

Bioremediation of Atrazine- and BTX- Contaminated Soils: Insights Through Molecular/Physiological Characterization

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Pietermaritzburg

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To `Mè and Ntate

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Declaration

I do hereby declare that the work documented in this thesis, unless otherwise indicated, is the result of original research based on (innovative) proposals with my supervisors.

A handwritten signature in black ink, appearing to read 'TK Ralebitso', written over a dotted line.

Theresia Komang Ralebitso BA BSc(Hons)

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Abstract

Most natural products and xenobiotic molecules, irrespective of their molecular or structural complexity, are degradable by some microbial species/associations within particular environments. Atrazine- and selected petroleum hydrocarbon (benzene, toluene and *o*-, *m*- and *p*-xylene (BTX))-degrading associations were enriched and isolated from atrazine- and petroleum hydrocarbon (PHC)-contaminated KwaZulu-Natal loamy and sandy soils, respectively. In total, eight pesticide- and forty BTX-catabolizing associations were isolated. Electron microscopy revealed that, numerically, rods constituted the majority of the populations responsible for both atrazine and PHC catabolism. Cocci and, possibly, spores or fungal reproductive bodies were observed also. For the BTX-catabolizing associations, the population profiles appeared to be dependent on the enrichment pH and the molecule concentration.

After combining selected associations, to ensure that all the isolated species were present, batch cultures were made to determine the optimum pH and temperature for growth. With an atrazine concentration of 30 mg l^{-1} , the highest specific growth rates, as determined by biomass (OD) changes, were recorded at 30°C and pH 4 although the rates at 25°C and pH 5 were comparable. For the BTX (50 mg l^{-1})-catabolizing associations, the highest growth rates were recorded at pH 4 for the four temperatures (15, 20, 25 and 30°C) examined. The sole exception was *p*-xylene with the highest specific growth rate recorded at pH 5 and 30°C .

Batch and continuous (retentostat) cultivations in the presence/absence of methanol and under C- and N-limited conditions were used to investigate the impacts of the solvent and the catabolic potentials of a combined atrazine-catabolizing culture (KRA30). In general, different degradation rates were recorded for the culture in response to element limitation. Addition of citrate as the primary carbon source effected atrazine (100 mg l^{-1}) degradation rates comparable to that of *Pseudomonas* sp. strain ADP while succinate addition effected herbicide co-metabolism. Carbon supplementation may, therefore, be considered for site amelioration practices.

To complement conventional culture-based microbiological procedures, molecular techniques were employed to explore the diversities and analyze the structures of the microbial communities. In parallel, anaerobic microbial associations which targeted atrazine were also characterized. The soil DNA isolation/characterization protocol adopted consisted of a clean-up step followed by the polymerase chain reaction (PCR) and 16S rDNA fingerprinting by denaturing-gradient gel electrophoresis (DGGE). The preliminary results suggested that despite different, but chemically similar, petroleum hydrocarbon molecules, the common selection pressures of the primary enrichments effected the isolation of similar and complex aerobic microbial associations. Some similar numerically-dominant bands characterized the aerobic and anaerobic atrazine-catabolizing associations although distinct differences were also recorded on the basis of the enrichment/isolation pH value and the concentration of

the herbicide. Cloning and sequencing were then used to identify some of the numerically-dominant and non-dominant association members.

Community-level physiological profiling (CLPP) for physiological fingerprinting was made with Biolog EcoPlates and highlighted the differences in the isolated aerobic atrazine-catabolizing associations depending on the enrichment pH and molecule concentration.

Logarithmic-phase cultures of the combined atrazine- and BTX-catabolizing associations were used to explore the association profiles following pH and temperature optimization. Although some common numerically-dominant components were maintained, differences in numerical and, possibly, activity dominance were observed in the 16S rDNA profiles in response to changes in pH and temperature. This indicated that environmental parameter optimization and characterization of catabolic association structure must precede bioaugmentation so that control of key variables will facilitate maintenance of the dominant site-specific species.

Following KRA30 cultivation in the presence/absence of methanol and under carbon- and nitrogen-limited conditions, the population fingerprints showed that the presence of methanol effected shifts in species numerical dominance and, possibly, changes in atrazine catabolic capacity. Also, Coulter counter results, optical density readings and 16S rDNA characterization by DGGE indicated that degradation rate changes were accompanied by shifts in species numerical/activity dominance within the association. Although N-limitation effected the highest rates of herbicide catabolism, a potential versatility of the combined association for bioaugmented and/or biosupplemented remediation with acceptable rates regardless of any elemental limitation was recorded. To determine if the contaminated and pristine source soils contained comparable catabolic populations and, thus, offered potential for intrinsic bioremediation, PCR-DGGE was used to characterize the populations in comparison with the enriched/isolated associations. Some similar dominant bands characterized the contaminated soils and the enriched/isolated associations. The significance of this, in relation to a possible correlation between numerical and activity dominance in the component species, is discussed with respect to the use of PCR-DGGE to identify natural attenuation potential and monitor sustained intrinsic and enhanced (bioaugmented and biosupplemented) bioremediation.

