of less impact for the receiving waters. In the last decades it has become more and more apparent that not only the Carbon- and Energy-Sources are having an impact, but also the nutrients contained in the wastewater. Especially Nitrogen and Phosphorus present major issues, because elevated concentrations of these nutrients can give raise to accelarated growth of algae and other photosynthetic aquatic life within the receiving waters. This in turn can cause eutrophication, loss of oxygen from the water, and can have as far reaching consequences as turn the whole ecosystem of the receiving waters over. Most industrialised countries have very strict regulations and limits on the concentrations of nutrients being allowed to be discharged into receiving waters. Consequently there is a high emphasis on research and applied technology to deliver nutrient-removal systems for new and existing wastewater treatment plants.

Of great interest beside the chemical-mechanical removal of nutirents is the biological removal, because these systems have proven to be very effective in the removal of Carbon- and Energy-Sources from wastewater. The main mechanisms for biological nutrient removal are:

1. Assimilation,
2. Nitrification/Denitrification,
3. Enhanced Biological Phosphorus Removal (EBPR).

Assimilation is the uptake of Phosphorus and Nitrogen into the cells during cell growth. Under normal circumstances the uptake of the nutrients represents only a small percentage of the total concentration within the wastewater. Although, especially in aerobic wastewater treatment designs the excess biomass will have to be disposed of. The total nutrient concentration removed through the Assimilation Process is relatively small.

The Nitrification/Denitrification Process is well known and consists of an aerobic step, in which microorganisms convert organic Nitrogen into the Ammonium form, and in turn this is oxidised via Nitrite to Nitrate. The second step occurrs in anoxic environments, and the respective micro-organisms use Nitrate as an electron acceptor in a redox-

recation, in which Nitrate is reduced to molecular Nitrogen, which in trun escapes as a gas from the wastewater.

The EBPR process has been studied in the recent years extensively, and it is understood that micro-organisms after an anaerobic period, or a period without Phosphate present, are subjected to an aerobic period with Phosphate in the wastewater, which results in an elavated uptake of the Phosphate into the cells. The physiology of the micro-organisms involved is normally divided into two steps:

1. An anaerobic phase, in which substrates (Volatile Fatty Acids) are accumulated and stored inside the cells.
2. An aerobic phase, where the internally stored substrate is used for growth and phosphate uptake.

As with the assimilation process , the removal of biomass (ie activated sludge) from the treatment system has a large impact on the total Nutrient-Removal form the wastewater treatment system.

The typicall biological nutrient removal system in a wastewater plant includes three distinct steps or areas:

1. Anaerobic Step, for the selection for Phosphate-removal bacteria and the cell-internal storage of substrates.
2. Anoxic Step, for the Denitrification Process and Phosphate uptake.
3. Aerobic Step, for the Nitrification Process and Phosphate uptake.

The sequence of Steps 2 and 3 is fluent and through recirculation of biomass interchangable.

In this work, a full-scale wastewater treament plant with biological nutrient removal operating for a Dairy-factory is presented.

2 Recalcitrance of 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)-ehtylene (DDE) to Cometabolic Degradation by Pure Cultures of Aerobic and Anaerobic Bacteria

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2.1 Abstract

Pure cultures of aerobic and anaerobic bacteria capable of oxidation and reductive dehalogenation of chloroethylenes, and aerobic bacteria involved in biodegradation of polychlorinated biphenyls (PCBs) were screened for their ability to co-metabolize the persistent pollutant 1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ehtylene (DDE). Bacterial culture expressing methane monooxygenase (*Methylosinus trichosporium*), propane monooxygenase (*Mycobacterium vaccae*) and biphenyl 2,3-dioxygenase enzymes (*Pseudomonas fluorescens* and *Rhodococcus globerulus*), as well as bacteria reductively dechlorinating chloroethylenes (*Acetobacterium woodii* and *Clostridium butyricum*) could not degrade DDE. Cell-free extracts of *M. trichosporium*, *M. vaccae*, *P. fluorescens* and *R. globerulus* were also unable to transform DDE, indicating that cell wall and membrane diffusion barriers were not biodegradation limiting. These studies suggest that these bacteria can not degrade DDE, even when provided with co-subrates that induce chlorophenyl- and dichloroethylene-group transforming enzymes.

2.2 Introduction

In contaminated soils 1,1-Dichloro-2,2-bis*(p*-chlorophenyl)ehtylene (DDE) is the most persistent and frequently encountered degradation product of 1,1,1-trichloro-2,2-bis*(p*-chlorophenyl)ethane (DDT) (Helling *et al.* 1971). It is formed photochemically (Maugh 1973) and by bacterial (Subba-Rao and Alexander 1985) and abiotic dehydrochlorination (Boul 1995). Photooxidation and volatilization are probably responsible for the rapid loss of DDT and its residues from tropical soils (Hussein *et al.* 1994), but in temperate climates DDT residues and particularly DDE can persist for longer periods. A significant proportion of the DDT applied to a New Zealand pasture was present as DDE 27 years after the last application (Boul *et al.* 1994). The resistence of DDT to microbial attack has been ascribed to its structure having both *para* aromatic and aliphatic chlorine substituents (Kapoor *et al.* 1973). Despite its recalcitrance, DDT has been shown to be co-metabolically degraded by natural micro-organisms in soils and laboratory cultures (Wedemeyer 1966; Subba-Rao and Alexander 1985; Nadeau *et al.* 1994). Reports of DDE breakdown by micro-organisms, however, are rare (Menzie 1974; Bumpus *et al.* 1993). Ledford and Chen (1969) reported degradation of DDE by *Geotrichum candidum* and *Brevibacterium linens*, both isolated from surface ripened cheese, but the products were not identified.

Co-metabolism is likely to be important for the microbial degradation of chlorinated compounds such as DDE. It has been observed that the microbial enzymes of primary substrate oxidation pathways can fortuitously transform many chlorinated aromatic and aliphatic hydrocarbons (Furukawa *et al.* 1979; Little *et al.* 1987). We investigated bacteria capable of degrading compounds with structural similarity to DDE, to determine whether they possessed enzymes with sufficient relaxed specificities to enable parallel degradation of this compound. The analogues chosen (Figure 2.1) were 1,1-dichloroethylene (DCE) representing the chlorinated aliphatic DDE side chain, and 4,4'-dichlorobiphenyl (DCB), the chlorinated ring system. The appropriateness of the biphenyl skeleton as an analogue for DDT residue degradation has been recently demonstrated by Nadeau *et al.* (1994), in the 4-chlorobiphenyl degrading strain of Alcaligenes eutrophus. A broad range of aerobic and anaerobic bacteria able to co-metabolise either DCE or biphenyl were thus tested for their ability to transform DDE under optimised culture conditions.

2.3 Materials and Methods

Chemicals and Materials

DCE, DDE, trichloroethyelen (TCE), and biphenyl were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. All other chemicals were analytical grade and obtained from Sigma Chemical Co., St Louis, MO, USA, unless specified otherwise. ^{14}C-p,p'-DDE was synthesized from radio-labeled DDT as described elsewhere (Fawcett *et al*. 1981; Thiele *et al*. 1996). Gases were purchased from New Zealand Industrial Gases. Butyl stoppers were from Bellco Glass Inc., Vineland, NJ, USA.

Organisms and Culture Conditions

Obligate anaerobic bacteria, *Acetobacterium woodii* DSM 1030, *Clostridium acetobutylicum* DSM 8052, *C. butyricum* DSM 552, *Methanosaeta concilii* DSM 3671, and *Methanobactericum formicicum* DSM 1535 were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany. *Methylosinus trichosporium* NCIB 11131 was obtained from the National Collection of Industrial Bacteria, Aberdeen, UK. *Mycobacterium vaccae* JOB5 ATCC 29678 was obtained from the American Type Culture Collection, Rockville, MD, USA. Additional methanogenic cultures were enriched on methanol and formate using granular anaerobic sludge as inoculum (Thiele *et al*. 1990). *Rhodococcus globerulus* P6 previously designated as *Acinetobacter* sp. Strain P6 (Furukawa *et al*. 1978) and *Pseudomonas fluorescens* MMI were obtained from Division of Microbiology National Research Center for Biotechnology, Braunschweig, Germany.

Pressure bottles (160 ml) with butyl rubber stoppers and aluminium crimp seals with N_2/CO_2 (95/5 vol/vol) head space were used to grow obligately anaerobic bacteria. Media and stock solutions were prepared anaerobically according to Kenealy and Zeikus (1981). The final pH of the medium was 7.0. Culture bottles were incubated at 37° C at 80 rpm on a gyrotory shaker.

The methanotroph, *M. trichosporium* was grown in sealed 160 ml serum bottles in minimal salts medium (Fogel *et al*. 1986) with 50/50 methane/air headspace. *M. vaccae* was grown in 160 ml serum bottles containing a 0.25% (w/v) yeast extract medium under

a 50/50 propane/air atmosphere. Cultures of the polychlorinated biphenyl (PCB) degrading bacteria *R. globerulus* P6 and *P. fluorescens* MMI were grown in modified M9 minimal salts medium (Abril *et al.* 1989) supplemented with biphenyl (0.5 g/L) as a growth substrate. The cultures (100 ml in 500-ml Erlenmeyer flasks closed with Teflon covered screw caps) were incubated at 30° C at 150 rpm.

Biotransformation of 1,1-Dichloroethylene

M. trichosporium, *M. vaccae* and obligate anaerobes were grown in 10-ml batches in sealed autoclaved pressure tubes (Bellco Glass Inc., Vineland, NJ, USA), with appropriate media and head space. DCE was added from concentrated stock solutions to a final concentration of 150 mg/L in the culture medium. Abiotic DCE losses, including those due to sampling from the experimental cultures, were determined using dechlorination-inhibited methanogenic cultures (Belay and Daniels 1987) with comparable biomass concentrations to live controls incubated under identical conditions. Methanogens included M. concilli, M. formicum and two methanogenic enrichments maintained at 120 mg/L DCE (Belay and Daniels 1987). A 0.5-ml liquid sample was withdrawn from duplicate cultures at 0, 100, 175 and 250 h after the addition of DCE and immediately extracted at 0° C with 0.1 ml of toluene. Cells were separated by centrifugation (10 min at 13,000 g) and toluene phase directly analysed for DCE by capillary gas chromatography. Protein from the cell pellet was determined colorimetrically (Hartee 1972). The effect of TCE on DCE removal was tested under similar conditions by adding 200 mg/L TCE to the culture medium with 150 mg/L DCE.

Assay for Co-metabolic DDE Transformation

Sterile pressure tubes, media, and head-space conditions identical to the DCE bio-transformation tests were used. Bacteria were pre-cultured in 160-ml pressure bottles and concentrated tenfold by centrifugation under aseptic and, where required, anaerobic conditions. For each bacterial culture the following treatments were incubated for 30 days:

live cells with ^{14}C-DDE at a final concentration of 100 mg/L (9.5 KBq/ml culture);
autoclaved cells amended with ^{14}C-DDE as killed cell controls;
live cells with carrier solvent only as an untreated control;
medium only with ^{14}C-DDE as an uninoculated control to account for abiotic losses.

The initial cell protein concentrations after the addition of DDE were 250-300 mg/L culture. All treatments and controls were performed in duplicate (5-ml medium/pressure tube) and whole cultures were sacrificed at each time point. At 0 and 30 days after the addition of DDE the cultures were analysed in duplicate for DDE and cellular protein. DDE was extracted from the culture tubes with 5 ml chloroform with an extraction efficiency of 100%.

Assay for PCB-degrading Bacteria for DDE Co-metabolism

R. globerulus P6 and *P. fluorescens* MMI were grown in 1-L baffled Erlenmeyer flasks supplemented with biphenyl crystals as the sole carbon source in the medium. The cells were harvested at exponential phase, concentrated by centrifugation (6,000 g for 10 min), washed with minimal salts medium, re-centrifuged, and re-suspended in fresh minimal salts medium to a final cell protein concentration of 400-430-mg/L culture.

Assays of cultures were performed as follows. Aliqouts from stock solutions of DDE alone and in combination with biphenyl were added to sterilised culture tubes (26 ml volume) to provide a final concentration of 100 mg/L each in the culture medium. The carrier solvent was evaporated to dryness and 5-ml portions of the bacterial cultures were added to give a cell protein concentration of 400-430-mg/L culture. Treated autoclaved cultures served as dead cell controls. An additional set of treated cultures was amended with 0.02% (v/v) of Triton X-100 to determine whether surfactant addition affected the degradation of DDE. All treatments and controls were incubated in darkness for 30 days at 30° C at 150 rpm on a gyrotory shaker. At the beginning and end of the incubation period, duplicate cultures were extracted in chloroform (as described above) and extracts analysed for DDE and biphenyl by high-performance liquid chromatography (HPLC).

The location of DDE within the culture tubes inoculated with *M. vaccae* and *M. trichosporium* was determined after 30 days incubation. The entire contents in the test tube were centrifuged (9,000 g for 10 min) and divided into cell pellet, aqueous phase, and residual (glass associated) portions. Each portion was solvent extracted with chloroform and the DDE content analysed by HPLC.

Cell-Free Assays

M. trichosporium, *M. vaccae*, *R. globerulus* and *P. fluorescens* were harvested by centrifugation (4,000 g for 20 min at 4° C), washed with KH_2PO_4 buffer (pH 7.2) and re-suspended in the same buffer amended with 2 mM $MgCl_2$. The cells were placed on ice and disrupted (10 X 30 s) with a model W 185 Sonifier cell disrupter (Heat Systems Ultrasonics Inc., Plain View, NY, USA). The resultant homogenate was centrifuged (19,000 g; 30 min; 4° C) and the supernatant used as a crude cell-free extract. Assays with *M. trichosporium* and *M. vaccae* were performed in 15-ml Serum vials containing 200 mmol potassium phosphate buffer (pH 7.3), 0.2 mmol NADH, and 0.4 ml of extract (containing 1.4-1.6 mg protein) in a total of 1 ml (deBont *et al.*1979). An appropriate air/gas mixture was provided and ^{14}C-DDE (9.5 KBq) added to a final concentration of 100 mg/L DDE and incubated for 18 h. Controls were performed with heat inactivated (20 min at 90° C) enzyme extracts.

Cell extracts from *R. globerulus* and *P. fluorescens* were prepared from cells grown in the presence of biphenyl as described above. The assay mixture in 1 ml of phosphate buffer contained 1.0 mM NADH, 1 mM flavin adenine dinucleotide (FAD), 100 mg ^{14}C-DDE (9.5 KBq), and cell extract (1.1-1.3 mg protein). Positive controls containing biphenyl instead of DDE, and heat inactivated enzyme controls were also included. Reaction was determined as substrate consumption at the end of the 18-h incubation period measured by HPLC and scintillation counting.

Analytical Methods

Chloroform extracted DDE, presumptive DDE degradation intermediates, and biphenyl were separated by HPLC on Ecosil C8 (Alltech Assoc. Inc., Deerfield, IL, USA). An isocratic elution phase (80:19:1 methanol/Milli-Q water/acetic acid, v/v/v) was used. Detection was by UV absorbance at 235 nm. Peak areas were integrated using DP800 software (GBC Scientific, Auckland, New Zealand) and quantitated by comparison with authentic external standards. HPLC enabled separation of the following presumptive DDE degradation intermediates:

1-chloro-2,2-bis(*p*-chlorphenyl0ethylene (DDMU)

1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethyane (DDD)

2,2-bis(*p*-chlorophenyl)ethanol (DDHO)

4,4'-dichlorobenzophenone (DBP)

2,2-bis(*p*-chlorophenyl) acetic acid (DDA)
diphenylethylene (DPE)
with the retention times of 9.8, 7.8, 5.1, 6.0, 5.1 and 7.2 min respectively. DDE had a retention time of 11.3 min. The lower detection limit for the above compounds were in the range of 0.05-0.01-mg/L culture. Gases (methane and propane) were determined from headspace samples (1 ml) as described elsewhere (Stroempl and Thiele 1996).

Levels of DCE in the culture were determined from toluene extracts by direct capillary gas chromatography and flame ionization detection with a Carbon Layer Open Tubular (CLOT) column (15 m X 0.52 nm i.d., Supelco, Bellafonte, PA, USA). Detector temperature was 250° C with an injector port temperature of 110° C and H_2 carrier gas flow rate of 15 ml/min, held for 10 min, and cooled at 35° C /min to 35° C). The retention time of DCE was 0.6-0.99 min. Peaks were identified and quantified by comparison with authentic standards.

Radioactivity Measurements

$^{14}CO_2$ production in culture tubes incubated with ^{14}C-DDE was determined by removing duplicate 2-ml gas samples with a gas-lock syringe and injecting these into sealed vials containing 2 ml of 0.1 M NaOH. Vials were shaken for 1 h to allow absorption of $^{14}CO_2$ (Fogel *et al*. 1986), and the resultant solutions transferred to vials containing 18 ml of scintillant.

Culture extracts were subjected to HPLC analysis as described above to determine whether labelled DDE degradation products were present. Column eluate was fractionated (at time intervals of 1-3, 3-6, 6-9, 9-10.5, 10.5-14 min) and the ^{14}C present was quantified using a Phillips model PW 4700 Scintillation counter with an automatic quench correction protocol.

2.4 Results

Co-metabolic DCE Removal

To confirm the DCE degrading ability of the chosen bacterial cultures, we incubated *Methylosinus trichosporium* (aerobic methane oxidiser), *Mycobacterium vaccae* (aerobic propane oxidiser), Acetobacterium woodii, *Clostridium butyricum*, and *Clostridium acetobutylicum* (fermentative obligate anaerobes) with DCE and their respective primary carbon substrates for 10 days. Cultures were extracted at 0, 100, 175, and 250 h after addition of DCE. All cultures removed DCE, the most effective being *M. vaccae* followed by *M. trichosporium*, *C. acetobutylicum*, A. woodii, and *C. butyricum* (Figure 2.2A). *M. vaccae* which expresses propane monooxygenase, and *M. trichosporium* which expresses methane monooxygenase, removed about 60 and 50% of DCE respectively. The obligate anaerobes, *C. acetobutylicum*, *A. woodii* and *C. butyricum* removed about 50, 40 and 30% respectively. Removal of up to 17% DCE in the inactive controls containing DCE-inhibited methanogenic bacteria (Belay and Daniels 1987) accounted for all sampling losses including abiotic removal and adsorption. With the exception of the controls, all cultures grew in the presence of 150 mg/L DCE as evidenced by increased cellular protein (Figure 2.2B). Growth was similar whether DCE was present or not, but the addition of 200 mg/L TCE suppressed DCE removal by the cultures (data not shown).

Test for Co-metabolic DDE Degradation by DCE Degrading Bacteria

Four cultures (*M. trichosporium*, *M. vaccae*, *A. woodii*, and *C. butyricum*) were evaluated for their ability to co-metabolize DDE while growing on primary carbon substrates. Similar conditions to those of the DCE removal assay were used, except DCE was replaced by ^{14}C-DDE and the incubation was extended to 30 days. All cultures grew significantly as shown by increases in cellular protein (Table 2.1), indicating that the experimental conditions were appropriate to induce the enzymes hypothetically capable of DDE co-metabolism. None of the cultures however, transformed DDE over the 30-day incubation period and no mineralisation to $^{14}CO_2$ was observed. HPLC of culture extracts at the end of the incubation period did not detect any degradation intermediates. Isotope recovered from the HPLC fraction corresponding to the DDE peak accounted for >99.9% of the added radioactivity.