Chapter 1: Introduction

1.1 Soil Contaminants

1.1.1 Atrazine

Atrazine [2-chloro-4-(ethylamino)-6-(*isopropylamino*)-*s*-triazine] ($C_8H_{14}ClN_5$) is one of the most environmentally-prevalent *s*-triazine-ring herbicides since it is used worldwide for the control, through interruption of the light-driven flow of electrons in photosynthesis (Esser *et al.*, 1988; Tasli *et al.*, 1996), of pre- and post-emergence broadleaf and grassy weeds in major crops such as maize (*Zea mays*), sorghum (*Sorghum* spp) and sugarcane (*Saccharum officinarum*) (Goodrich *et al.*, 1991; Pick *et al.*, 1992; Seiler *et al.*, 1992; Mandelbaum *et al.*, 1993; Yanze-Kontchou and Gschwind, 1995; de Souza *et al.*, 1996; Sparling *et al.*, 1998). The United States Environmental Protection Agency (EPA) (1991) reported that since the 1980s, the annual pesticide usage had been holding steady at 500 million kg of active ingredients. Thus, 363 million kg of atrazine were applied between 1980 and 1990 in the USA alone (Yanze-Kontchou and Gschwind, 1995) and, as a consequence, contamination of water supplies by this herbicide has become an international concern.

Although extensive toxicological investigations by numerous researchers have effected different conclusions (Langan and Hoagland, 1996; Allran and Karasov, 2000; Anon, 2000; Diana *et al.*, 2000), the potential toxicity of atrazine motivates continuous research. Atrazine has been classified a Class C/possible human carcinogen (Loprieno *et al.*, 1980; Biradar and Rayburn, 1995). This classification was confirmed by the work of Biradar and Rayburn (1995) who observed chromosomal damage of Chinese hamster ovary cells exposed to atrazine (0.005, 0.014 and 0.080 μ M) over a period of 48 hours. In the same study, analysis of Illinois public water supplies revealed that the concentrations deemed safe by the EPA, although not severe enough to reduce the number of Chinese hamster ovary chromosomes per frequency distribution typical of a known clastogen, demonstrated the potential of the pesticide as a clastogen. Atrazine dealkylation metabolites are also regulated compounds and may pose potential health risks (Roy and Krapac, 1994; Shapir *et al.*, 1998). The need to elucidate the fates of these transformation products

has, therefore, been realized also (Brouwer *et al.*, 1990; Roy and Krapac, 1994; Kolpin *et al.*, 1998).

The herbicide has a solubility of approximately 30 mg l⁻¹ and a half-life of 70 d in soil although half-lives as low as 15 d and as high as 100 d have been reported (Protzman *et al.*, 1999). Atrazine decomposition products are relatively persistent in soil and their detection in surface- and ground-water has prompted some environmental concerns (Shannon and Unterman, 1993; Sparling *et al.*, 1998; Gebendinger and Radosevich, 1999). For example, detection of low concentrations in groundwater in many areas in New Zealand invoked fears that any increase in its concentration, and the concentrations of its catabolic intermediates, would compromise water quality (Sparling *et al.*, 1998).

1.1.2 Petroleum Hydrocarbons

Although a biosynthetic source has been suggested as the origin of aromatic hydrocarbons in the environment, its contribution to the concentrations of these compounds in soil, sediment and water was deemed insignificant (Gibson and Subramanian, 1984). The accidental release of petroleum hydrocarbons (PHCs) into the environment, principally from leaking oil storage tanks and fractured pipelines, is widespread (Atlas and Cerniglia, 1995; Kao and Wang, 2001). For example, the EPA estimated that about 25% of the two million underground storage tank systems located at 700 000 facilities in the USA may be leaking (Hinchee and Ong, 1992). The result is significant contamination of soil and groundwater with the most soluble and mobile components (benzene, toluene and xylene (BTX)) posing particular threats (Corseuil and Alvarez, 1996; Solano-Serena *et al.*, 1999). Their acute toxicities and genotoxicities have mandated the classification of the BTX compounds as priority pollutants by many governments (Shen, 1998; Tsao *et al.*, 1998; Kao and Wang, 2001). The USA drinking water standards for benzene, toluene and xylenes are 5 µg l⁻¹, 2 mg l⁻¹ and 10 mg l⁻¹, respectively (Allen-King *et al.*, 1994). Whyte *et al.* (1999) reported that petroleum hydrocarbon contaminants of soil and water can enter biological food webs and, thus, threaten indigenous organisms and the human population. Although not studied extensively, the cytotoxicities of BTX metabolites have been observed *in vitro* and, therefore, necessitate further investigation (Shen, 1998).

1.2 Adsorption/Desorption of Contaminants in Soil

1.2.1 Pesticides (*Atrazine*)

Pesticides are known generally for their low aqueous solubilities and tendency to remain sorbed in soil (Mata-Sandoval *et al.*, 2000). Extensive investigations have, therefore, been made on their adsorption/desorption to/from soil components. Sorption affects other processes such as transport, degradation, volatilization and bioaccumulation which, in turn, determine pesticide fates including irreversible binding and persistence in the environment (Gao *et al.*, 1998). The desorption processes are equally important particularly for quantifying pesticide transport and, therefore, establishing a mass balance.

Some researchers have explored the importance of organic matter, particle size, “ageing” and pH on the sorption and transport of atrazine (Huang *et al.*, 1984; Roy and Krapac, 1994; Gao *et al.*, 1998; Sluszný *et al.*, 1999) while others have investigated the role of colloids (Celis *et al.*, 1998; Sprague *et al.*, 2000). Moreau-Kervéan and Mouvet (1998) stated that the main organic components involved in the adsorption of atrazine were humic substances, especially humic acids, while clays and oxyhydroxides were the inorganic constituents responsible. Other researchers have investigated the impacts of modifying soil organic matter, via, for example, land application of treated sewage sludge, on atrazine sorption, bioavailability, mobility and, thus, microbial degradation (Masaphy and Mandelbaum, 1997; Sluszný *et al.*, 1999). Generally, the adsorption/desorption of *s*-triazine herbicides to humic acids and clays is determined by various physical and chemical mechanisms which may act together or individually. Such interactions are dependent upon the solute and key environmental determinants such as pH (Ward and Weber, 1968; Moreau-Kervéan and Mouvet, 1998). The effects of hydrophobic bonding, Van der Waals forces, hydrogen bonds, charge transfer and triazine protonation on the sorption properties of *s*-triazine herbicides have been investigated for some soil types (Moreau-Kervéan and Mouvet, 1998).

The role of tillage and irrigation practices on atrazine sorption to different soil types in different geographical regions has also been researched extensively (Azevedo *et al.*, 2000; Gaynor *et al.*, 2000; Hang *et al.*, 2000) with different results and conclusions.

1.2.2 BTX Compounds

Physical properties of the low molecular weight aromatic hydrocarbons, including BTX molecules, indicate that they should be localized in the atmosphere. Their detection in soil, therefore, is probably due to adsorption to soil organic matter (Gibson and Subramanian, 1984). Freijer (1996) stated that full control of the decomposition rates of pollutants requires knowledge of biodegradation kinetics and soil transport processes while Zhang and Bouwer (1997) concluded that biodegradation rates were affected by both the extent and rate of sorption. For example, sorption to the soil matrix should affect the apparent hydrocarbon concentrations while low gas diffusion rates might cause reduced oxygen concentrations and, therefore, lower the mineralization rates. Additionally, sorption is one of the mechanisms of hydrocarbon loss from the aqueous phase which can be attributed erroneously to transformation (Allen-King *et al.*, 1996).

As with atrazine, soil organic matter or organic carbon content was identified as the major component which controls hydrocarbon molecule sorption (Jin and O'Connor, 1990; Zhang and Bouwer, 1997). The researchers recorded that toluene sorption was increased in sludge-supplemented soil although the degree of sorption was still dependent upon the soil type, the type of the sludge organic matter and the soil clay content.

For both atrazine and BTX molecules, low water solubility of the contaminant and sorption of the molecule to regions of the soil particle, including the soil pores, which are inaccessible to microbial species, are potential causes of low biodegradation rates. A combination of bioventing, air sparging or thermal treatment (1.4.1) with biofiltration (1.4.1) could, however, be adopted for the entrapment and deodorization of the volatilized contaminant(s).

Alternatively, enhanced water solubility and increased mass transfer of the contaminant(s) to the water phase by the addition of surfactants, such as sodium dodecyl sulphate (Sanchez-Camazano *et al.*, 2000) and/or inorganic nutrients, such as Inipol EAP22 (Atlas, 1995), via percolation (1.4.1), are possible methods for increasing the rates of biodegradation (Churchill *et al.*, 1995; Alexander, 1999). Also, surfactant additions in amounts which are above the critical micellar concentration can enhance the apparent aqueous solubility of hydrophobic organic compounds (Mata-Sandoval *et al.*, 2000). Lindoerfer *et al.* (1992) demonstrated that addition of

surface-active compounds, such as glycolipid biosurfactant mixed with Tween 80, to a mixed culture of indigenous microorganisms enhanced the rate of hydrocarbon biodegradation. High treatment dosages (9 kg biosurfactant tonne⁻¹ soil) were, however, required.

Although effective, synthetic surfactants have some limitations. They could: inhibit microbial activity on pollutants solubilized in their micellar phase (Rouse *et al.*, 1994); be bacteriostatic and/or bactericidal; and have considerable affinity for soil surfaces and, therefore, accumulate and pollute subsurface environments (Lee *et al.*, 1995; Chin *et al.*, 1996). To circumvent these limitations, some researchers (Banat, 1995; Lin, 1996; Desai and Banat, 1997) have examined the use of biosurfactants, such as: guanidinium fatty acids, to promote the degradation of petroleum hydrocarbons (Nelson *et al.*, 1996); and rhamnolipids, to solubilize pesticides such as atrazine, trifluralin and coumaphos (Mata-Sandoval *et al.*, 2000).