To determine the bio-availability of DDE in the system, cultures of *M. trichosporium* and *M. vaccae* were separated after a 30-day incubation into aqueous, cell and "glass bound" fractions. Most DDE was recovered in the chloroform extract of the cell pellet (88-91%) with the remainder either associated with the culture vessel surface (5-8%) or the aqueous phase (4%).

Co-metabolism of DDE by DCB Degrading Bacteria

The potential of two DCB degrading bacteria, *R. globerulus* and *P. fluorescens* to co-metabolise DDE during aerobic biphenyl degradation was examined (Table 2.1). Incubation of *P. fluorescens* and *R. globerulus* cells, pre-cultured on biphenyl, in the presence of DDE either alone or in combination with biphenyl for 30 days, did not result in DDE degradation. Biphenyl was completely degraded while DDE was un-transformed (Table 2.1).

The apparent solubilities of water insoluble organic compounds can be enhanced by surfactants such as Triton X-100 (Kile and Chiou 1989). We therefore supplemented cultures with 0.02% (w/v) Triton X-100 to determine whether this facilitated DDE degradation. There was no observed effect of this treatment as 100% of the DDE was recovered unchanged while biphenyl was again degraded (Table 2.1).

DDE Degradation by Cell-free Extracts of Bacteria

HPLC analysis of the crude cell-free extracts incubated with ^{14}C-DDE for 18 h revealed that all recovered radio-activity was associated with the DDE fraction and no labelled metabolites were detected. Cell extracts of *M. trichosporium* and *M. vaccae* were able to oxidise methane and propane respectively (data not shown). Biphenyl was totally degraded by the cell extracts of *P. fluorescens* and *R. globerulus*.

2.5 Discussion

The ability of pure cultures of *Methylosinus trichosporium*, *Mycobacterium vaccae*, Acetobacterium woodii, *Clostridium acetobutylicum*, and *C. butyricum* to remove DCE from the culture medium was confirmed. The strains exhibited considerable differences in their ability to degrade DCE, indicating that there may be differences in the enzyme systems involved in DCE degradation. Lack of significant DCE removal in controls suggested that this removal was by microbial transformation rather than cell sequestration or abiotic degradation. The aliphatic portion of DDE is structurally analogous to DCE (Figure 2.1). Removal of DDE was not observed in DCE degrading cultures despite the use of higher cell densities and prolonged incubation period than in experiments with DCE alone. The enzymes of DCE degradation in both the aerobic and anaerobic bacteria studied could not therefore transform DDE. The sensitivity of the radio-tracer assay (100 ng/L) would have detected a DDE transformation rate of less than 5 ng/L/h. Thus, any undetected DDE transformation would be very small.

Cell-free extracts of pure bacterial cultures of bacteria have been shown to reductively dechlorinate DDT (Wedemeyer 1966), pentachlorophenol (Mohn and Tiedje 1992), and tetrachloroethylene (Neumann *et al.* 1994, 1995). In addition, the enzymes for biphenyl and DCB degradation are located in the bacterial cytoplasm (Mondello 1989). To investigate whether DDE recalcitrance was due to cell wall and membrane barrier effects, we determined DDE degradation in crude cell-free extracts. HPLC analysis of the crude cell-free extracts of cells induced for biphenyl degradation incubated with ^{14}C-DDE for 18 h revealed that all recovered radioactivity was associated with the DDE fraction and no labelled metabolites were detected. Biphenyl was totally removed by the cell extracts of *Pseudomonas fluorescens* and *Rhodococcus globerulus* indicating that active biphenyl di-oxygenases were present. Cell extracts of *M. trichosporium* and *M. vaccae* were able to oxidise methane and propane respectively (data not shown), indicating the activity of methane and propane mono-oxygenases in the cultures. The absence of DDE transformation in these crude cell-free extracts, despite degradation of presumptive co-substrates, suggest that permeability limitations are not the primary cause of DDE persistence.

Bacterial uptake of organic compounds increases with hydrophobicity (Parosn *et al.* 1987). DDE is poorly water soluble (0.04-0.06 mg/L; Montgomery 1993) and a large part

of the added DDE was associated with bacterial cells. Generally, compounds must be accessible as free solutes in the cell cytoplasm to be accessible for enzymatic attack. Hydrophobic compounds like polychlorinated biphenyls (PCBs), DDT and chlorobenzenes are degradable by bacterial enzymes, however (Mohn and Tiedje 1992), and low aqueous solubility may not, therefore, limit biodegradation.

Since DCB and DDE share some common structural features (Nadeau *et al.* 1994), we tested the potential of two DCB degrading bacteria, for their ability to co-metabolise DDE when growing on biphenyl. *P. fluorescens* MMI can grow on mono-chlorobiphenyls, degrades mono-chlorobenzoates and can co-metabolise other PCB congeners (Megharaj, unpublished data). *R. globerulus* strain P6 is a well-studied PCB degrader capable of co-metabolic transformation of 4,4'-dichlorobiphenyl to 4-chlorobenzoate (Furukawa *et al.* 1978, 1979). It has been demonstrated that growth on biphenyl as a sole carbon source is necessary for the optimal PCB degrading activity and induction of PCB degrading enzymes (Mondello 1989). Incubation of biphenyl grown cells of *P. fluorescens* and *R. globerulus* in the presence of DDE, alone and in combination with biphenyl, and even in the presence of a surfactant, resulted in no degradation of DDE. The observation that 4,4'-dichlorobiphenyl (aqueous solubility 0.056 mg/L; Bruggemann *et al.* 1982) was degraded by *R. globerulus* in our experiments, but that DDE was un-tarnsformed suggests that low aqueous solubility is not the primary factor limiting DDE degradation.

The resistance of DDT to microbial degradation has been ascribed to its structure having both aromatic chlorine substituents at *para* positions and chlorines on the aliphatic moiety (Kapoor *et al.* 1973). Helling *et al.* (1971) also suggested the chlorination of both phenyl rings of DDT as a reason for the recalcitrance to metabolism of DDT and its analogues by a Hydrogenomonas strain. *R. globerulus* tested here degraded 4,4'-dichlorobiphenyl which was *para* chlorinated on both aromatic rings but could not degrade DDE. In addition, the biphenyl 2,3 dioxygenase of Alcaligenes eutrophus could oxidise DDT (Nadeau *et al.* 1994) despite *para* chlorination on both aromatic rings. This implies that aromatic chlorination pattern alone cannot explain the persistence of DDT residues. There are four pairs of unsubstituted vicinal aromatic carbon atoms in the DDE molecule (Figure 2.1) potentially available for bacterial di-oxygenase attack. The observed lack of DDE degradation by broad specificity mono-oxygenases and biphenyl 2,3-di-oxygenases despite the activity of such enzymes in whole cells and cell extracts of *R. globerulus*, *P. fluorescens*, *M. vaccae* and *M. trichosporium* against DCB and DCE

suggests that the 1,1-diphenylethenyl structure of DDE (Figure 2.1) may also contribute to its recalcitrance. The relative resistance of the un-chlorinated diphenylethylene (DPE) molecule to aerobic biodegradation was recently observed in ethylbenzene degrading bacterial communities from aromatic hydrocarbon contaminated soils (Thiele *et al.* 1996). The degradation of the un-chlorinated phenyl ring of 1-*(p*-chlorophenyl)-1-phenylethylene by a Pseudomonas sp. demonstrated that bacterial degradation of the diphenylethenyl structure is bio-chemically possible (Francis *et al.* 1976). This culture was enriched on diphenylethane as the sole carbon source and the activity was unfortunately later lost. DPE removal activity was also observed in aerobic/anaerobic sequenced batch reactors (Stroempl and Thiele 1996) but this activity was also transient. Thus microbial degradation of the unusual 1,1-diphenylethenyl skeleton may be a bottleneck for DDE degradation. The selection of new aerobic bacteria with unusual oxygenase enzymes that allow attack on the 1,1-diphenylethenyl structure could thus provide new possibilities for aerobic co-metabolic degradation of DDE.

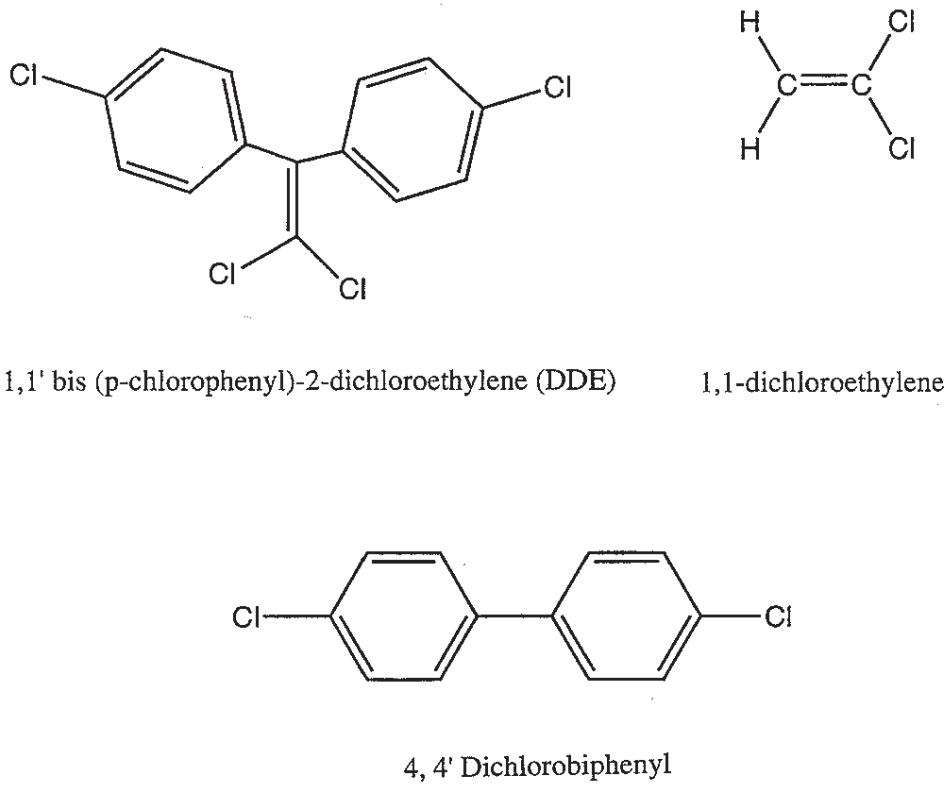
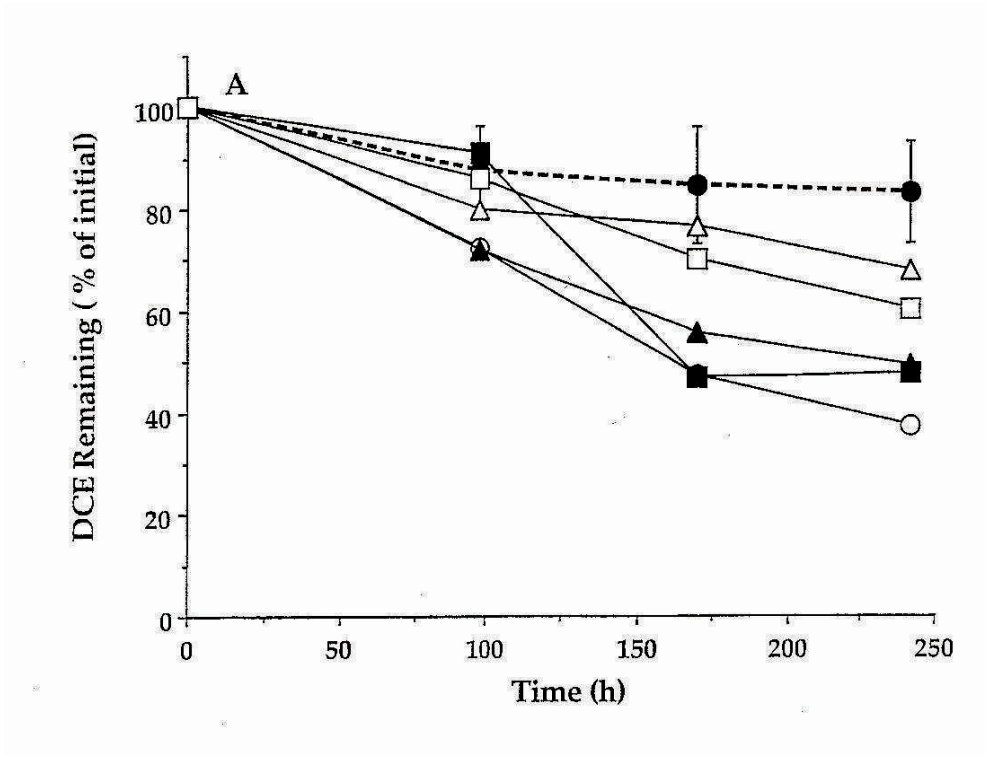


Fig. 1. Structural formulae of DDE and its analogues

Figure 2.1: Structural Formula of DDE and its analogues.


A
DCE Remaining (% of initial)
100
80
60
40
20
0
0
50
100
150
200
250
Time (h)

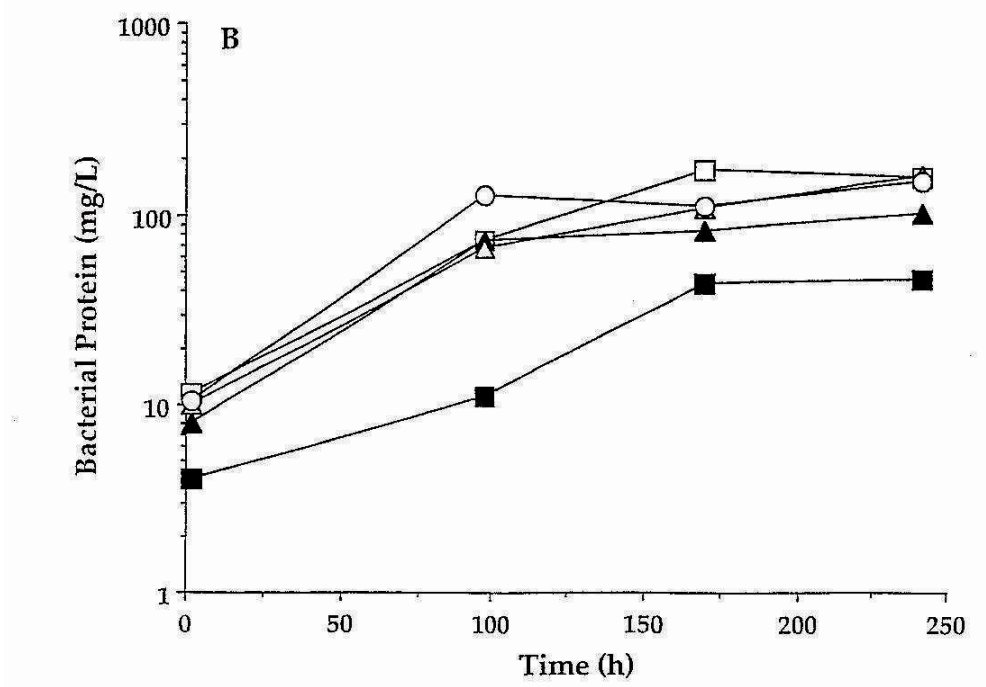

B
Bacterial Protein (mg/L)
1000
100
10
1
0
50
100
150
200
250
Time (h)

Figure 2.2: (A) Relative removal of 1,1-dichloroethylene (DCE 150 mg/L) by growing pure cultures of various bacteria. (B) Growth of bacteria in the presence of DCE (150 mg/L). Symbols:
M. Vaccae (○)
M. trichosporium (■);
C. butyricum (△);
C. acetobutylicum (▲);
A. woodii (□);
And controls of inactive methanogenic cultures lacking ability to degrade DCE●).
Bars represent SD of means (n = 4).
Sampling times were 100, 175, and 250 h after addition to respective cultures.

Table 2.1 Co-metabolic DDE-degradative ability of bacteria

Parameter	Organisms					
	M. trichospo-rium	*M. vaccae*	*P. fluorescens*	*R. globerulus*	*A. woodii*	*C. butyricum*
% DDE recovered						
Live cells (14C-DDE)b	99.9	99.9	102.0	99.8	99.0	99.9
Autoclaved (14C-DDE)b	99.9	99.9	100.0	101.0	100.0	99.8
Live cells + 0.02% TX-100 b	n.a.	n.a.	100.6	101.8	n.a.	n.a.
Live cells + 0.02% TX-100 + 100 mg/L BP b	n.a.	n.a.	101.6	102.4	n.a.	n.a.
Cell extracts						
Heat killed (14C-DDE)b	99.8	99.8	100.8	100.2	n.d.	n.d.
Cell growth (mg protein/30 days)						
DDE addition	210	310	n.d.	n.d.	150	150
Control (no DDE)	230	320	n.d.	n.d.	180	160

a Means of at least duplicate experiments (average deviation of the mean less than 3%)
b Fraction corresponding to the DDE peak; Biphenyl was completely degraded by *P. fluorescens* and *R. globerulus*
Values expressed as percentages in relation to uninoculated control. DDE was added at 100 mg/L and radioactivity of 9.5 KBq/ml culture; Sampling time was at 720 h (30 days); TX-100, surfactant Triton X-100; BP, Biphenyl; n.a., not applicable; n.d., not determined.

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3 Screening for anaerobic bacteria in New Zealand sediments to degrade the polychlorinated chemicals, PCB and DDE

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3.1 Introduction

During World War II DDT (Figure 3.1) was introduced as a pesticide for control of insect-borne diseases like malaria. PCBs (Figure 3.3) were first introduced well before World War II as lubricants, fire retardents and electrical insulators, because of their non-flammable and heat-resistant properties. Both chemicals represent families of stable, highly water insoluble industrial chemicals. These chemicals can accumulate in anaerobic river sediments or attach to soil particles when brought into the environment. Mackay (Mackay and Wolkoff 1973) pointed out that compounds like PCBs have a high molecular weight and low vapour pressure, and thus evaporation rates should be low. A factor that is often overlooked are the high activity coefficients of these compounds in water that cause unexpectedly high equilibrium vapour partial pressures and thus high rates of evaporation from aqueous solutions. PCBs and DDT have thus been distributed even into the Arctic and Antarctic regions by atmospheric deposition. Recent work on DDT in tropical soils has shown, that in all cases the half lives for DDT and DDE (Figure 3.2) were between 2 and 9 month mainly due to losses by volatilisation-dissipation, whereas in temperate zones the half lives for these chemicals are between 2 and 35 years. Ballschmitter et al (Ballschmitter and Wittlinger 1991) reported on the inter hemisphere exchange of PCBs, DDT, DDE and other organochlorines. They found that the overwhelming part of the PCB input into the global environment takes place in the Northern Hemisphere.