1.3 Chemical Extraction of Soil Pollutants

Soxhlet extraction (Naudé *et al.*, 1998), immunosorption, sonication (Llompart-Vizoso *et al.*, 1997), agitation (Meney *et al.*, 1998), supercritical fluid (Phelps *et al.*, 1996; Shows and Olesik, 2000) and ethyl acetate micro-method extraction (Steinwandter, 1992), microwave-assisted extraction (Pastor *et al.*, 1997) and alkaline digestion (Pearce *et al.*, 1995) have been adopted to address the recognized challenges which sorption poses to effective/successful remediation programmes and, more particularly, to the recovery of pollutants prior to analysis. Each technique can be used either in combination with biological amelioration or as an inbuilt step in alternative chemical treatment methods (Pearce *et al.*, 1995; Sabik *et al.*, 1995; Del Valle *et al.*, 1996; Martín-Esteban *et al.*, 1997). According to Hawthorne (1990), an ideal extraction method should: be rapid, simple and inexpensive to perform; yield quantitative recovery of the target analytes without loss or degradation; yield a sample that is ready immediately for analyses without additional concentration or class fractionation steps; and generate no additional laboratory wastes. Also, the efficacy of an extraction method is dependent on certain principal factors such as: soil composition; ageing; pH; matrix particle size; moisture content; organic matter content; and the octanol-water partition coefficient (K_{ow}) of both the solvent and the target molecule/pollutant (Dean, 1996; Johnston, 1996; Llompart-Vizoso *et al.*, 1997).

In general, each extraction method has its merits and limitations. The choice of a particular method is, therefore, determined by the: accepted methodology and detection limits; application of the acquired information; capital and consumable costs; duration of the extraction and analysis; and regulatory/policy guidelines which differ between countries (Miller *et al.*, 1991; Tsipi and Hiskia, 1996).

Since the sustained effectiveness of natural attenuation (or any remediation technology) must be demonstrated continually, regular site evaluations and analyses of results are crucial practices (Rittman, 2000). Clearly, chemical extraction is essential to monitor bioremediation programmes since the actual concentrations of pollutants in environments undergoing amelioration, and the endpoint concentrations in remediated soils, may be otherwise underestimated or measured inaccurately. However, where proven cost-effective, applicable in terms of scale and efficient, the impacts of solvents (*e.g.* methanol, ethyl acetate) or detergents used for pollutant extraction/solubility on the biodegradation of the target molecules warrant substantial consideration. It has been shown that the use of ethanol as a gasoline solvent inhibited BTEX biodegradation and also, possibly, increased the pollution plume length due to a possible decrease in sorption-related retardation (Powers *et al.*, 2001). Pollutant extraction with organic solvents may continue despite the emerging trend towards reducing/eliminating their use (Rodrigues *et al.*, 1998; Bouaid *et al.*, 2000). Thus, competitive degradation, with the organic solvents providing labile carbon, may also necessitate investigation.

1.4 Microbial Intervention in Soil Remediation

Several researchers have reported or reviewed (Ralebitso *et al.*, 2002) the isolation of microorganisms which are able to: dealkylate atrazine in a carbon-limited medium (Bheki and Kahn, 1986); mineralize and use atrazine as a sole carbon and energy source (Mandelbaum *et al.*, 1995; Radosevich *et al.*, 1995; Yanze-Kontchou and Gschwind, 1994; Topp *et al.*, 2000a; b); utilize the heterocyclic nitrogen (Bichat *et al.*, 1999); and, in the presence of supplemental carbon, mineralize atrazine and its metabolites as a source of nitrogen (Mandelbaum *et al.*, 1993; Assaf and Turco, 1994a; b). Success stories, such as the mineralization of >94% of atrazine-C (50 μgml^{-1}) and the isolation of a microorganism, *Agrobacterium radiobacter* strain J14a, which was able to dealkylate, dehalogenate and mineralize the s-triazine ring when

the molecule was used as a nitrogen source, were reported by Struthers *et al.* (1998). By screening some *Rhodococcus* strains, which were known to be ubiquitous in soil and had diverse biodegradative capabilities, Behki *et al.* (1993) identified the strain TE1 with a 77-kb plasmid with an atrazine-degrading capacity. Other workers reported microbial associations (Mandelbaum *et al.*, 1993; Alvey and Crowley, 1996; de Souza *et al.*, 1998a) and fungal species (Donnelly *et al.*, 1993; Mougin *et al.*, 1994; Gorbatova *et al.*, 2001) which mineralized atrazine. Generally, one of the aerobic degradation pathways compiled from many field and laboratory studies, as reviewed by Cook (1987) and Erickson and Lee (1989), is known to be hydrolytic rather than oxidative and consists of four main steps: dehalogenation; *N*-dealkylation; deamination; and ring cleavage (Figure 1.1).

Laboratory studies (and field tests) of oil-compromised soils and petroleum wastes have shown that the constituent hydrocarbon molecules are susceptible to microbial catabolism (Atlas, 1981; Freijer, 1996; Mallakin and Ward, 1996; Chen and Taylor, 1997; Piffner *et al.*, 1997; Salanitro *et al.*, 1997; Yerushalmi and Guiot, 1998; Deeb and Alvarez-Cohen, 1999; Vanderberg *et al.*, 2000; Kao and Wang, 2001) and the specific biodegradative pathways have been reviewed briefly by Shen (1998). Thus, bioremediation, which was pioneered for the petroleum industry, has become a popular alternative to chemical or physical remediation because of its relatively low costs and minimal impacts on the environment (Newcombe and Crowley, 1999).

1.4.1 Bioremediation as a Treatment Option

Most natural products, irrespective of their molecular or structural complexities, are degradable by some microbial species within particular environments (Knackmuss, 1996). The growing importance of environmental protection has brought into increased focus the need for new technologies to treat and dispose wastes. Bioremediation, although not a novel technology with its usage spanning at least 30 years (Ryan *et al.*, 1991; Newcombe and Crowley, 1999), has been identified as an appropriate technology because its application has provided a way for safe and efficient environmental amelioration (Stegmann *et al.*, 1994; Korda *et al.*, 1997). Through contaminant-/substrate-specific treatments, bioremediation can reduce the concentrations of single compounds or mixtures of biodegradable materials (Korda *et al.*, 1997). The technology often entails the use of indigenous microorganisms to treat

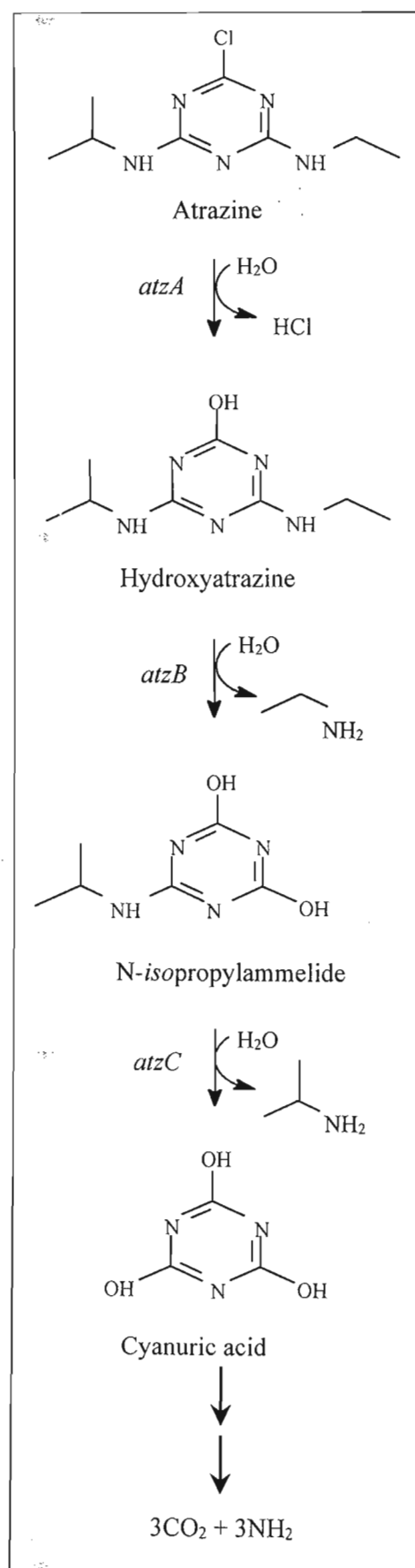


Figure 1.1: Aerobic atrazine catabolic pathway elucidated with *Pseudomonas* sp. strain ADP (de Souza *et al.*, 1998a).

chemically-compromised soil and groundwater and, thus, reinstate these finite resources.

In bioremediation, prokaryotic species may be used in combination with eukaryotes (including higher plants). Radwan *et al.* (1998), for example, reported that the phytoremediation of the oil-contaminated Kuwaiti desert also involved bacterial species. The workers postulated that a combination of the degradative activities of rhizospheric hydrocarbon-utilizing microorganisms with uptake and biotransformation by select desert plants had facilitated some amelioration of the polluted sand.

It is worth noting that, unlike hydrocarbons, other soil contaminants, such as heavy metals and radionuclides, cannot be removed by degradation. Rittman (2000) reported, however, that natural attenuation can immobilize them by precipitation or irreversible sorption. The potential to exploit filamentous fungi to remove metals has been recognized also (Gray, 1998). For example, fungal species such as *Schizophyllum commune* and *Armillaria* spp have been found to translocate ^{134}Cs , ^{137}Cs , ^{65}Zn and ^{109}Cd and, thus, remove them from contaminated soils (Gray, 1998).

Eweis *et al.* (1998) stated that bioremediation effectiveness was influenced by environmental, physical and chemical factors. For example, the rate of microbial catabolism during bioremediation depends on: the rate of target molecule transfer to the cell (mass transfer); and the rate of chemical uptake and metabolism (the intrinsic activity of the cell) (Bosma *et al.*, 1996). Therefore, the availability of a chemical for biodegradation is determined by the rate of mass transfer relative to the intrinsic activity of the microbial cell. Vogel (1996) postulated that environmental conditions play a pivotal role in determining biological activity whether of indigenous, added or cultured indigenous microorganisms returned to the soil (bioaugmentation). Specifically, Corseuil and Alvarez (1996) determined that successful bioremediation depended on:

1. The presence of microorganisms with potential to biodegrade the target compound;
2. The accessibility of target pollutants to the microorganisms;
3. The induction of appropriate degradative enzymes;
4. The availability of electron acceptors;
5. The availability of inorganic nutrients;
6. Adequate pH;

7. Adequate temperature;
8. The absence of toxic substances; and
9. A faster biodegradation than migration rate.

The term bioremediation covers a wide range of systems which utilize microorganisms to degrade, detoxify and immobilize organic pollutants (Ryan *et al.*, 1991). It also has several definitions which differ according to the researcher. Two of the definitions which apply to this study are:

1. “A managed or spontaneous process in which biological, especially microbiological, agents catalyze/act on pollutant compounds thereby remedying or eliminating environmental contamination”; and
2. “The use of natural, enhanced or genetically-engineered microorganisms to improve environmental quality by exploiting their ability to treat hazardous (including toxic) or merely offensive compounds at contaminated sites” (Lees, 1996).