Although PCBs, DDT and DDE show very little direct toxicity their accumulation and bio-magnification throughout the food chain is a concern. A term often used in the literature is the "sink", which highlights the fact that these organochlorines are accumulating in biota and sediments and are found in higher concentrations further up the food chain. Eggshell thinning related to DDT and especially DDE caused reproductive failure in raptors and fish-eating birds (Hickey and Anderson 1968); (Wiemeyer and Porter 1970). Hileman (Hileman 1994) reviewed the effect of "environmental oestrogens" in regard to reproductive abnormalities and cancer. "Oestrogen mimics" are a diverse group of chemicals that have no obvious structural similarity, but attach themselves to oestrogen receptor cells. Examples would be DDT, DDE and PCBs. There is also some evidence that DDE reacts with androgen receptors in some juvenile mammals, leading to a feminisation of male individuals (Kelce et al. 1995). DDE has been suggested as the most persistent DDT residue in New Zealand (Solly and Shanks 1974); (Copplestone et al. 1973) which has been confirmed in more recent work (Thiele et al. 1997); (Megharaj et al. 1997a); (Megharaj et al. 1997b) (Boul 1995).

PCBs were manufactured and sold as complex mixtures with different average chlorination levels. Product numbers were assigned that usually reflected the degree of chlorination by either the average number of chlorine's/biphenyl or the weight percentage chlorine in the mixture. For example, Aroclor 1260 (12 carbon atoms and 60 % chlorine) Clophen A 60 (average of 6 chlorine's per biphenyl, (Abramowicz 1990). The detailed characterisation of chemical composition changes in environmental PCB extracts is made possible by this unique composition of PCB commercial mixtures, like fingerprints (Figure 3.5). Thus, an environmental altered Aroclor has been shown to have a new congener distribution (and GC pattern) that is characteristic for the transformation process (Brown et al. 1984). Detailed analysis of that degradation pattern may elucidate the chemical nature of the transformation.

Aerobic biodegradation of PCBs is generally limited to molecules with 5 or fewer chlorine's with two adjacent un-substituted ring carbon atoms (Bedard et al. 1987b); (Bedard et al. 1987a); (Furukawa et al. 1979); (Furukawa 1982). There is no convincing evidence for the total degradation of Aroclor 1260 under aerobic conditions. The process of anaerobic reductive dehalogenation of PCBs mediated by bacteria has been confirmed in a number of laboratories with sediments from many distinct aquatic systems throughout the world (see review by (Abramowicz 1990). It has been shown that Vitamin B12 in the culture medium can also catalyse the reductive dehalogenation of

PCBs (Assaf-Anid et al. 1992). PCBs in New Zealand habitats occur either at very low levels (under 1 ppb), most likely related to atmospheric deposition, or occur in areas of high population with light industry, where run off from electrical equipment, paint thinners or hydraulic fluids might have occurred (Burggraaf et al. 1994); (Holland et al. 1993); (Buckland 1995); (Jones 1995); (Wilcock 1995).

3.2 Experimental Design

DDT is aerobically transformed to DDE and accumulates in environments around the world. Due to the previous widespread use of DDT in New Zealand, one would expect a dechlorination to DDE. If Vitamin B12 is a necessary catalyst to dehalogenate PCBs, then in its' absence there should not be any dehalogenation. In this work the experimental test systems were wrapped to exclude any photochemical degradation and no additional Vitamin B12 or any other catalyst (like Ferredoxin, Thioredoxin, etc.) was added. Therefore, all known abiotic chemical and photochemical degradation/dechlorination possibilities were reduced to an absolute minimum. Methanogens and other organisms produce Vitamin B12 and release this compound at cell lysis. The production of Vitamin B12 is a biotic process and thus depends on the living micro organisms. In contrast the dehalogenation of organochlorines catalysed by Vitamin B12 is an abiotic process, therefore the whole sequence can be regarded as abiotic, but biologically mediated.

The hypothesis addressed in this work, regarding PCBs, was, whether bacteria from sediments in New Zealand are involved in reductive dehalogenation of PCBs, as extensively described in work based on the heavily polluted methanogenic freshwater sediments in the USA and Europe. These sediment types also occur in New Zealand but without an extensive PCB pollution. Could New Zealand sediment bacteria carry out PCB dehalogenation? With long term usage of DDT in New Zealand and contamination of marine sediments (Mapua) and pasture soils (Winchmore) for over 30 years, additional questions arise regarding new microbial adaptation to DDT/DDE degradation. Adaptation and effective reductive dehalogenation of DDT by bacteria has been shown overseas, but did this occur in New Zealand?

Although the enzymatic processes leading to the aerobic dechlorination and the adaptation processes required to obtain these enzymes are understood, the same can not

be said about the microbial reductive dehalogenation under anaerobic conditions, which prevail in freshwater and marine sediments.

The sediments used in the organochlorine screening experiments originated from four different sites within New Zealand, they consisted of freshwater sediments, soil samples, and an estuary sediment. The freshwater samples were collected from the Waikato river (site 1 in Figure 3.4), and from an anaerobic pond (site 2 in Figure 3.4) in the North Island. The soil samples were collected from known PCP contaminated sites in the Canterbury region (site 3 in Figure 3.4), whereas the estuary sediment was sampled from Mapua, Waimea Inlet in the Nelson region (site 4 in Figure 3.4). To acquire the inoculum of the different sediments a technique was applied similar to techniques used by a number of authors (Quensen III et al. 1990); (Morris et al. 1992a); (Morris et al. 1992b); (Van Dort and Bedard 1991); (Ye et al. 1992).

By using micro organisms present in anaerobic sediments, from a variety of New Zealand environments under defined laboratory conditions, the aim was to determine whether the dechlorination of PCBs and DDE under strict anaerobic conditions was possible.

If the reductive dechlorination of PCBs is a co-metabolic process that does not require adaptation then all these sediments should be able to dehalogenate these chemicals under methanogenic conditions. This is because of the common eco-physiological pathways in anaerobic sediments and environments (Thiele et al. 1988); (Thiele 1988). Conversely, if the process of dehalogenation needs a long term adaptation or exposure of the micro organisms to the chemicals, the sediments used here should not be comparable to work obtained with highly contaminated sediments overseas. This is because these New Zealand sediments have had negligible exposure to PCBs.

The following conditions and controls were compared to determine the metabolic potential of the microbial attack on DDE, PCB-mix and Aroclor 1260 for the respective inocula (sediments and soils):

Condition 1 Incubation with carbon and energy sources, that have been shown to produce PCB dehalogenation in other sediments, alone (inoculated control).

Condition 2 Incubation with respective carbon and energy sources and environmentally significant levels of the respective organochlorines.

Condition 3 Incubation with respective carbon and energy sources and environmentally significant levels of the respective organochlorines but killed by autoclavation (autoclaved control).

Condition 4 Incubation with respective carbon and energy source without sediments but with environmentally significant levels of the respective organochlorines (un-inoculated control).

Growth and metabolism in condition 1 will prove that the conditions applied were conducive for growth and physiological metabolism of the respective inocula. A comparison of 2 and 3 will show, whether the respective inocula were capable of removing the respective organochlorines. Comparing 3 and 4 will indicate if there was any occurrence of unspecific, irreversible binding of the respective organochlorines to the cells in the culture. The importance of condition 3 is to show if there was any non-biological (= abiotic) removal of the respective organochlorines from the culture.

Condition 1 had four different carbon and energy sources and six different inocula, which amounts to 24 cultures.

Condition 2 and 3 had four different carbon and energy sources, six different inocula and three different organochlorines, which amounts to 72 cultures or controls each.

Condition 4 had duplicates of four different carbon and energy sources and three different organochlorines, which amounts to 24 controls.

To check the growth of the cultures, the head spaces of the inoculated controls (but not of the organochlorine spiked experiments) were monitored for produced methane gas every month. On the assumption that these controls would react the same way as the cultures spiked with organochlorine pollutants, all were fed with carbon and energy sources according to the methane production in the controls.

3.3 Materials and Methods

All culture and analytical techniques have been described in Megharaj and Strömpl (Megharaj et al. 1997b); (Strömpl and Thiele 1997) the only difference was the exclusion of Vitamin B12, because Vitamin B12 by itself is able to reductively dehalogenate PCBs (Assaf-Anid et al. 1992).

Organochlorines were injected to attain a final concentration of:

DDE (Riedel de Haën, AG, Seelze, Germany)		200 ppm
Aroclor 1260 (CHEM Service, West Chester, PA, USA)		200 ppm
PCB mix consisting of the congeners		
(all from Prochem GmbH, Wesel, Germany)		
3,3',4,4'-Tetratchlorobiphenyl	Ballschmitter number 77	20 ppm
2,2',4,4',6,6'-Hexachlorobiphenyl	Ballschmitter number 155	10 ppm
2,2',3,4,4',5,5'-Heptachlorobiphenyl	Ballschmitter number 180	5 ppm
2,2',3,3',4,4',5,5',6,6'-Decachlorobiphenyl	Ballschmitter number 209	20 ppm

These were dissolved in DCE (1,1-Dichloroethylene) as inert, non-degradable solvent and 0.3 ml of the specific mixture was injected with a glass syringe.

To test whether environmental contamination in the sediments could interfere with any reductive dehalogenation, the samples from known highly contaminated sources (Pulp & Paper Mill pond and Mapua) were also diluted to 1/10 of the inoculum size.

Gibson (Gibson and Sewell 1992) reported that some fermentation products, such as lactate, butyrate, ethanol, propionate, crotonate and toluene, stimulated dehalogenation activity of TCE in an anaerobic aquifer microcosm. In the work here presented, ethanol, butyric acid, ethanol/lactic acid mixtures and butyric acid/lactic acid mixtures were utilised as carbon and energy sources. The final concentration of the added carbon and energy sources was 100 mM, with an addition of 50 mM NaOH to buffer the pH, which was (at time point zero) between 7.2 and 7.0. After 3 months incubation the pH was stable, between 7.2 and 6.8. Unfortunately after 6 months the pH of a number of experiments had dropped to a pH range of 7.0 to 4.0**.** The DCE solvent had the advantage that it evaporated into the head-space of the pressure bottles at the incubating temperature minimising solvent interference.

All culture bottles were completely wrapped with aluminium foil, to assure that photochemical degradation, or photochemical induction of degradation were excluded. All bottles were incubated at 37˚ C and not shaken unless directly before sample taking. Samples of 4 ml were taken at time points zero, three and six months during the experiment. These samples were stored in glass vials with Teflon seals and screw caps and kept at -20° C in the dark prior to analysis.

From each sample a sub-sample was centrifuged. The pellet was used for duplicate protein analysis, using the Lowry Protein determination (Hartree 1972). The aqueous phase of the sub-sample was used for duplicate determinations of the carbon and energy sources concentrations (lactic acid, butyric acid and ethanol). Another portion of the sub-sample was used for chloroform extraction, to determine the organochlorine concentration by a reversed phase HPLC, and selected samples were further analysed with FID-GC and ECD-GC.

Evaluation of chromatograms for significant changes

Samples of each combination of inocula, carbon and energy sources and organochlorines for each condition (Condition 1 - 4) were extracted and duplicate reversed phase HPLC analysis for the time points zero and six months were undertaken. The obtained chromatograms were compared for changes occurrent in retention time, new peaks or shifts in the chromatographic profile. A high emphasis was placed on a shift of peaks from high retention times to low retention times, because this would have indicated dechlorination from higher chlorinated compounds to lower chlorinated compounds (see Figure 3.5). The same scrutiny was applied to all four conditions, and then Condition 2 and 3 were compared directly. A significant change indicating biological PCB transformation was defined as one that could be observed from time point zero to six months in Condition 2 but not in Conditions 1, 3 or 4 and with a significance level of $p = 0.05$ or 95% confidence for the student-t-test.

All conditions which produced significant changes in Condition 2 when compared with the appropriate controls from Condition 1, 3, and 4 were re-examined by extraction and subsequent analysis in duplicates for time point zero and six months with the FID-GC. This was also done for a number of non-significant changes and their respective controls to confirm the results from the reversed phase HPLC analysis.

For the experiments from Condition 2, with DDE as added organochlorine, the same scrutiny was applied for the FID-GC chromatograms as for the reversed phase HPLC analysis.

For the experiments from Condition 2, with PCB-mix as added organochlorine, in addition to the above procedure, the individual peak areas were standardised (in % of total peak area) for the four PCB congeners and compared with the respective control conditions by student-t-test. For Aroclor 1260 conditions the chromatograms were divided in three groups of peaks, peaks 1-30, peaks 31-40 and peaks 41-71, these groups were compared by student-t-test with the respective control conditions.

The detection limits for the reversed phase HPLC were lower than for the FID-GC, but the resolution of the profiles for the Aroclor's was higher using the capillary column FID-GC technique.

3.4 Results and Conclusions

For the DDE conditions two experiments which produced significant changes were found with the HPLC technique but could not be verified in further analysis. These were the Waikato sediments with Lactic acid/Ethanol and Mapua sediments with Lactic acid/Butyric acid as carbon and energy sources (see Table 3.1 and 3.2). The general increase in culture protein showed that only a few cultures were not active. Note that the p-value 0.002 for the reversed phase HPLC, experiment 50 is not highlighted. This peak was the DDE peak in the chromatogram and a higher concentration was extracted at time point six months that resulted in this p-value. With the findings of Megharaj and Strömpl (Megharaj et al. 1997a); (Megharaj et al. 1997b); (Strömpl and Thiele 1997) this result confirmed the recalcitrance of DDE towards microbial attack. Besides the work by Bumpus and Barr (Barr and Aust 1994); (Bumpus et al. 1993), the microbial degradation of DDE has not been shown. Even the aerobic attack by *Phanaerocheata chrysosporium* on DDE occurred at a low level and low rate. Based on the mechanism how the white rot fungus succeeded to degrade these chemicals one could come to the conclusion that the excreted enzymes and the resulting radicals point toward the third hypothesis, ie. an abiotic but biologically induced pathway.

Significant changes in congener profiles for the Aroclor 1260 experiments were found with all four different carbon and energy sources in the Waikato sediments (see tables 3.3 – 3.5). The increase in protein showed that all cultures were active. These changes were found in the first group of peaks 1-30, which represent lowly chlorinated peaks. Tables 3.4 and 3.5 show a pooling of data from inocula with similar carbon and energy sources. This was undertaken to retest these findings for convincing statistical significance. The expected removal of highly chlorinated congeners in the third or second group of peaks could not be observed. Therefore the data did not provide consistent evidence for reductive dechlorination of PCB in sediments from New Zealand. It has to be said that Aroclor 1260 dechlorination is rarely observed overseas and at slow rates, because the high degree of chlorination of each congener represents a difficult problem for microbial attack.

Significant changes for the PCB-mix experiments were found in all four different carbon and energy sources from the Mapua sediments, the Ethanol and Lactic Ethanol

experiments from the Waikato and the 1/10 diluted Mapua experiments (see tables 3.6 – 3.8). The increase in protein showed that all cultures were active. The changes found were in the relative proportion of the different peaks, no new peaks could be identified. Tables 3.7 and 3.8 show a pooling of data from inocula with similar carbon and energy sources. This was undertaken to retest these findings for convincing statistical significance.

A high rate reductive dechlorination as achieved with sediments from the Hudson river or other sediments exposed to PCBs over long term could not be shown in this work. Luckily New Zealand appears not to have a major problem with PCB contamination, but it does not answer the question which mechanism is responsible for the reductive dehalogenation of PCBs. DDE seems to be a even larger problem, because all attempts to break it down either with pure cultures at aerobic or anaerobic conditions, sequence batch aerobic/anaerobic conditions, or anaerobic conditions with sediments were not successful.

Table 3.1: Results of the analysis for DDE and new peaks conducted with various detection methods. + represents a significant change, (+)represents a change probably caused by biological material and not a change of the organochlorine, - represents no significant change.

	reversed phase HPLC	FID-GC	ECD-GC	GC-MS	Protein increase mg/L
Waikato Ethanol, DDE (49)	-	-			86.22
Waikato Lactic/Ethanol, DDE (50)	+	+	-		40.70
Waikato Butyric DDE (51)	-	-			65.62
Waikato Lactic/Butyric DDE (52)	-				77.53
Kinleith Ethanol, DDE (53)	-				261.93
Kinleith Lactic/Ethanol, DDE (54)	-				-
Kinleith Butyric DDE (55)	-				-
Kinleith Lactic/Butyric DDE (56)	-				48.75
Kinleith 1/10 Ethanol, DDE (57)	-				33.17
Kinleith 1/10 Lactic/Ethanol, DDE (58)	-				64.76
Kinleith 1/10 Butyric DDE (59)	-				-
Kinleith 1/10 Lactic/Butyric DDE (60)	-				16.60
Mapua Ethanol, DDE (61)	-				625.14
Mapua Lactic/Ethanol, DDE (62)	-				24.91
Mapua Butyric DDE (63)	-	-			52.99
Mapua Lactic/Butyric DDE (64)	+	+	+	-	1460.26
Mapua 1/10 Ethanol, DDE (65)	-				45.62
Mapua 1/10 Lactic/Ethanol, DDE (66)	-				-
Mapua 1/10 Butyric DDE (67)	-				-
Mapua 1/10 Lactic/Butyric DDE (68)	-				82.36
PCP-sites Ethanol, DDE (69)	-				57.54
PCP-sites Lactic/Ethanol, DDE (70)	(+)				110.35
PCP-sites Butyric DDE (71)	-				93.66
PCP-sites Lactic/Butyric DDE (72)	-				73.61

Table 3.2: Results from the student-t-test comparing chromatograms of experiments spiked with DDE. The peak areas at the same retention time were compared for duplicate chromatograms from time point zero and time point six months. The significant changes are highlighted and the significance level for the student-t-test was set at p = 0.05 or at a 95% confidence interval.

	reversed phase HPLC p values	FID-GC p-values
Waikato Ethanol, (49)	0.060	0.126
Waikato Lactic/Ethanol, (50)	0.002	0.046
Mapua Lactic/Butyric (64)	0.482	0.201
PCP-sites Ethanol, (69)	0.500	nd

Table 3.3: Results of analysis for Aroclor 1260 and changes in the chromatographic profile conducted with two detection methods. + represents a significant change, (+) represents a change probably caused by biological material and not a change of the organochlorine, - stands for no significant change, and -/+ represents significant changes in some part(s) but not the whole chromatogram.

	reversed phase HPLC	FID-GC	Protein increase mg/L
Waikato Ethanol, Aroclor 1260 (73)	**-**	**- / +**	21.65
Waikato Lactic/Ethanol, Aroclor 1260 (74)	**-**	**- / +**	60.97
Waikato Butyric Aroclor 1260 (75)	**-**	**- / +**	102.49
Waikato Lactic/Butyric Aroclor 1260 (76)	**+**	**- / +**	98.53
Kinleith Ethanol, Aroclor 1260 (77)	**-**		502.14
Kinleith Lactic/Ethanol, Aroclor 1260 (78)	**-**		367.99
Kinleith Butyric Aroclor 1260 (79)	**-**		262.39
Kinleith Lactic/Butyric Aroclor 1260 (80)	**-**		249.70
Kinleith 1/10 Ethanol, Aroclor 1260 (81)	**(+)**	**-**	49.05
Kinleith 1/10 Lactic/Ethanol, Aroclor 1260 (82)	**-**		39.61
Kinleith 1/10 Butyric Aroclor 1260 (83)	**-**		110.72
Kinleith 1/10 Lactic/Butyric Aroclor 1260 (84)	**-**		88.75
Mapua Ethanol, Aroclor 1260 (85)	**-**	**-**	940.26
Mapua Lactic/Ethanol, Aroclor 1260 (86)	**-**	**-**	70.10
Mapua Butyric Aroclor 1260 (87)	**-**	**-**	68.58
Mapua Lactic/Butyric Aroclor 1260 (88)	**-**	**-**	27.65
Mapua 1/10 Ethanol, Aroclor 1260 (89)	**-**	**-**	58.61
Mapua 1/10 Lactic/Ethanol, Aroclor 1260 (90)	**(+)**	**-**	20.24
Mapua 1/10 Butyric Aroclor 1260 (91)	**-**	**-**	92.61
Mapua 1/10 Lactic/Butyric Aroclor 1260 (92)	**-**	**-**	64.53
PCP-sites Ethanol, Aroclor 1260 (93)	**-**		119.78
PCP-sites Lactic/Ethanol, Aroclor 1260 (94)	**-**		151.77
PCP-sites Butyric Aroclor 1260 (95)	**-**		152.86
PCP-sites Lactic/Butyric Aroclor 1260 (96)	**-**		113.58

Tables 3.4: Results from the student-t-test comparing chromatograms of experiments spiked with Aroclor 1260. The peak areas were grouped into three groups as defined by the Aroclor 1260 standards and compared for duplicate chromatograms from time point zero and time point six months. To increase the statistical significance, treatments with similar carbon and energy sources were grouped (eg Ethanol combined with Ethanol/Lactic, and Butyric combined with Butyric/Lactic) in comparison with the respective autoclaved controls. The significant changes are highlighted and the significance level for the student-t-test was set at p = 0.05 or at a 95% confidence level.