Bioremediation, while not a panacea, can be applicable to the treatment of a broad variety of organic contaminants (Ryan *et al.*, 1991) and is often the treatment of choice for the following reasons:

1. Waste products and hazardous chemicals can be mineralized into water and carbon dioxide (and biomass) and, thus, negate the need to move the contaminants from one site or medium to another;
2. It can be very cost-effective. Adding fertilizer, other nutrients (biosupplementation), plants and even selected microorganisms (bioaugmentation) to contaminated soil/water, or otherwise manipulating the microbial environment, can often be much less expensive than alternative processes such as incineration, the use of adsorbents and catalytic destruction (Protzman *et al.*, 1999);
3. The technology is versatile and may be applicable when other processes cannot be used (Goodrich *et al.*, 1991), for example, to treat complex/highly toxic materials such as polychlorinated biphenyls (PCBs), chlorinated solvents and chlorofluorocarbons (CFCs) which, until recently, were regarded as recalcitrant;
4. It has also been proposed as a cleanup technology in extreme and remote environments, such as the High Arctic region and the Alpine Glacier area,

where it was deemed to be the most logistically- and economically-favourable solution to hydrocarbon contamination (Whyte *et al.*, 1999; Juck *et al.*, 2000); and

5. It also lends itself to possible future use of genetically-engineered microorganisms with superior degradative enzymes and pathways (Shao *et al.*, 1995; Lees, 1996; Strong *et al.*, 2000). Vogel (1996) stated that microorganisms which are fast-acting, short-lived, mobile, adhesive, resilient and inexpensive with a wide range of degradative activities represented the ideal for bioaugmentation.

Blackburn and Hafker (1993) classified the available bioremediation techniques into three categories, namely *in situ*, *ex situ* solid and *ex situ* slurry, while Eweis *et al.* (1998) highlighted *in situ*, solid phase or bioreactor treatments as the physical conditions involved in bioremediation processes. The authors added that the bioremediation of contaminated soils, gases and water necessitated the use of considerably different treatment processes. Nonetheless, it is accepted widely that *in situ* bioremediation entails the treatment of the contaminated soil and the associated groundwater in place without excavation while *ex situ* treatments are often preceded by excavation (Blackburn and Hafker, 1993; Eweis *et al.*, 1998). Examples of *in situ* techniques include: bioaugmentation; biostimulation; landfarming; thermal treatment/enhancement; pump-and-treat systems (*e.g.* bioslurping); percolation; bioventing and air sparging; and electrochemistry.

Bioaugmentation. This is the direct application of indigenous (obtained via enrichment/isolation from the contaminated site), non-indigenous (obtained from an inoculum supplier) or genetically-engineered microorganisms to initiate and/or increase the rate of contaminant biodegradation (Leavitt and Brown, 1994; Otte *et al.*, 1994; Atlas, 1995).

Biostimulation. This is effected by the addition of oxygen, water and nutrients such as nitrogen, phosphorus and trace metals to promote the population of indigenous catabolic microorganisms (Leavitt and Brown, 1994; Margesin and Schinner, 2001). Addition of nutrients, such as nitrate, in *in situ* remediation programmes must, however, be approached with caution to prevent possible further contamination.

Landfarming. This treatment, which is also known as solid-phase treatment or land treatment, entails spreading the wastes in thin layers on soil and tilling to incorporate them into the soil matrix, thus, providing sufficient aeration to promote and optimize microbial activity (Eweis *et al.*, 1998).

Thermal Treatment/Enhancement. By the use of steam, heated water, radio frequency (RF) or electrical resistance (AC) heating, the temperature-dependent properties of contaminants *in situ* are altered, thus facilitating their mobilization, solubilization and removal. As a consequence, volatile and semi-volatile organic and inorganic contaminants may be vaporized, removed by vacuum extraction and treated. An excellent understanding of the hydrogeological conditions on site is essential for this approach (<http://www.gwrtac.org/html/techs.html>, 04-iv-01).

Pump-and-Treat Systems. In these processes, contaminated water is pumped from the polluted zone for treatment and possible reinjection into the aquifer after the addition of oxygen, nutrients and, sometimes, catabolic microorganisms. Alternatively, following treatment the reclaimed water may be discharged to a sewer or a land disposal site (Eweis *et al.*, 1998).

Percolation. Essential nutrients for bioremediation are added as a solution to the soil surface and allowed to migrate or flow through the vadose zone to the top of the contaminated aquifer. This type of bioremediation is limited to shallow aquifers because of the time required for effective migration into the contaminated zone (Eweis *et al.*, 1998).

Bioventing. This technique combines conventional advective soil venting, which takes advantage of the high volatility of some pollutants, and biodegradation of the less volatile high molecular weight compounds (Reisinger *et al.*, 1994; Van Eyk, 1994). Eweis *et al.* (1998) highlighted bioventing as an alternative approach to the excavation and *ex situ* treatment of less volatile biodegradable contaminants with Henry's law coefficients <0.1 . Solid matrices and shallow water tables may minimize the diffusion of gases and, thus, limit the effectiveness of this method.

Air Sparging. Oxygen is added to contaminated aquifers which are often oxygen limited. Sparging can be attained by forcing air into the aquifer or raising the water surface in the aquifer by air-lift action. Nutrient injection, such as the addition of ammonia to provide nitrogen, may be required. Also, nutrient percolation may be feasible in shallow aquifers and sandy soils (Eweis *et al.*, 1998).

Electrochemistry. This is the application of an electric field to soil and results in the direct movement of ions by electromigration, the flow of pore fluid by electroosmosis, ionic changes to the contaminants bonding to soil, and migration of charged particles, including microorganisms, by electrophoresis. Thus, pore fluid, bacteria and organic molecules may be moved relative to each other and so increase the contact between the contaminant(s) and the catabolic species (Rajeshwar and Ibanez, 1997).

Soil-treatment units, compost piles and engineered biopiles are examples of *ex situ* solid-phase techniques.

Soil-Treatment Units/Land-Treatment Units. This technique is also known as engineered landfarming. Excavated contaminated soil is treated as in landfarming but in engineered units which are designed to contain or minimize the transport of contaminants but maximize the treatment efficiency (Eweis *et al.*, 1998).

Compost Piles. This is an aerobic biological process in which wet organic solids, such as agricultural residue, garden and kitchen waste, municipal solid waste and sewage sludge (together with the soil contaminants) are oxidized to biologically-stable forms such as humus. Aeration, temperature, moisture content and pH are the parameters whose optimization is critical for efficient composting (Eweis *et al.*, 1998).

Engineered Biopiles. These are static compost piles which are engineered or designed to optimize the mixture composition, air distribution within the pile and the water-holding capacity and, therefore, facilitate rapid and extensive contaminant removal (Eweis *et al.*, 1998; von Fahnestock *et al.*, 1998). Aeration is often attained through a positive mode (forced aeration) or a negative mode (applied vacuum). The latter is, generally, preferred since gaseous emissions of volatile compounds are minimized

and can be treated, together with any off-gases from the piles, by biofiltration (Eweis *et al.*, 1998).

Bioreactors, ponds or lagoons exemplify *ex situ* slurry approaches which are defined by the creation and maintenance of a soil slurry as the bioremediation medium in which the raw materials are continually mixed and transformed biochemically into desirable and/or less undesirable by-products (Blackburn and Hafker, 1993; Eweis *et al.*, 1998).

Bioreactors. These can be classified according to: their mode of operation which may be batch, fed-batch or continuous; and the type of electron acceptor (aerobic, anaerobic or microaerophilic). The fundamental requirements for maximizing the biodegradation rate include: provision/maintenance of environmental conditions (pH, temperature); residence time; and substrate availability. The optimal environments created in bioreactors enhance microbial activity on a large scale and, thus, the required efficiency, in terms of the residual contaminant concentration, can be attained (Armenante *et al.*, 1998). Although versatile, with applications for treatment of various wastes, bioreactors are highly mechanized and, thus, require high capital and operation and maintenance costs (Eweis *et al.*, 1998).

Biofiltration. A final type of *ex situ* bioremediation is the biofiltration of gases removed by air sparging. This process involves the use of biofilters, which are closed packed-bed reactors, through which contaminated air is either blown or drawn. The packing surfaces facilitate adhesion of biofilms of microbial associations, extracellular polysaccharides and bound water (Matteau and Ramsay, 1997; Abumaizar and Kocher, 1998; Eweis *et al.*, 1998; Bibeau *et al.*, 2000).

Together with the above imposed bioremediation strategies, natural environments have the capacity to self-ameliorate or to undergo natural attenuation *in situ* (intrinsic bioremediation) (Zablotowicz *et al.*, 2001). Since this is a knowledge-based technology, it mandates consideration of three basic steps which are needed to document the evidence that it protects humans (and the environment) from unacceptable exposure risks:

1. Development of a conceptual model of the site's hydrogeology and biogeochemical reactions;
2. Analysis of site measurements to quantify the attenuation process by changes in the contaminant concentration and the reaction footprint; and
3. Establishment of a long-term monitoring programme to document the evidence that natural attenuation continues as expected (Rittmann, 2000).

Strong evidence has emerged that the right biotreatment, combined with a thorough knowledge of its limitations, can be highly effective in remediating a contaminated site (Lees, 1996; Wackett, 1997; Eweis *et al.*, 1998). Hence, it is important to identify some of the possible limitations of site microbial bioremediation. These include:

1. The length of time required;
2. Its limitation to biodegradable compounds;
3. Inhibition by toxic components; and
4. The long-term effects of the additions of microorganisms and nutrients are not well known and are insufficiently understood. Furthermore, more empirical evidence is required to provide knowledge to fill the gaps of how this technology can be effective under one set of circumstances but yet quite inappropriate under another (Stegmann *et al.*, 1994).