Aroclor 1260 FID-GC peak number	Waikato Ethanol (73) - Waikato Lactic/Ethanol (74)	Autoclaved Controls
1 - 30	***0.01***	0.44
31 - 40	0.97	0.73
41 -71	0.48	0.50

Aroclor 1260 FID-GC peak number	Waikato Butyric (75) - Waikato Lactic/ Butyric (76)	Autoclaved Controls
1 - 30	***0.03***	0.25
31 - 40	072	0.90
41 -71	0.28	0.50

Aroclor 1260 FID-GC peak number	Mapua Ethanol (85) - Mapua Lactic/Ethanol (86)	Autoclaved Controls
1 - 30	0.27	0.47
31 - 40	1. 00	0.10
41 -71	0.29	0.12

Tables 3.5: Results from the student-t-test comparing chromatograms of experiments spiked with Aroclor 1260. The peak areas were grouped into three groups as defined by the Aroclor 1260 standards and compared for duplicate chromatograms from time point zero and time point six months. To increase the statistical significance, treatments with similar carbon and energy sources were grouped (eg Ethanol combined with Ethanol/Lactic, and Butyric combined with Butyric/Lactic) in comparison with the respective autoclaved controls. The significant changes are highlighted and the significance level for the student-t-test was set at $p = 0.05$ or at a 95% confidence level.

Aroclor 1260 FID-GC peak number	Mapua Butyric (87) - Mapua Lactic/ Butyric (88)	Autoclaved Controls
1 - 30	0.15	0.24
31 - 40	0.68	0.37
41 -71	0.26	0.08

Aroclor 1260 FID-GC peak number	Mapua 1/10 Ethanol (89)- Mapua 1/10 Lactic/Ethanol (90)	Autoclaved Controls
1 - 30	0.75	0.83
31 - 40	0.81	0.75
41 -71	0.92	0.84

Aroclor 1260 FID-GC peak number	Mapua 1/10 Butyric (91) - Mapua 1/10 Lactic/ Butyric (92)	Autoclaved Controls
1 - 30	0.20	0.42
31 - 40	011	0.40
41 -71	0.74	0.53

Table 3.6: Results of analysis for the PCB-mix and new peaks conducted with two detection methods. + represents a significant change, (+)represents a change probably caused by biological material and not a change of the organochlorine, - represents no significant change, and -/+ represents significant changes in some part (s) but not the whole chromatogram.

	reversed phase HPLC	FID-GC	Protein increase mg/L
Waikato Ethanol, PCB mix (97)	-	- / +	646.99
Waikato Lactic/Ethanol, PCB mix (98)	+	- / +	118.72
Waikato Butyric PCB mix (99)	+	-	55.20
Waikato Lactic/Butyric PCB mix (100)	-	-	101.57
Kinleith Ethanol, PCB mix (101)	-		1028.82
Kinleith Lactic/Ethanol, PCB mix (102)	-		104.66
Kinleith Butyric PCB mix (103)	-		980.37
Kinleith Lactic/Butyric PCB mix (104)	-		237.30
Kinleith 1/10 Ethanol, PCB mix (105)	-		94.22
Kinleith 1/10 Lactic/Ethanol, PCB mix (106)	-		40.36
Kinleith 1/10 Butyric PCB mix (107)	-		70.52
Kinleith 1/10 Lactic/Butyric PCB mix (108)	-		35.99
Mapua Ethanol, PCB mix (109)	-	- / +	22.36
Mapua Lactic/Ethanol, PCB mix (110)	+	- / +	18.26
Mapua Butyric PCB mix (111)	(+)	- / +	1491.57
Mapua Lactic/Butyric PCB mix (112)	-	- / +	27.51
Mapua 1/10 Ethanol, PCB mix (113)	-	- / +	16.52
Mapua 1/10 Lactic/Ethanol, PCB mix (114)	-	- / +	23.91
Mapua 1/10 Butyric PCB mix (115)	-	-	1696.60
Mapua 1/10 Lactic/Butyric PCB mix (116)	+	-	55.76
PCP-sites Ethanol, PCB mix (117)	-		166.11
PCP-sites Lactic/Ethanol, PCB mix (118)	-		896.73
PCP-sites Butyric PCB mix (119)	-		200.07
PCP-sites Lactic/Butyric PCB mix (120)	-		96.55

Tables 3.7: Results from the student-t-test comparing chromatograms of experiments spiked with the PCB-mix. The peak areas were compared for duplicate chromatograms from time point zero and time point six months. To increase the statistical significance, treatments with similar carbon and energy sources were grouped (eg Ethanol combined with Ethanol/Lactic, and Butyric combined with Butyric/Lactic) in comparison with the respective autoclaved controls. The significant changes are highlighted and the significance level for the student-t-test was set at p = 0.05 or at a 95% confidence level.

Ballschmitter PCB congener number	Waikato Ethanol (97)- Waikato Lactic/Ethanol (98)	Autoclaved Controls
77	0.293	0.256
155	***0.002***	0.979
180	***0.019***	0.651
209	***0.014***	0.533

Ballschmitter PCB congener number	Waikato Butyric (99)- Waikato Lactic/ Butyric (100)	Autoclaved Controls
77	0.939	0.105
155	0248	0.990
180	0.979	0.117
209	***0.014***	0.152

Ballschmitter PCB congener number	Mapua Ethanol (109)- Mapua Lactic/Ethanol (110)	Autoclaved Controls
77	***0.048***	0.343
155	***0.022***	0.884
180	0.330	0.687
209	0.888	0.669

Tables 3.8: Results from the student-t-test comparing chromatograms of experiments spiked with the PCB-mix. The peak areas were compared for duplicate chromatograms from time point zero and time point six months. To increase the statistical significance, treatments with similar carbon and energy sources were grouped (eg Ethanol combined with Ethanol/Lactic, and Butyric combined with Butyric/Lactic) in comparison with the respective autoclaved controls. The significant changes are highlighted and the significance level for the student-t-test was set at p = 0.05 or at a 95% confidence level.

Ballschmitter PCB congener number	Mapua Butyric (111)- Mapua Lactic/ Butyric (112)	Autoclaved Controls
77	0.922	0.071
155	***0.024***	0.337
180	0064	0.530
209	0.073	0.329

Ballschmitter PCB congener number	Mapua 1/10 Ethanol (113)- Mapua 1/10 Lactic/Ethanol (114)	Autoclaved Controls
77	***0.029***	0.330
155	***0.002***	0.583
180	0.057	0.320
209	0.133	0.862

Ballschmitter PCB congener number	Mapua 1/10 Butyric (115)- Mapua 1/10 Lactic/ Butyric (116)	Autoclaved Controls
77	0.238	0.363
155	0.751	0.264
180	0.224	0.841
209	0.725	0.897

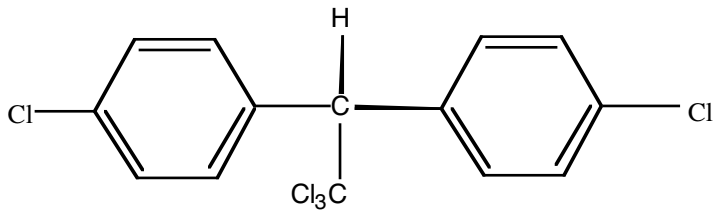

Figure 3.1: 1,1,1-trichloro-2,2-bis (p-chlorophenyl)ethane (DDT)

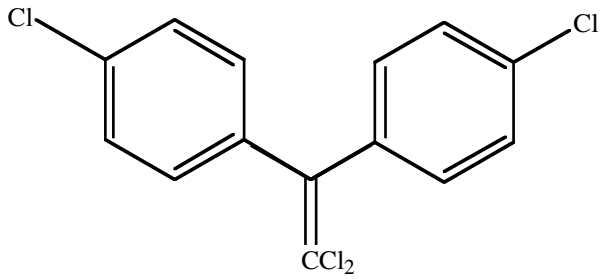

Figure 3.2: 1,1-bis (p-chlorophenyl)-2-dichloroethylene (DDE)

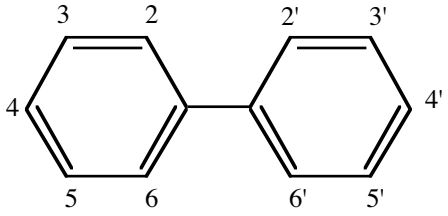

Figure 3.3: Biphenyl structure with all ten possible sites for chlorine attachment

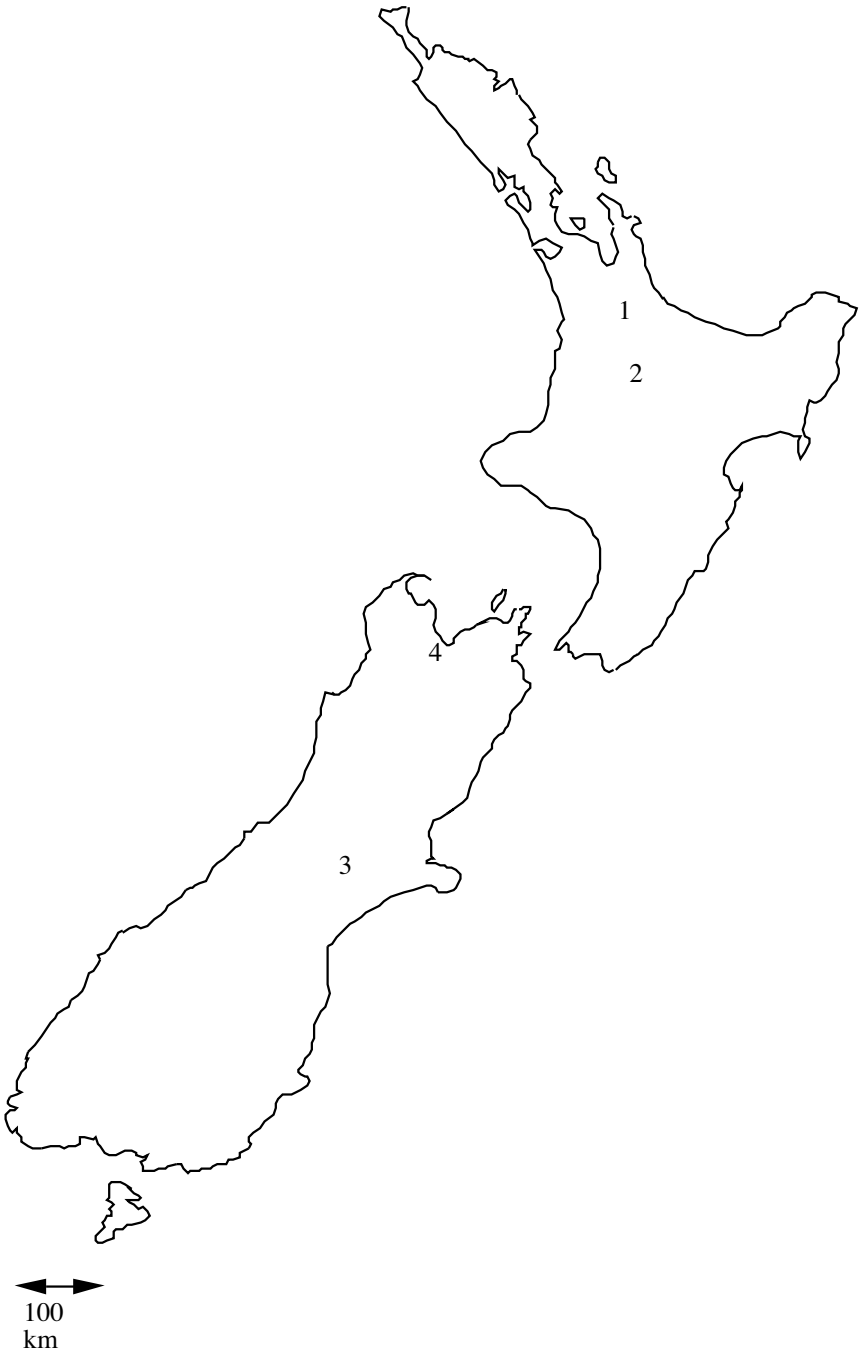

Figure 3.4: Map of New Zealand with sampling sites.

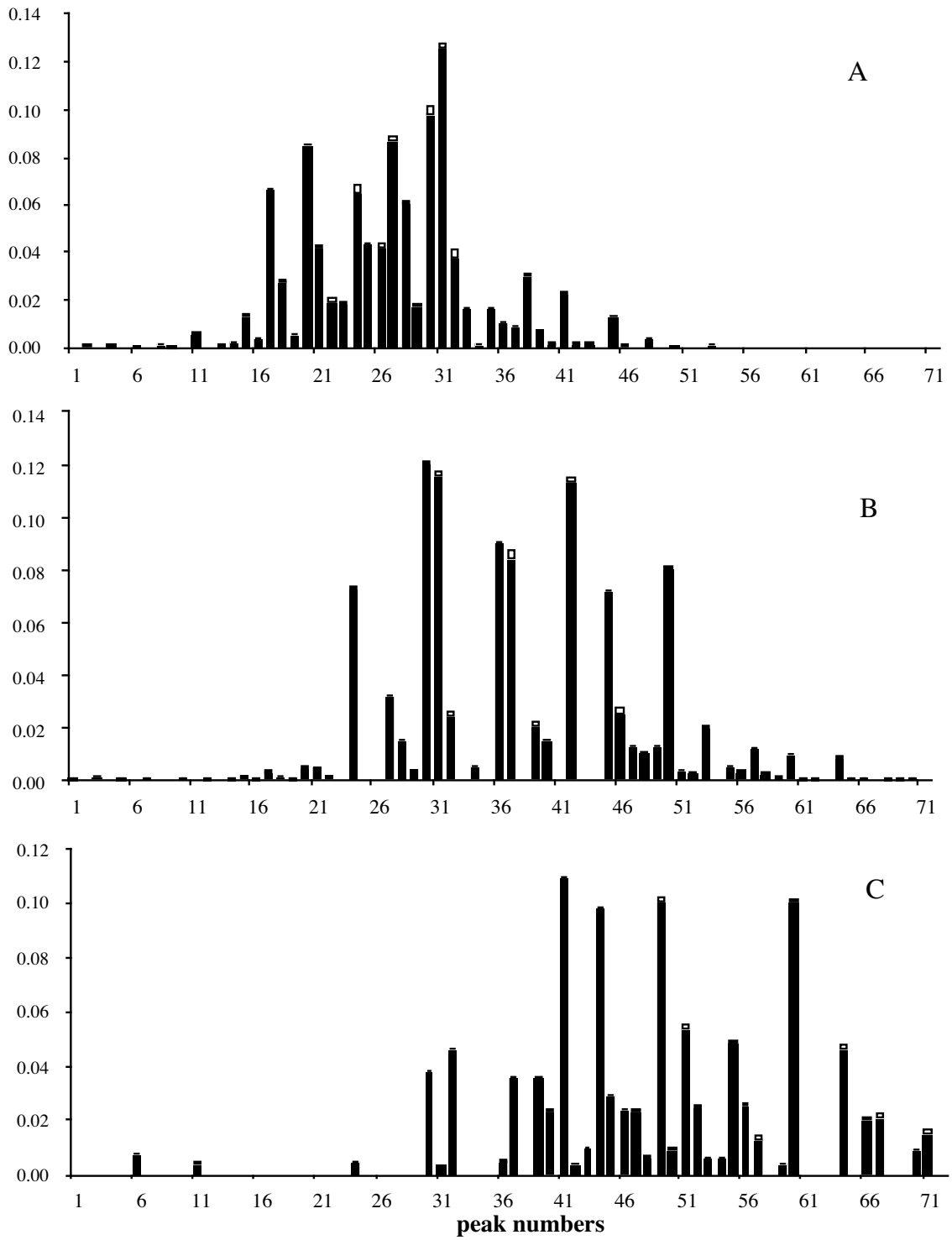

Figure 3.5: Triplicate 1000 ppm Aroclor 1248 standards with standard deviation (A), triplicate 1000 ppm Aroclor 1254 standard with standard deviation (B), and triplicate 1000 ppm Aroclor 1260 standard with standard deviation (C). The value for each peak is expressed as parts per thousand of the sum of all peak areas in each separate chromatogram. The peak number was given according to the retention time of peaks found in the Aroclor standards 1260, 1254 and 1248.

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4 Granulation of Saale- and Waikato-River-Sediments in UASB Reactors

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4.1 Introduction

One of the anaerobic systems to treat waste water is the concept of the Upflow Anaerobic Sludge Blanket (UASB)reactors. It has been used successfully around the world for different types of waste waters, at a variety of COD levels, with a number of different temperature- and pH-conditions and with different process regimes. UASB's were first designed and operated at the Agricultural University of Wageningen in 1987 (Zeeuw de, 1987). The concept was aimed at treating low and medium strength waste water at high volumetric loading rates. The core of the high performance of these type of waste water treatment is always the granule or pellet, which allows a high loading rate and good settling abilities, because of its dense bacterial biomass. Weimin (Weimin *et al.*, 1987) reviewed the different seed sources utilised by a variety of researchers so far. Most conveniently granules were used to start up new UASB reactors, up to 10 - 30% of the reactor volume is required as inoculum (Hickey *et al.*, 1991). The advantage of utilising granules as inoculum is, that the start up time for a new waste water composition is reduced. Fully grown granules are not always readily available and instead digested sewage sludge, cow manure and sewer slurry have been used. But all seed sources have the same problem, that they are not neccessarily readily available all around the world. Weimin (Weimin *et al.*, 1987) tested therefore the applicability of aerobic activated sludge as seed. In this work anaerobic river sediments have been utilised as inoculum source. These originated from the river Saale in Germany and the river Waikato in New Zealand.

4.2 Materials and Methods

Bacterial protein was determined colorimetically (Hartree, 1972). The analytical methods for the Volatile Fatty Acid determination and the Gas analysis for N_2, CO_2 and CH_4 has been described previously in Strömpl (Strömpl and Thiele, 1996). For the biodegradation potential experiments anaerobic conditions were applied, the methods are described in Megharaj (Megharaj *et al.*, 1997).

Phenol and benzoic acid were assayed by taking 1 ml samples of the bacterial culture suspensions using syringes and hypodermic needles. Of these samples 200 µl sub-samples were injected into a system composed of two LC1500 HPLC pumps, an LC1600 auto sampler, and a LC1200 variable wavelength UV-visible detector (ICI Ltd., Australia). The HPLC system was interfaced to a computer using a DP800 data interface (ICI Ltd., Australia). The detector was operated at a wavelength (l) of 230 nm. The column utilised was 250 x 4.6 mm C-8 reversed phase column packed with 10 µm Econosil (Alltech Associates Inc., IL, USA). The column was operated at ambient room temperature with 10 times 4.6 mm C-8 guard column packed with 10 µm Econosil (Alltech Associates Inc., IL, USA). For the separation of phenol and benzoic acid a linear gradient of 10 % methanol was applied for 2 min and rose then to 60 % methanol in 4 min and to 100 % methanol in 1 min. The linear gradient of 100 % methanol was applied for 1 min and decreased to 10 % within 1 min, which was held for 1 more min. Individual compounds were identified from chromatograms on the basis of peak retention time by comparison with those obtained using a mixed standard with previously determined retention times and (DP800 version 2.0 software, 1989 ICI Australia). To compensate for loss of column integrity (the margin of error in the column), phenol- and benzoic acid-standards were analysed each time the system was used for analysis..

Preparation of media for the cultivation of anaerobic bacteria in Upflow Anaerobic Sludge Blanket (UASB) reactors

Before the sediments from the Saale and Waikato rivers were placed into the reactors they were sieved through a mesh to free them of larger plant material and sand particles. The Saale sediment had been stored at 4° C for one year. 500 ml of each sediment were used as inoculum, then the reactor was filled completely with start up medium and flushed with nitrogen for 30 minutes. The reactor content was pumped in a recycle loop

for 24 hours and when the first gas production could be registered the medium was switched over to PPBM-medium.

Two UASB reactors were operated with sediments from the Saale River, Germany, and the Waikato River, New Zealand. Both sediments were taken down stream from Pulp and Paper Mill plants and municipal landfills with leachate discharge. The Saale sediment had been stored in the cold room at 4° C for one year prior to use.

Size distribution for UASB granules and river sediments

Samples of the reactor were taken from the bottom and river sediment samples were taken from the storage container. These were spread in a Petrie dish and if necessary diluted with water. As a standard a dice with 5 mm length was added. Photos were taken of this arrangement and then scanned into a computer. The scans were processed with the program "Image" to analyse size distribution of particles or granules in the samples.

Biodegradation potential for UASB granules and river sediments

Five mililiter samples of the sludge were taken from the bottom of the reactor and five mililiter river sediment samples were taken from a storage container. These were disintegrated and inoculated into pressure bottles with anaerobic PPBM medium. Two mililiter of this suspension was used as inoculum for biodegradation potential experiments. The different suspensions of granules and river sediments were inoculated with a variety of carbon and energy sources and incubated at 37° C in a water bath. Samples were taken every hour for 6 hours and were analysed for bacterial protein and the energy and carbon sources. The energy and carbon sources and their final concentrations in the culture bottles were:

Acetic acid	30 mM
Lactic acid	10 mM
Propionic aid	10 mM
Benzoic acid	10 mM
Phenol	10 mM

Scanning electron microscopy

I thank Richard Easingwood from the Department of Microbiology, University of Otago for obtaining all of the Scanning Electron Micrographs. The procedure to prepare the granules was described elsewhere (Thiele *et al.*, 1990).

Experimental design

Two reactors were operated with sediments from the Saale river, Germany, and the Waikato river, New Zealand. Both sediments were obtained down stream from Pulp and Paper Mill plants. The two experiments for the granulation of river sediments were undertaken to determine the possible use of river sediments for the inoculation of UASB reactors to obtain granules. A feeding regime utilising high strength waste water, with lactic acid as main carbon and energy source, and low volumetric loading rates was applied.

After the start up procedure a time period had to be taken in account for the settling and stabilising of each reactor. The following parameters were measured regularly:

- date / time	
- pH internal	
- pH external	
- total effluent	L/d
- CH_4	%(v/v)
- CO_2	%(v/v)
- gas meter reading	L/d
- VFAs(volatile fatty acids) effluent	mM
- protein effluent	g/L
- total reactor protein	g/reactor
- samples of the bottom of the reactor	

4.3 Results

The following graphs are showing these results and give an indication for the event of granulation. Light microscopy and fluorescent light microscopy was used to establish that there were no granules in the sediments used for inoculation and that the granules were the places of active methanogenesis.

Over the time period of maintaining the experiment samples of the bottom of the reactors were taken regularly. These samples were in some cases used for size distribution analysis. In the majority of cases these samples were simply analysed under a light microscope or binocular for signs of granules. The findings show the increase in size of granules to correlate to wash out events shown in Figure 4.4 and 4.10. For UASB Saale the wash out occurred at 2300h and for UASB Waikato at 4200h. One can find at these times, a negative yield in the total protein production in relation to the accumulative protein wash out. Beforehand only pregranules, were found but granules shown as in Figure 4.5 – 4.7 and 4.11 – 4.13 were only obtained after the wash out. Pregranules were detected from 1000h in UASB Saale and 2300h in UASB Waikato onwards, this coincided in both cases with a decrease in the HRT (hydraulic retention time).

This work was designed to determine the use of anaerobic river sediments as source of inoculum to produce granules in an UASB reactor. The feed used for this design was mainly lactic acid at a high COD level, 21.8 gCOD/L. The feed consumption was regulated by pH-auxostate mode. The volumetric loading rate was kept at low levels, for UASB Saale at about 0.005 gCOD/L and UASB Waikao at about 0.003 gCOD/L.

Figure 4.1 for UASB Saale and Figure 4.2 for UASB Waikato show the comparison of the original sediments with the granules obtained from these sediments at the end of each experiment. Under the microscope it was possible to determine that the original sediments had no granules whatsoever and that the particles seen there were mainly parts of plant material and sand particles. The granules consisted of dense biomass, which was determined by fluorescent light microscopy and protein determination. The granules can be seen in the EM-photos Figures 5-7 for UASB Saale and Figures 8-10 for UASB Waikato. A shift from total suspended solids (TSS) to volatile suspended solids (VSS) and an increase in the protein content from each sediment to the according granules was recorded. This is an indication for the wash out of inert material from the reactor and an

increase in active biomass. These different methods combined established that a successful granulation had occurred.

Figures 4.5-4.7 show the granules obtained from UASB Saale. Figure 4.6 and 4.7 show close ups from the granule surface. One can see in Figure 4.6 Methanothrix like bacteria and in Figure 4.7 Methanosarcina like bacteria, the first type was predominant. Figures 4.8-4.10 shows the granules from UASB Waikato. The close ups in figures 4.9 and 4.10 show a similar picture as for UASB Saale. Figure 4.9 shows Methanothrix like bacteria (long filamentous bacteria), relative short specimen were predominant. Figure 4.10 shows Methanosarcina like bacteria. In both cases it can be noticed that at the end of the experiment the bulk of the bacteria was found at the bottom of the reactor. The ER photos were taken from samples which originated also from this location. Both findings together allow the conclusion that a granulation has occurred successfully.

The biodegradation potential of the original sediments was compared against the obtained granules in Table 4.1 for UASB Saale and in Table 4.2 for UASB Waikato. The t-test with a significance level of 5% showed that there was a significant increase of biodegradation potential for lactic acid and propionic acid for UASB Saale. All other carbon and energy sources for UASB Saale and all tested carbon and energy sources for UASB Waikato showed no significant difference. The total reactor protein showed an overall increase of bacterial protein in both experiments. With other words the bacteria existing in the sediment were grown to a higher cell density. Even if the biodegradation potential did not increase for the granules, the higher cell density would grant a faster degradation of the feed or specific carbon and energy sources.

4.4 Discussion and Conclusions

This work was designed to determine the use of anaerobic river sediments as source of inoculum to produce granules in an UASB reactor. The feed used for this design was mainly lactic acid at a high COD level, 21.8 gCOD/L. The feed consumption was regulated by pH-auxostate mode. The volumetric loading rate was kept at low levels, for UASB Saale at about 0.005 gCOD/L and UASB Waikato at about 0.003 gCOD/L.

Wiegant (Wiegant, 1987) proposed a negative selection against Methanosarcina like bacteria, to favour Methanothrix like bacteria, which would result in stronger granules. Colleran (Colleran, 1987) in his review found that Wiegant did not exceed a 200mg/L acetic acid when selecting for Methanothrix, which equals 3.333 mM. Grotenhuis (Grotenhuis *et al.*, 1991c) found a threshold level for acetic acid for Methanosarcina of 0.2 mM and Methanothrix of 0.01 mM. Below these levels no growth of the bacterial species would occur. Wu (Wu *et al.*, 1993) found threshold levels of 0.4 - 1.0 mM for acetic acid for Methanosarcina and below 0.01 mM for acetic acid for granules predominated by Methanothrix. In this work the average level for acetic acid for UASB Waikato was about 2.5 mM and 6-8 mM of acetic acid for UASB Saale. In the case of UASB Waikato the levels were below the value indicated by Wiegant but above the values stated by the other researchers. The acetic acid concentration level for UASB Saale was higher than the threshold levels at all times. Nevertheless the observation by electron microscopy was made that Methanothrix like bacteria were predominating the granules obtained from river sediments.

The biodegradation potential of the original sediments was compared against the obtained granules in Table 4.1 for UASB Saale and in Table 4.2 for UASB Waikato. The t-test with a significance level of 5% showed that there was a significant increase of biodegradation potential for lactic acid and propionic acid for UASB Saale. All other carbon and energy sources for UASB Saale and all tested carbon and energy sources for UASB Waikato showed no significant difference. The total reactor protein, figures 3 and 4, showed an overall increase of bacterial protein in both experiments. With other words the bacteria existing in the sediment were grown to a higher cell density. Even if the biodegradation potential did not increase for the granules, the higher cell density would grant a faster degradation of the feed or specific carbon and energy sources.

Weimin (Weimin et al., 1987) pointed out that the use of other seed material than granules might be a major option for countries where access to granules is difficult. Here we took this idea even further by using a different loading scheme to the one proposed by Lettinga and gained granules from anaerobic river sediments. These sediments originated from the river Saale in Germany and the river Waikato in New Zealand. By using river sediments as seed source one might be able to combine two advantages. First anaerobic river sediments are readily available if a slow flowing river or backwater of a river can be located. Secondly, if there is a pollutant influx into the river (eg. from pulp and paper industries, sugar refineries, communal waste water discharges) bacteria in an anaerobic river sediment downstream of this pollution input might be adapted to this particular contamination. In the literature the biological break down of certain chemical pollutants has only been described for anaerobic bacteria. One example would be the anaerobic reductive dehalogenation of PCBs by bacteria from Hudson river sediments under methanogenic conditions. It is not fully understood whether this process is a catabolic pathway, for which a selection should be possible, or if it is a cometabolic pathway, for which selection is not possible. If such a river sediment would be granulated, a higher cell density of these bacteria would be obtained, and either of the above mentioned possibilities would gain from a higher cell density. In addition anaerobic river sediments are readily available if a low flowing river or backwater of a river can be located.

The conclusion of this work is that it was possible to granulate two different river sediments in UASB reactors. The main feed source was lactic acid and the reactor was run in a pH-auxostate mode. The feeding regime applied consisted of a high strength waste water, a low volumetric loading rate, and a high superficial loading rate with a recycle of the effluent. In both reactors granules were found after a negative growth yield occurred.

The findings show the increase in size of granules to correlate to wash out events shown in Figures 3 and 4. For UASB Saale the wash out occurred at 2300h and for UASB Waikato at 4200h. One can find at these times a negative yield in the total protein production in relation to the accumulative protein wash out. Beforehand only pregranules, a term used by other researchers, were found but granules shown as in Figures 5-7 and 8-10 were only obtained after the wash out. Pregranules were detected from 1000h in UASB Saale and 2300h in UASB Waikato onwards.

These results have intiated further research within the Waste Technology Research Group, Department of Microbiology, University of Otago. The results will be published in conjunction with J. H. Thiele in a scientific magazine in 2002.

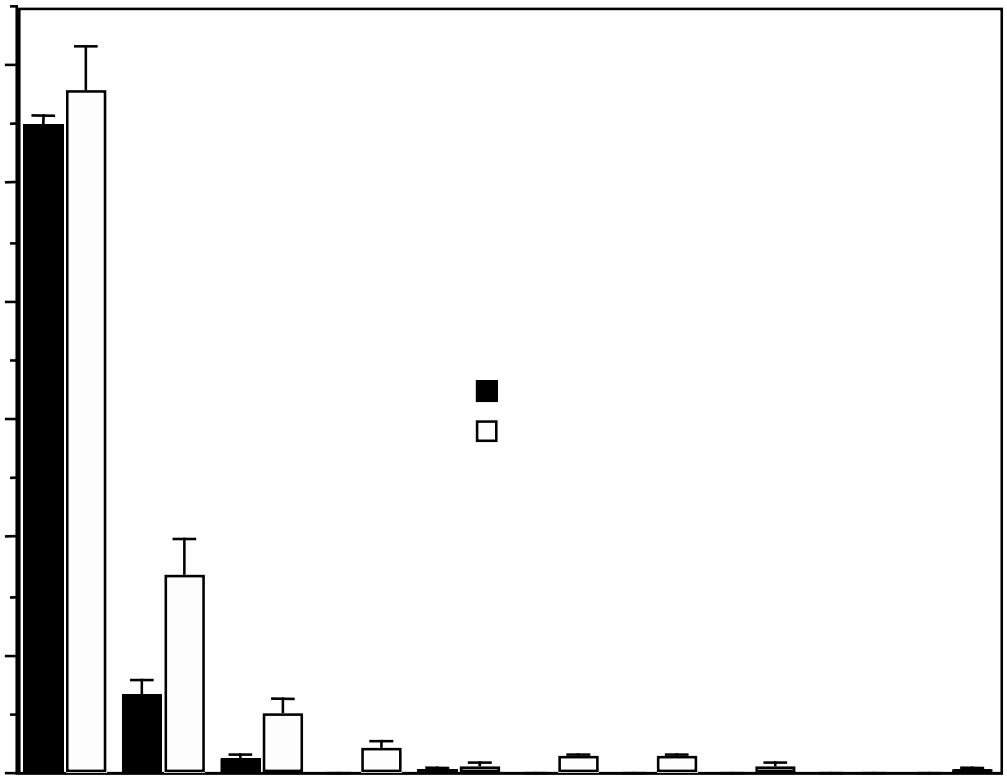

Figure 4.1: Size distribution, comparison between Saale sediment and UASB Saale sludge bed contests. These data represent particle ranges from 0 to 2.5 mm diameter. Here the original sediment was compared against the granules obtained after 4500 h of operating UASB Saale. The particles found in the original sediment were mainly parts of plants and sand particles, whereas the granules consisted of active bacterial biomass. Over the time of the experiment a shift from smaller particles to larger granules and an overall increase in the number of granules can be identified.

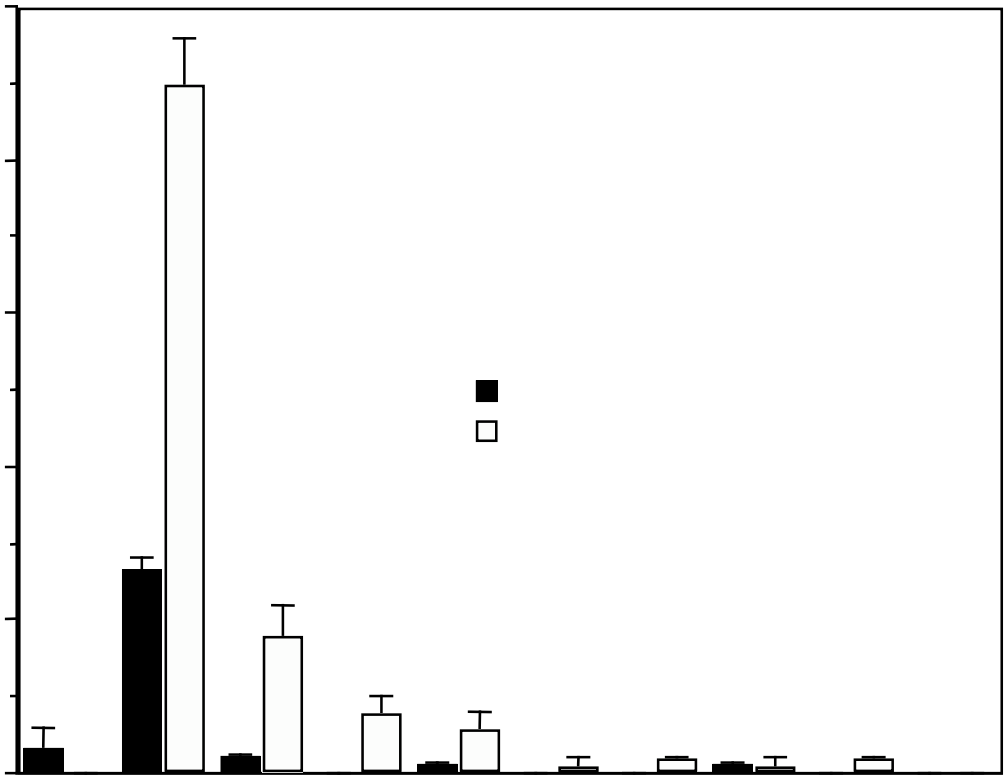

Figure 4.2: Size distribution, comparison between Waikato sediment and UASB Waikato sludge bed contest. These data represent particle ranges from 0 to 2.5 mm diameter. Here the original sediment was compared against the granules obtained after 6200 h of operating UASB Waikato. The particles found in the original sediment were mainly sand particles, whereas the granules consisted of active bacterial biomass. Over the time of the experiment a shift from smaller particles to larger granules and an overall increase in the number of granules can be identified.

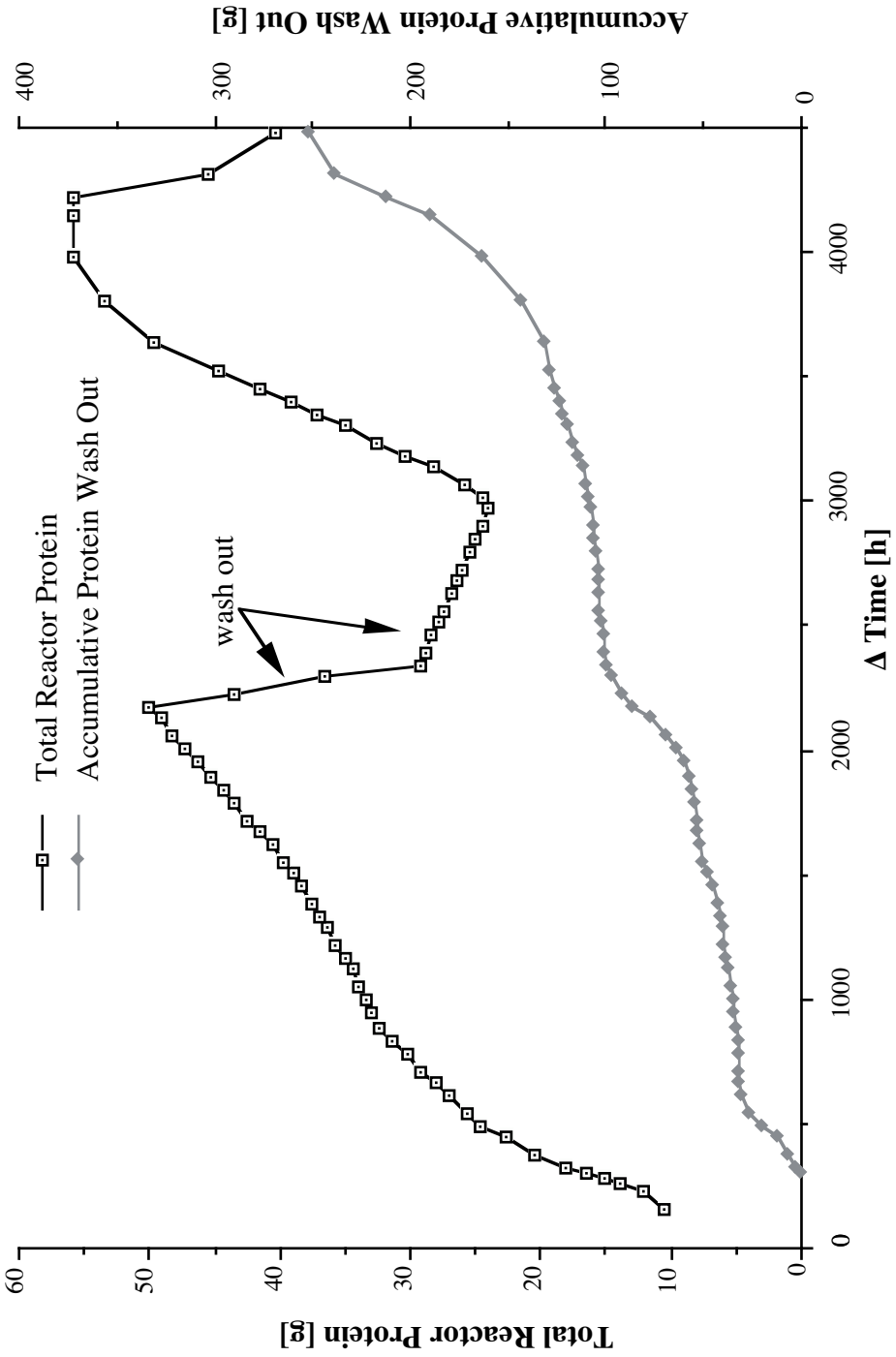

Figure 4.3: Total reactor protein and accumulative protein wash out of UASB Saale over time. The total reactor protein shows the fluctuations of bacterial protein caused by a major wash out at 2300 h and a slow wash out period from 2300 to 3000 h. Pregranules were found at about 1000 h, the first granules were found after 3000 h. The accumulative protein wash out shows how much bacterial protein was discard from the reactor.

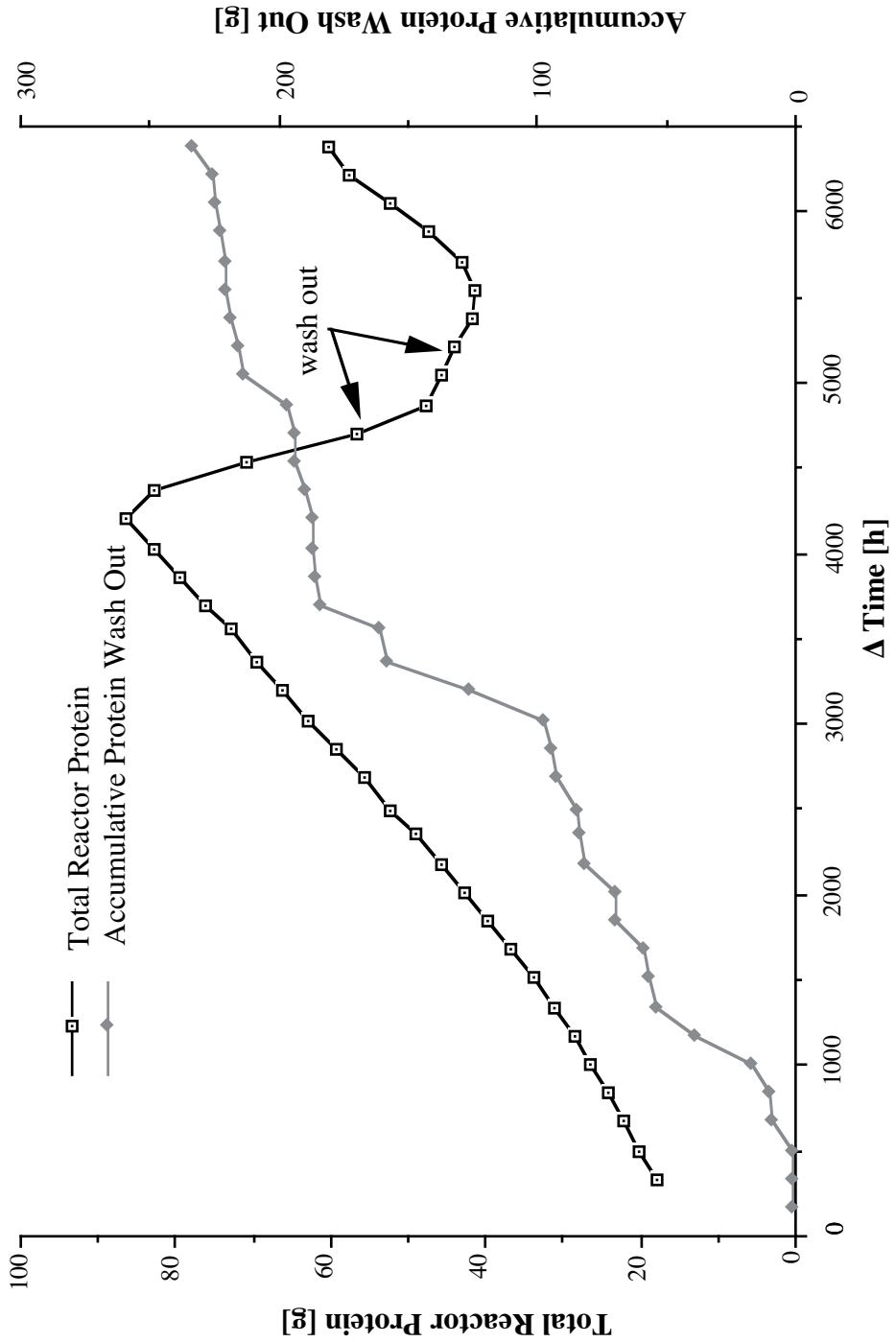

Figure 4.4: Total reactor protein and accumulative protein wash out of UASB Waikato over time. The total reactor protein shows the fluctuations of bacterial protein caused by a major washout at 4200 h and a slow wash out period from 4200 to 5400 h. Pregranules were found at about 2500 h, the first granules were found after 5400 h. The accumulative protein wash out shows how much bacterial protein was discard from the reactor.

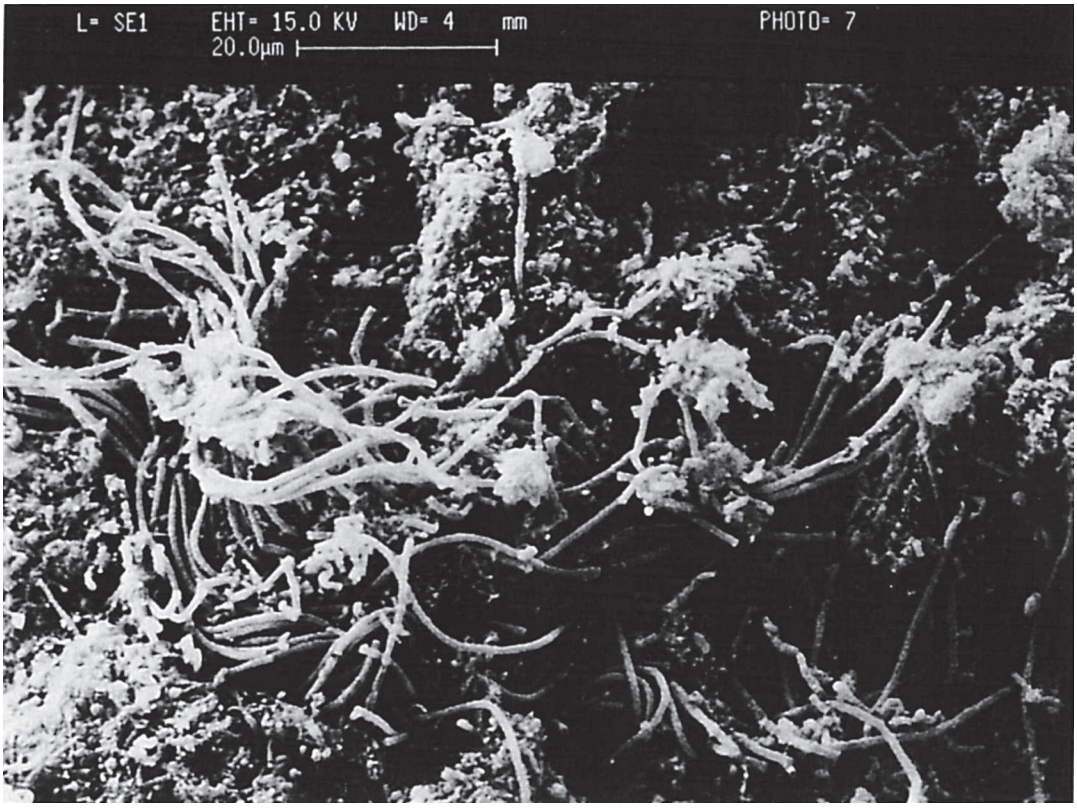

Figure 4.5: UASB Saale ER photo 2 granule

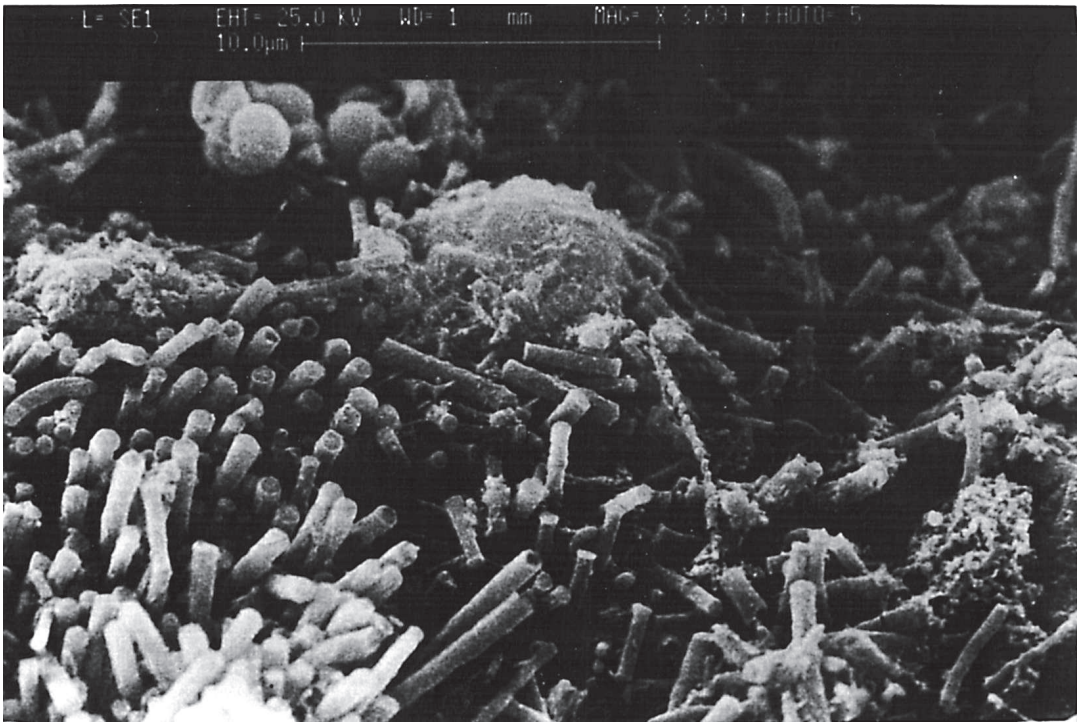

Figure 4.6: UASB Saale ER photo 5 granule surface close up, Methanothrix like bacteria

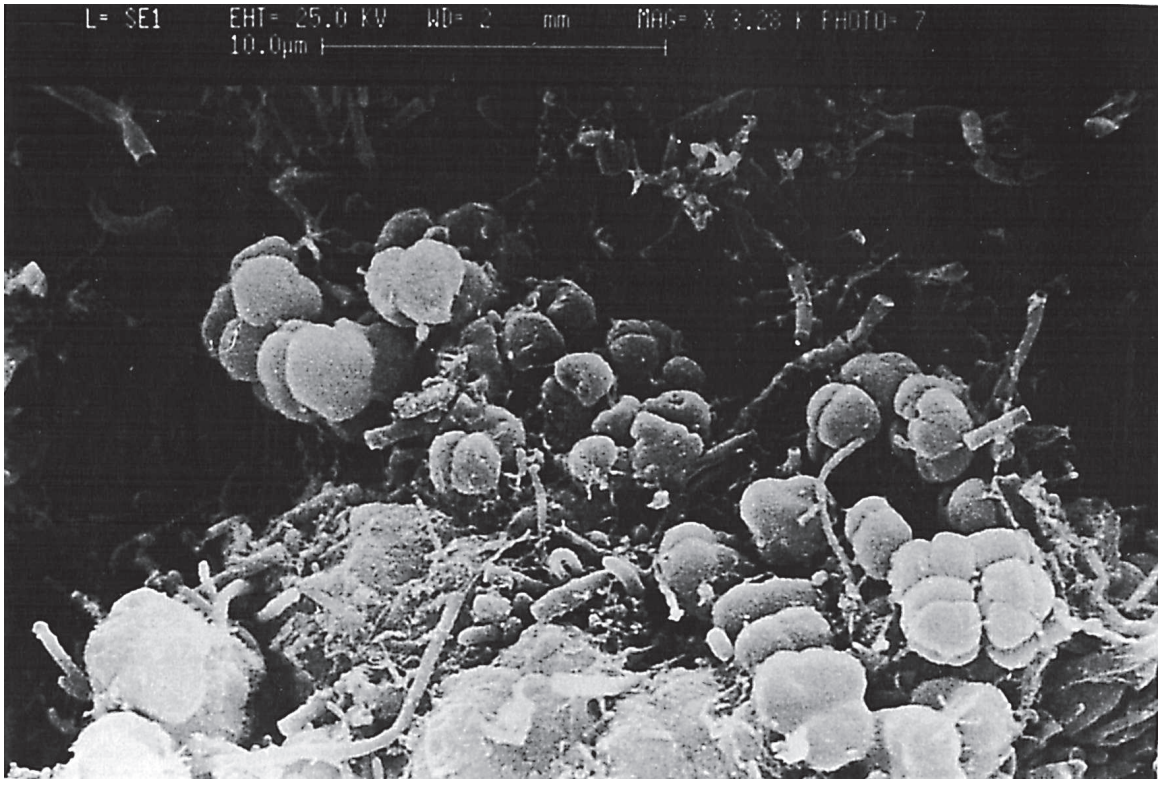

Figure 4.7: UASB Saale ER photo 7 granule surface close up, Methanosarcina like bacteria

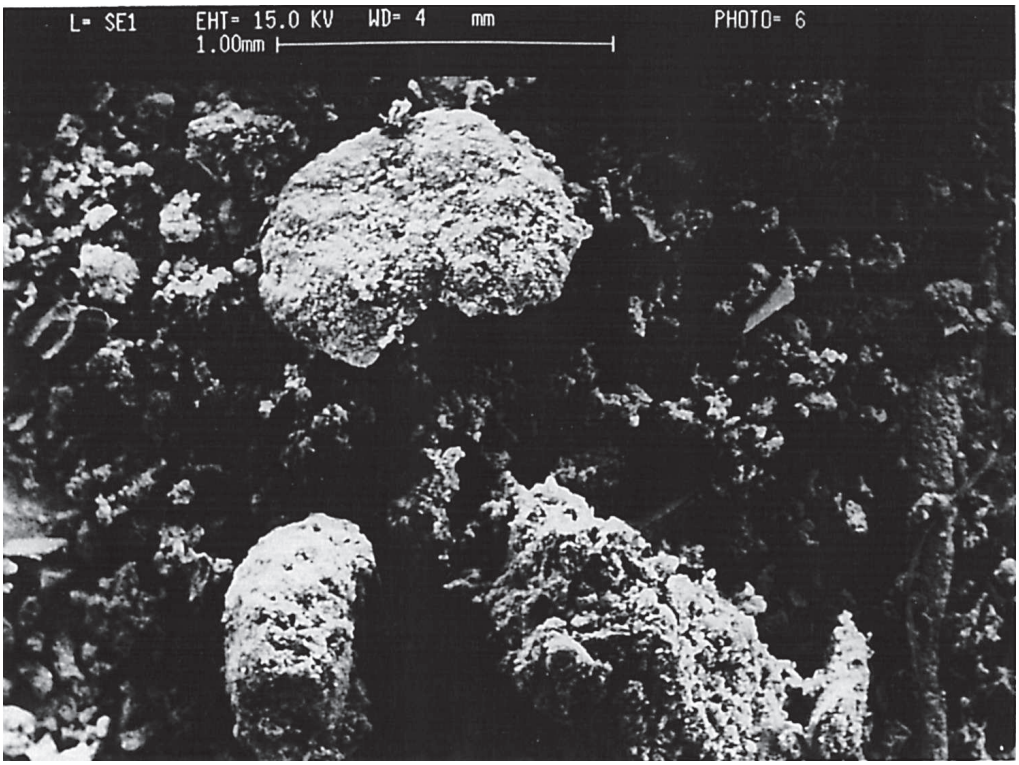

Figure 4.8: UASB Waikato ER photo 6 granules

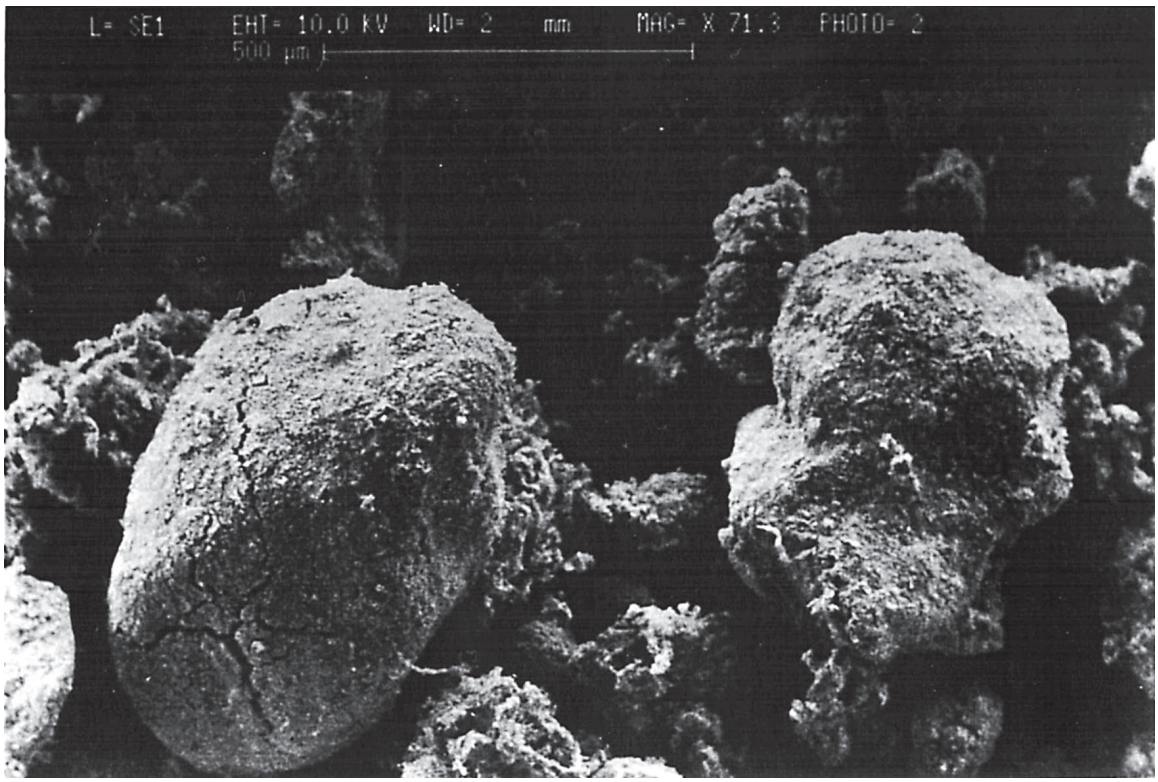

Figure 4.9: UASB Waikato ER photo 7 granule surface close up, Methanothrix like bacteria

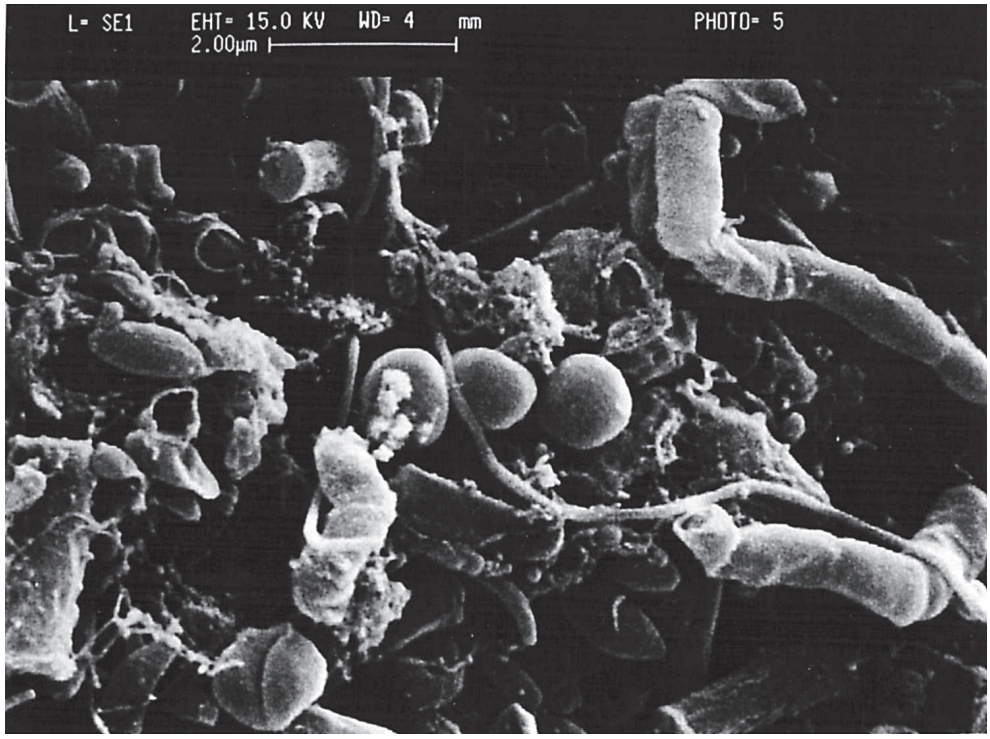

Figure 4.10: UASB Waikato ER photo 5 granule surface close up, Methanosarcina like bacteria

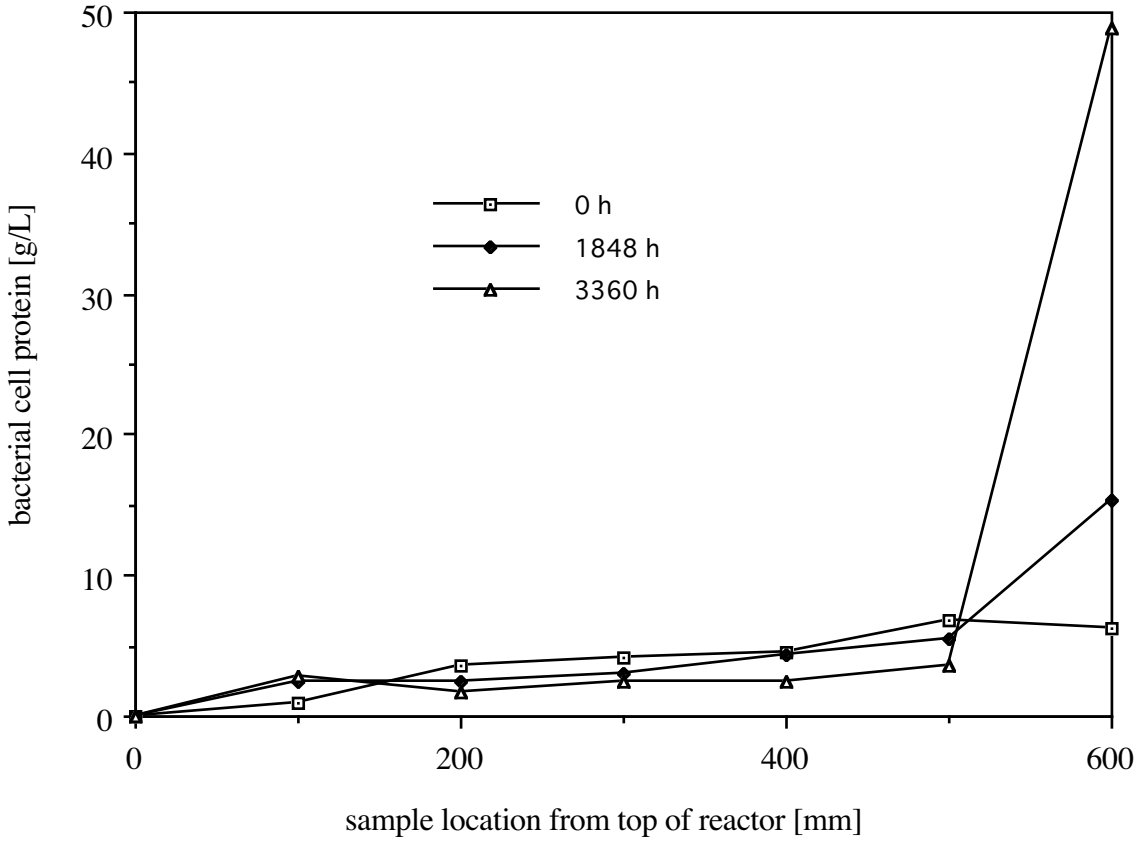

Figure 4.11: Distribution of bacterial cell protein within the reactor for UASB Saale. As described in Materials and Methods, samples were taken from the top at every 100 mm of the reactor height. This gave an indication where in the reactor the bacteria were predominantly located. At 0 hours the bacterial distribution was almost even whereas at 3360 hours the bacterial biomass predominated at the bottom of the reactor.

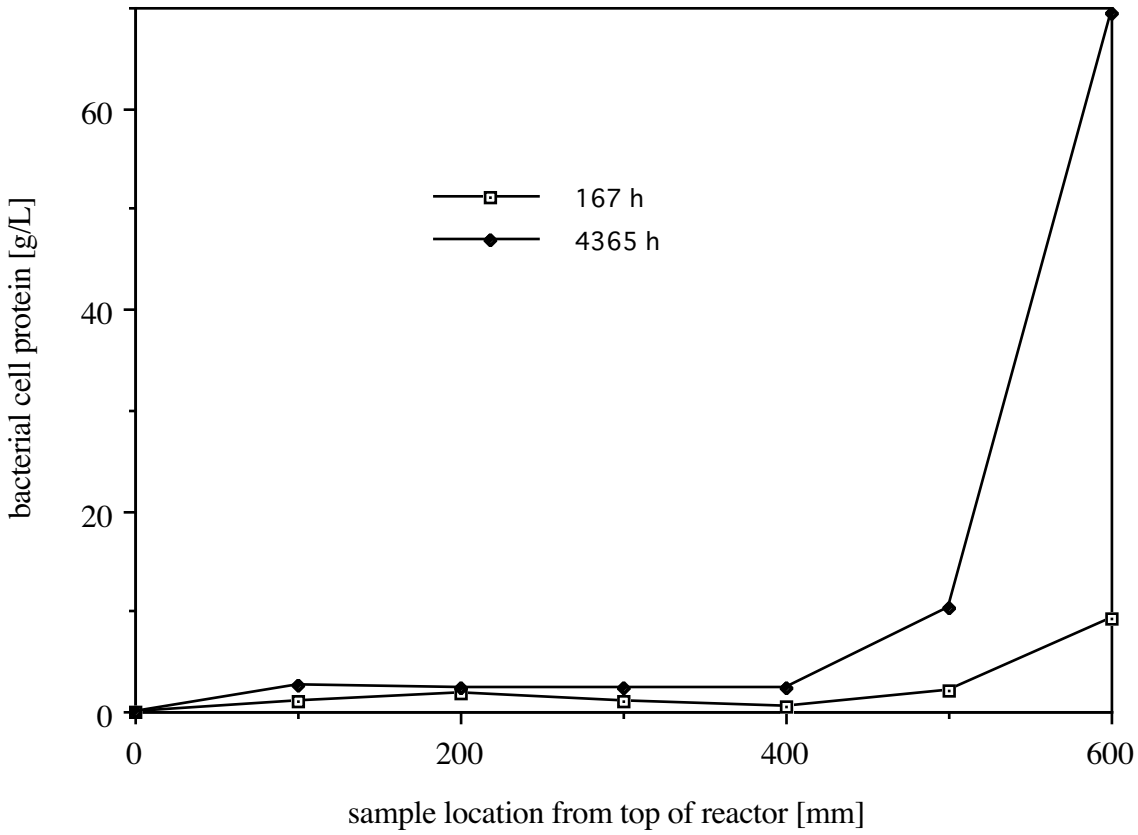

Figure 4.12: Distribution of bacterial cell protein within the reactor for UASB Waikato. As described in Materials and Methods, samples were taken from the top at every 100 mm of the reactor height. This gave an indication where in the reactor the bacteria were predominantly located. At 167 hours and at 4365 hours the bacterial biomass predominated at the bottom of the reactor, although the amount is much higher at 4365 hours. This shows the different behaviour of UASB Waikato in comparison to UASB Saale.

Table 4.1: Comparison of biodegradation potential for the Saale sediment and UASB Saale granules. The Saale sediment utilised was from the same batch as used for the granulation itself and was compared with disintegrated granules from the UASB reactor after successful granulation. The level of significance for the student-t-test here applied was a value of 5 % or lower.

	Saale sediment	UASB Saale	p values from t-test
lactic acid	0.52 ±0.15	0.95 ±0.19	***0.04***
acetic acid	1.0 ±0.2	1.11 ±0.32	0.64
propionic acid	0.39 ±0.09	0.61 ±0.1	***0.05***
benzoic acid	0.53 ±0.08	0.54 ±0.13	0.95
phenol	0.82 ±0.08	0.92 ±0.15	0.84

Table 4.2: comparison of biodegradation potential for the Waikato sediment and UASB Waikato granules. The Waikato sediment utilised was from the same batch as used for the granulation itself and was compared with disintegrated granule. The level of significance for student-t-test here applied was a value of 5% or lower.

	Waikato sediment	UASB Waikato	p values from t-test
lactic acid	0.26 ±0.08	0.34 ±0.02	0.2
acetic acid	0.87 ±0.27	0.9 ±0.76	0.95
propionic acid	0.25 ±0.04	0.25 ±0.04	0.86
benzoic acid	0.29 ±0.11	0.31 ±0.11	0.38
phenol	0.18 ±0.04	0.22 ±0.06	0.4
butyric acid	0.49 ±0.16	0.68 ±0.31	0.42

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4.6 Appendix

Start up medium for UASB Saale and UASB Waikato

50	mM	formic acid
100	mM	$NaHCO_3$

in double distilled water adjusted with HCl to pH 7.2

PPBM-Medium

Acid stock for UASB Saale first 450 hours

588.9 g	lactic acid (90 %)	5.884	mM /L	30.503	gO_2/ L
28.8 g	benzoic acid	10	mM /L	2.4	gO_2/ L
1.9 g	phenol	1	mM /L	0.224	gO_2/ L
		sum of		33.127	gCOD/L

Acid stock for UASB Saale and UASB Waikato.

Na-lactic acid and lactic acid were purchased in bulk from PURAC biochem, Holland, at food grade, heat stable.

300	g	Na-lactic acid (60 %)	1.998	M/ L	}	19.16	gO_2/ L
170	g	lactic acid (90%)	1.698	M /L			
28.8	g	benzoic acid	10	mM /L		2. 4	gO_2/ L
1.9	g	phenol	1	mM /L		0.224	gO_2/ L
			sum of			21.8	gCOD/L

In each case the acid stock was filled up with double distilled water till 2 L volume was reached. Then the mix was autoclaved for 30 minutes and after cooling added to the following.

17.5	L	double distilled water
20	g	NH_4Cl
2	g	$CaCl_2$
4	g	$MgCl_2$
0.46	g	$FeSO_4$
200	ml	Trace Elements II
200	ml	Vitamin Solution A
20	ml	Resazurin

66.8 ml K_2HPO_4 (50 g /L stock solution)

After the addition of the acid stock the medium was filled up with double distilled water until 20 L volume was reached.

UASB laboratory reactor design and operational parameters

A conceptional drawing of the reactor design used in these experiments is shown in Figure 4.13. The recycle pump (3) was recycling the reactor content at 18.4 L/h the superficial loading rate was 2.22 m/h. The reactor was operated in a pH auxostate mode, which means that the acidic carbon source was dosed in when the pH exceeded a certain setpoint due to microbial acid consumption. When the bacteria were using up the feed the pH was rising (measured at the pH-electrode (5)) and via the pH meter (6) the food pump (4) was regulated. Typical for the UASB reactor design is the separation of liquid-solid and gaseous phase, here achieved in a funnel (7). The gas produced, was measured in the gas meter (9) and samples for the determination of the gas composition were taken in 7. The accuracy of pH electrode (5) was regularly checked against an external measurement. At the top (ceiling) of the reactor was a sealable outlet that was used for sample taking. These samples taken at regular intervals were analysed for bacterial cell protein and volatile fatty acids. At regular intervals a lance was lowered into the reactor through this outlet and duplicate samples of 1.6 ml were taken at 100 mm, 200 mm, 300 mm, 400 mm, 500 mm and 600 mm from the top of the reactor and analysed for bacterial cell protein. These data were used for distribution profiles of the bacterial cell mass within the UASB reactors. The same set up was used to check for granules.

UASB reactor measurements

reactor height	650	mm
reactor diameter	102.5	mm
reactor working volume	5.4	L
reactor cross section	82.52	cm^2
recycle rate	18.4	L/h
superficial loading rate	2.22	m /h * (recycle rate) reactor surface

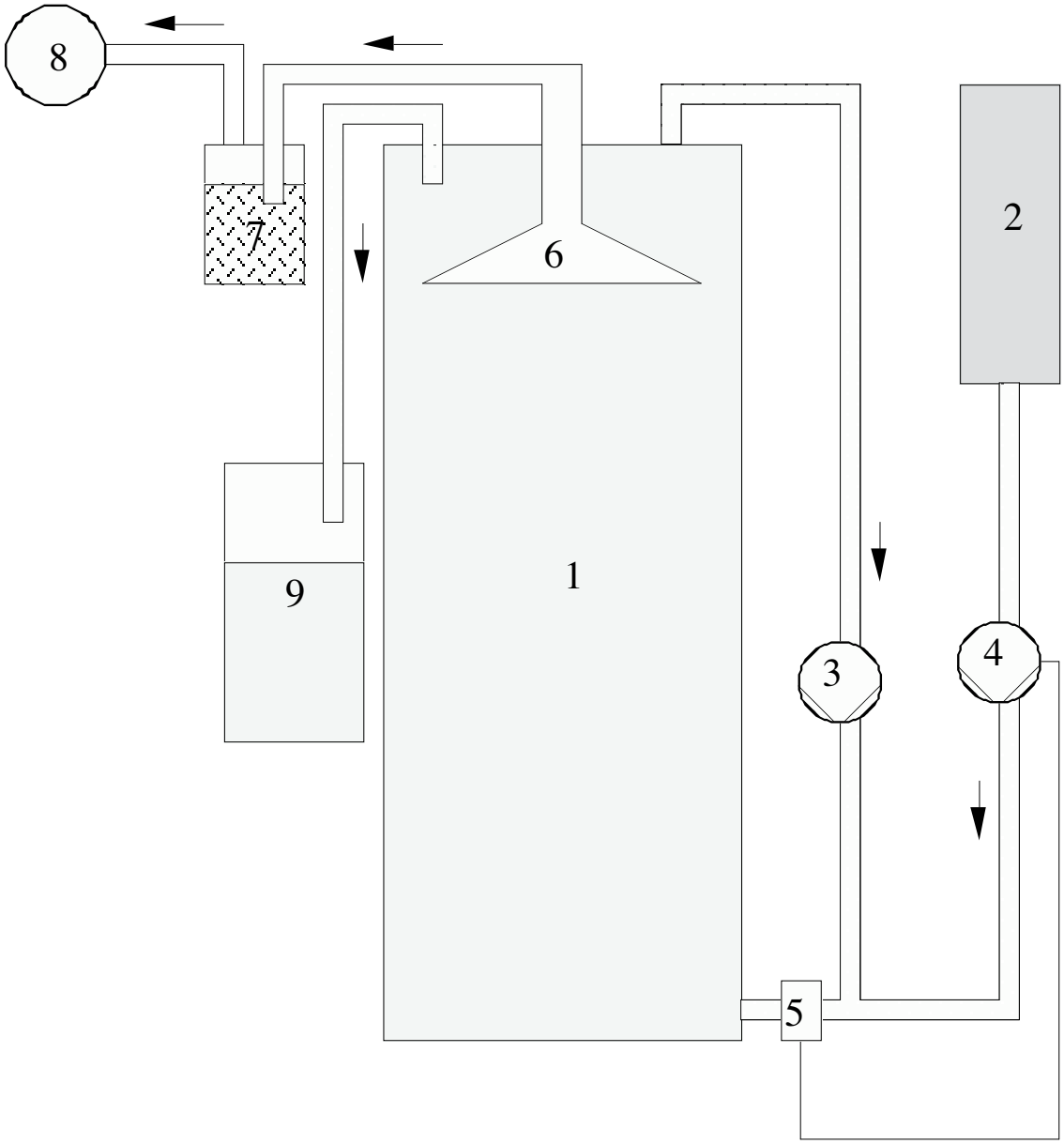

Figure 1
1, reactor vessel
2, food container
3, recycle pump
4, food pump
5, pH-meter
6, gas outlet (separation of gas -liquid-solid phase)
7, gas washing
8, gas meter
9, effluent tank

Figure 4.13: Conceptual drawing of UASB reactors.

5 Commissioning of newly constructed Wastewater Treatment Plant and Implementation of Biological Nutrient Removal Processes for the NZMP Edendale Site

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5.1 Introduction

The NZMP - Edendale site is located in Edendale (Southland, New Zealand) on State Highway One, 40 kilometers north of Invercargill. Activities undertaken at the site include the manufacture of milk powders, cheese, casein, milk fats, whey products and lactose.

The plant was originally established as a cheese factory in 1882 and has expanded rapidly in recent years. In 1992/93, the company was processing 500 m3 of milk on peak day, this volume increased to 4600 m3 of milk on peak day in the 2000/01 season.

In 1996 a Wastewater Treatment Plant was build and commissioned in the 1997/98 season. From 1997-2001 the plant has shown good nutrient removal rates. Theoretical calculations from plant data show that often higher concentrations of Nitrogen and Phosphorus have been removed from the wastewater than can be accounted for by assimilation into bio-mass. The processes that increase the removal of nutrients from biological treatment systems are nitrification/de-nitrification and enhanced biological phosphorus removal.

5.2 Methods

Sampling

Flow proportional 24 hour composite samples were taken before/after the DAF and after either Clarifier 1 or Clarifier 2 depending on the discharge mode.
Grab samples were taken from both Ponds and both Clarifiers.

Analytical Methods

Samples of the Balance Tank Effluent, the DAF Effluent, the Mixed Liqour of the Ponds, the Clarifier Effluent and the Activated Sludge were analysed for the following parameters:

pH
Conductivity
Total Suspendid Solids (TSS)
Total Solids (TS)
Chemical Oxygen Demand (COD)
Total Nitrogen (TN)
Total Phosphorus (TP)
Dissolved Reactive Phosphorus (DRP)
Settleable Volume Index 30 minutes (SVI_{30})
Biochemical Oxygen Demand 5 days (BOD_5)

These analytical methods are described in: “Standard Methods for the Examination of Water and Wastewter (APHA, 1992)“.

5.3 The Wastewater Treatment Plant

The wastewater treatment system for the Edendale Dairy factory consists of two Irrigation Farms and a Wastewater Treatment Plant. Figure 5.1 shows a schematic of the different flows and treatment options. The wastewater arrives from the production plants in three streams, these are from the milkpowder flumes, the casein flumes, and the combined cheese, whey evaporation and lactose flumes. These three streams are flow-balanced in two Balance Tanks and then treated by dissolved air flotation (DAF). After the DAF-treatment the wastewater can either be irrigated or is further treated through two biological treatment ponds and a Clarifier for Activated Sludge Removal. At this point the treated wastewater can either be irrigated or Aluminium-Sulphate is added for final phosphorus- and suspended solids-removal before the treated wastewater is discharged to the Mataura River.

It is important to note, that due to cultural and political preferences the preferred option is to treat and/or discharge wastewater onto land rather than surface waters. The operation of the Dairy Plants is seasonal. In the here described timeframe the winter months June-July are the off-season and no wastewater from production facilities is processed. During the rest of the year treated wastewater can only be discharged to the receiving river when flowrates exceed a set limit. One part of the Dairy Plant operation is the discharge of Casein-Whey onto land. This operation is in direct competition for land and trucking resources with the removal and disposal of waste activated sludge (WAS) from the Wastewater Treatement Plant. The main buisness of the company is the processing of milk, therefore alternate ways for the management of RAS/WAS have to be applied.

Due to these reasons the operation of the Wastewater Treatment Plant becomes complex and repeated start-up and shut-off situations are common procedure. Periods of permitted river-discharge last from 2-14 days at a time, and only on five occasions from 1997-2001 did the river-discharge time exceed 20 days duration. This scenario has a strong effect on the management of the Wastewater Treatment Plant and the biological nutrient removal.

The biological treatment unit of the system consists of two ponds (14000 m^3 each) in series (occasionally parallel) followed by the first clarifier (3500 m^3). Pond I receives the majority of the wastewater and at times, Pond II may directly receive up to 20% of the wastewater. The ponds were originally designed to operate with an F:M ratio of 0.14

(based on BOD) and a Total Suspended Solids (TSS) concentration of 3000 mg/L. The plant was designed for 3000 mg BOD/L on average and 4000 mg BOD/L maximum, or 5000-6000 mg COD/L. The design wastewater flows were 3500-4000 m^3/day. This gives a design load of 12 t BOD/day or 22 t COD/day. Table 5.1 shows maximum and average COD loadings for the biological ponds since commissioning.

As shown in Figure 5.1, the return activated sludge (RAS) and the waste activated sludge (WAS) both pass through the same sludge holding tank. The RAS is sent back to the first pond using hydraulic pressure, this enters the pond near the DAF-treated wastewater entry point. The sludge returned to the pond may be taken from one of two points in the sludge holding tank. The first point is almost at the top of the tank, this configuration allows the sludge to thicken in the lower part of the tank for wasting. The second point is in the lower part of the tank, this results in the RAS being approximately equivalent to the WAS.

System Operation

The principal modifications to the design and operation of the plant since commissioning are:

- pumping of activated sludge to the first balance tank (see Figure 5.1),

- operating the ponds on a generally lower dissolved oxygen level,

- specific regions of lower dissolved oxygen levels (anoxic/anaerobic regions) in both ponds,

- elevated TSS concentrations in the ponds.

The capacity of the first balance tank is 360 m^3, of these approximately 250 m^3 volume are used for mixing and balancing. Approximately 50 m^3/day of activated sludge are pumped from the sludge holding tank to the first balance tank. The balance tank is never pumped below a minimum volume to retain a seed sludge for acidification and anaerobic digestion. The occurance of anaerobic sludge particularly in the first balance tank has been observed by Wastewater Treatment Plant staff on a number of occasions.

The Dissolved Oxygen (DO) concentrations in the ponds are kept relatively low (between 0.1 and 1.5 mg O_2/L) to ensure that there are anoxic/anaerobic regions to allow for the occurance of biological nutrient removal. The aerators are controlled in banks to allow fine control of the DO concentrations via automatic DO-probes in both ponds.

Table 5.2 shows the average removal rates and effluent concentrations achieved in this plant since commissioning.

Table 5.3 shows the average influent and effluent concentrations.

5.4 Results and Discussion

In general the composition of dairy wastewater will depend on the milk processing operation on each specific site. In the Edendale case the composition is determined by the wastes from the Milktreatment, AMF, Milkpowder, Casein, Cheese, and Lactose/Whey-Cheese plants. Beside the main Carbon-sources the nutrients, that are effecting the wastewater impact, are Nitrogen and Phosphorus, because these influence biological growth in receiving waters. Nitrogen and Phosphorus both originate from the milk proteins and cleanning agents/detergents used in milk processing facilities (Wanner 1994, US Department of Agriculture, 1976).

To remove Nitrogen and Phosphorus from the wastewater chemical, mechanical and biological processes are available. In Edendale the enhanced biological removal of the nutrients was demonstrated. The mechanisms include assimilation (Eckenfelder & Argaman, 1991), nitrification/denitrification (Rabinowitz et al., 1990; Eckenfelder & Argaman, 1991; Wanner, 1994; Bortone & Piccinini, 1991) and enhanced biological phosphorus removal (EBPR) (Stensel, 1991; Janssen & Rensink, 1987; Liu, 1998). Especially for the nitrification/denitrification and EBPR processes it has been demonstrated that a combination of anerobic and aerobic zones within the wastewater treatment process is absolutely necessary to achieve optimum removal rates. These processes need to be managed carefully and because the mechanisms for EBPR are not yet fully understood, this can become a difficult task in a full-scale industrial wastewater treatment plant complex.

The results for the Edendale plant have shown that the removal rates for COD, TN and TP are high and are higher than one would expect from such a plant if biological removal would not be included.

These results have intiated a further research study as a combined effort between the Edendale Site staff and staff from the New Zealand Dairy Research Institue in Palmerston North. This study was conducted in September and October 1999 and the results will be published in a conference paper in 2002.

Figure 5.1: NZMP – Edendale Wastewater Treatment Plant.

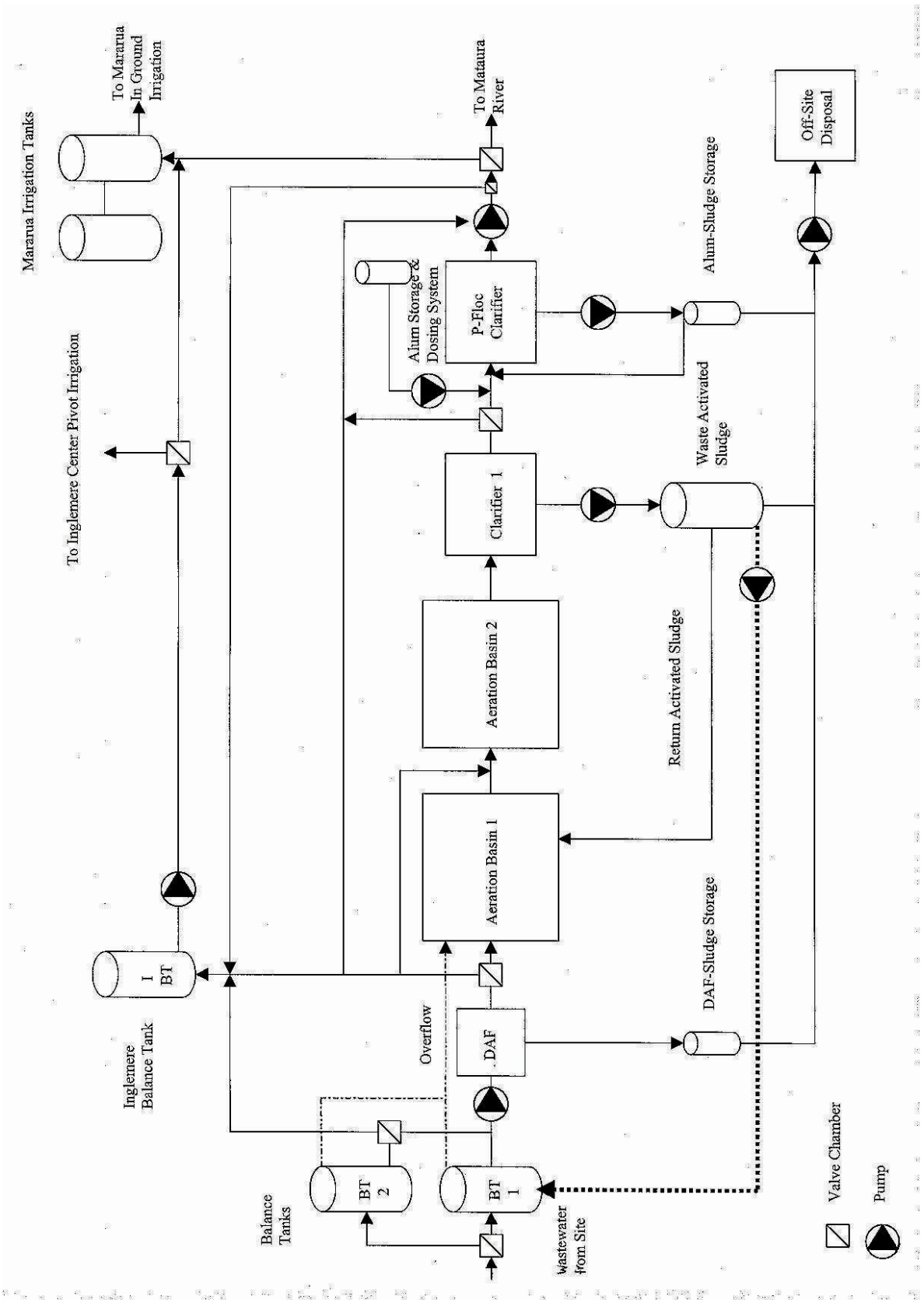

Table 5.1: Maximum and average COD loadings for the biological ponds since commissioning.

	COD maximum mg/L	**COD maximum t/day**	**COD average mg/L**	**COD average t/day**
1997/98	12,070	50.53	5,948	11.96
1998/99	12,400	41.30	5,256	11.00
1999/00	9,200	23.60	4,773	8.60
2000/01	9,130	27.80	5,360	10.00

Table 5.2: **Average removals and effluent concentrations.**

	COD		**TN**		**TP**	
	removal	**effluent (mg/L)**	**removal**	**effluent (mg/L)**	**removal**	**effluent (mg/L)**
1996/97		316		7		
1997/98	93%	315	92%	12	64%	29.3
1998/99	98%	74	85%	26	83%	6.7
1999/00	96%	186	88%	20	67%	11.0
2000/01	97%	132	90%	20	77%	8.2

Table 5.3: **Average influent and effluent concentrations (in mg/L).**

	COD			**TN**			**TP**		
	In-fluent	**Ef-luent**	**Re-moval**	**In-fluent**	**Ef-fluent**	**Re-moval**	**In-fluent**	**Ef-fluent**	**Re-moval**
1996/97		316			7				
1997/98	5425	315	5103	164	12	149	81.5	29.3	52.2
1998/99	5051	74	4978	149	26	122	40.2	6.7	33.5
1999/00	4773	186	4648	148	20	130	51.3	11.0	33.0
2000/01	4954	132	4811	216	20	201	21.2	8.2	23.4

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6. Discussion and Conclusions

The aim of this work was to investigate the ability of a variety of pure bacterial cultures and microbial anaerobic consortia to transform common environmental pollutants and nutrient removal through consortia of aerobic and anaerobic bacteria. The compounds chosen to study were DCE, DDE and PCBs for environmental pollutants and Nitrogen and Phosphorus for nutrient removal. DDE, a breakdown product of the widely used pesticide DDT, has been shown to be prevalent in various regions in New Zealand and overseas. Although the use of the chemical DDT was stopped in industrialised countries in the 1970's, DDE still remains in the soil and has an impact on agricultural and land-use. To date, no reports on the degradation of DDE by microbial cultures from New Zealand soils have been forthcoming. PCBs have been a major problem overseas especially in the USA and Europe. They do not represent a major source of contamination in New Zealand. Nitrogen and Phosphorus can be of major environmental concern, because they can be the cause of major eutrophication in wastewater receiving waters.

Only recently (1998) were pure bacterial cultures described that were able to degrade DDE. The breakdown of DDE was investigated here with pure bacterial strains and separately with anaerobic sediments and soils. In this work the under lying idea was to study the breakdown of compounds that have a similar chemical structure to each other. For example, DCE has some structural similarity to DDE, but is less complicated and less toxic. The aim was to attack DCE in the part of the structure that it has in common with DDE. Other publications have also used this approach by using DPE (non-chlorinated DDE derivative) instead of DCE (chlorinated). The results of this work demonstrated that the bacteria used here were able to degrade DCE under both aerobic and anaerobic culture conditions cometabolically. When DDE was added to the culture medium it appeared that the bacteria were also able to degrade DDE in a cometabolic reaction with the carbon and energy sources supplied. However, under these conditions an increase in the bacterial protein was not evident suggesting that the bacteria were either metabolically inactive or inhibited by DDE. The measurement of the disappearance of DDE from the culture medium was based on the loss of DDE concentration over time. To confirm these findings the experiments were repeated using a more sensitive assay based on [^{14}C]DDE. The radioactive labeled DDE would allow us to determine the fate of the breakdown products. Based on these experiments, it was found that the DDE was

attached to the bacterial cells. No breakdown products were detected and the concentration of DDE did not alter with time of incubation. The work emphasised that in regard to the analysis, a decrease in the concentration of the contaminant does not necessarily mean that this chemical was broken down by the bacterial cultures being investigated.

Hay and Focht in 1998 were the first to report the breakdown of DDE by a pure culture of *Pseudomonas acidovorans* strain M3GY. M3GY was able to grow on DPE but these cells could not degrade DDE. However, cells grown on biphenyl lead to the success of DDE degradation. This fact is surprising because the chemical structures of biphenyl and DDE share little similarity. It would be of further interest to determine whether biphenyl could induce the breakdown of DDE by the bacterial cultures used in this study. The observation that DDE was found to be attached non-specifically to the bacterial cells may have utility in practical application of *in situ* bioremediation.

The second part of this investigation was to enrich for anaerobic bacteria from New Zealand soils or sediments capable of breaking down PCBs or DDE. Since the exposure of the New Zealand environment to PCBs has been negligible, a PCB degradation was not expected, but on the other hand a DDE breakdown seemed likely because of long term exposure within this environment. The analysis used in the first investigation was adapted so that a breakdown of the compounds was only verified when breakdown products could be identified. The results of this work were also unsuccessful in terms of locating DDE-degrading bacteria from sediments. The mix of four PCB congeners was also not degraded and only some results with Aroclor 1260 showed a shift in the chromatographic profile. This shift was associated with lesser chlorinated PCBs, which can be degraded by aerobic bacteria more readily.

Quensen et al. in 1998 were able to enrich cultures from a marine sediment capable of degrading DDE. Their study was based on a number of samples from an isolated site. Instead of screening large geographical regions as in this study, a better approach might be to locate a hot spot for DDE contamination within New Zealand for further anaerobic breakdown studies. A hot spot would be a place where DDE can be found in the soil in high concentrations and was known to be contaminated in the past. Anaerobic consortia could be grown on a number of carbon and energy sources to get the optimal growth conditions (methanogenic conditions) and then [^{14}C] labeled DDE could be used to study the long term breakdown of DDE. Keeping the past in mind how DDT was applied to

pastures in New Zealand, one might expect to find a spot that would allow such a study. Once this location is found and a bacterial consortia able to degrade DDE anaerobically isolated, one could start to investigate the mechanisms behind this breakdown, which in turn would benefit the New Zealand and global community in regard to clean up strategies for contaminated sites.

The third investigation was into the granulation of river sediments in UASB reactors. The results show that it is possible to use New Zealand and German river sediments for granulation, which is positive because river sediments are much more readily available than for example UASB granules. Furthermore, river sediments contain bacteria that are adapted to or have been exposed to upstream contamination.

It would be worthwhile to combine the second and third investigations for example with sediments from the Hudson river in the USA that are able to degrade PCBs or still to be found sediments from New Zealand able to degrade DDE. These could be granulated and because of the higher cell density within the granules the investigation into the reductive dehalogenation could be increased, and the mechanisms could be investigated. Ultimately this approach should give an understanding of how the dehalogenation mechanisms for PCB, DDT or DDE work. This would allow a better understanding and the possibility to apply this knowledge to the clean up of contaminated sites.

The fourth investigation represents the commissioning of a full-scale industrial activated sludge wastewater treatment plant with the introduction/implementation of biological nutrient removal. It was demonstrated here that this plant has the ability to reduce wastewater loadings of Nitrogen and Phosphorus as well as carbon loadings (COD) at a large scale, which in turn allows to reduce the usage of chemical/mechanical floculation with Aluminium-Sulphate to a minimum.

This thesis has provided "minute bricks in the wall", but they can be used to build on or gain further understanding of the microbial-mediated degradation of xenobiotic compounds in New Zealand, and have given a model for biological nutrient removal for other wastewater treatment plants within the New Zealand Dairy Industry.

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