1.4.2 Carbon- and Nitrogen-Limited Pollutant Bioremediation

Bioremediation of sites contaminated with mixtures of pesticides (including *s*-triazines) has been proposed but little information exists on potential electron donor interactions and the influence of various nitrogen sources on catabolism (Alvey and Crowley, 1995; Ames and Hoyle, 1999). Also, a better understanding of the entire microbial community responsible for the degradation of a complex substrate and competition for nitrogen within the community is required. Such information could then be used to predict the rates of degradation of N-containing xenobiotic molecules in soil. Several authors (Assaf and Turco, 1994a; Alvey and Crowley, 1995; Grigg *et al.*, 1997; Ames and Hoyle, 1999; Gerbendinger and Radosevich, 1999; Abdelhafid *et al.*, 2000) have reported that the forms of C and N in a system were important factors affecting the mineralization of atrazine. The effects of N concentration on atrazine catabolism were most evident when nitrogen was supplied as inorganic nitrogen

rather than organic nitrogen via, for example, plant debris or *in situ* soil organic matter.

The release of petroleum hydrocarbons produces an environment with an abundant supply of carbon for microbial growth and metabolism (Nelson *et al.*, 1996; Margesi and Schinner, 2001). Thus, supplying oxygen, via biostimulation, landfarming or hydrogen peroxide (1.4.1), to the contaminated area should stimulate microbial degradation of the hydrocarbons by indigenous catabolic species. Gibson *et al.* (1998) demonstrated this approach by diffusing oxygen through silicone tubing to a BTEX-contaminated groundwater plume and, so, facilitating enhanced aerobic biodegradation. Under these conditions, however, insufficient assimilable nitrogen or, to a lesser extent, phosphorus is likely to limit the overall rate of biodegradation (Armstrong *et al.*, 1991; Nelson *et al.*, 1996). In situations where the concentrations of nitrogen and phosphorus are not rate limiting and a suitable electron acceptor is present, bioavailability may limit the rate of biodegradation of the contaminant(s) (Madsen, 1991). To overcome this, engineering solutions (Ryan *et al.*, 1991; Corseuil and Alvarez, 1996), such as pump, treat and reinjection or electrochemistry (1.4.1), may be considered particularly where the contaminants are adsorbed to soil components and the alternative of soil excavation would be difficult or extremely expensive.

1.5 Bioremediation Research Protocol Development

To develop a research protocol for bioremediation the following steps may be considered: enrichment/isolation of indigenous soil and aquatic catabolic microorganisms (Korda *et al.*, 1997); determination of the metabolic potentials of the isolated associations and optimization of the growth conditions (Tabak *et al.*, 1995); kinetic appraisal by use of laboratory bioreactors (Muller and Babel, 1996); molecular characterization (Thomas, 1996; Shapir *et al.*, 2000); and physiological profiling (Röling *et al.*, 2000a).

1.5.1 Enrichment and Isolation of Indigenous Soil Microbial Associations

Most environments support the growth of a wide range of microorganisms with different metabolic capabilities. Even complex xenobiotic compounds can be mineralized through the interactions of microbial associations (Senior *et al.*, 1976;

Slater and Lovatt, 1984; Knackmuss, 1996) with the numbers and types of microorganisms present in the local environment determining the biodegradative capacity. de Souza *et al.* (1998a) added a second tier when they postulated that bacteria of different genera, existing in close proximity, aided each other in growth and survival via gene transfer and metabolic cross-feeding.

A review of published literature suggested that microbial catabolism was the primary pathway for pesticide detoxification in terrestrial and aquatic ecosystems (Senior *et al.*, 1976; Cook, 1987; Shannon and Unterman, 1993; McBain *et al.*, 1996; Radosevich *et al.*, 1996). As introduced earlier (1.4.2), surface and subsurface releases of petroleum hydrocarbons produce environments which are laden with carbon (Nelson *et al.*, 1996). Several authors (Song and Bartha, 1990; Morgan, 1991; Atlas, 1995; Whyte *et al.*, 1999) have stated that hydrocarbon-degrading microorganisms are indigenous and their population sizes increase rapidly in response to oil inputs. This increased awareness of the capabilities of microorganisms to catabolize hydrocarbons stimulated the development of terrestrial bioremediation techniques (Freijer, 1996).

Laboratory studies of pollutant biodegradation are best made with microbial associations taken from the field with the results then used to predict *in situ* catabolic rates (Spain and van Veld, 1983). Inherent problems of laboratory studies must, however, be recognized (Madsen, 1991). Atlas (1995) reported that most microorganisms considered for bioremediation by microbial seeding/augmentation are obtained via enrichment cultures from previously-contaminated sites. The activation, through enrichment, of indigenous microbial associations results in acclimated, resistant and active biomass which, in turn, accelerates mineralization. Whyte *et al.* (1999) reported that the use of associations of enriched indigenous degradative microbial populations was particularly advantageous even in a cold-temperature site. They concluded that enriched indigenous populations should be acclimated to specific on-site conditions, soil characteristics and the nature of the contaminant(s).

1.5.2 Determination of Metabolic Potentials and Optimization of Growth Conditions

The fates of environmental pollutants are determined largely by abiotic processes, such as photooxidation (Konstantinou *et al.*, 2001), and by the metabolic activities of microorganisms (Knackmuss, 1996). Tabak *et al.* (1995) observed that there had been

an increased interest in understanding the fates of pollutants in soil and water systems and determining the mechanisms and rates of biodegradation. Intrinsic bioremediation is often limited by several factors such as the physicochemical and environmental conditions and, hence, directed biotechnology/bioremediation offers a way to eliminate the target molecule(s). Such technology is defined mainly by the principle of optimizing the environmental conditions so that the biodegradation occurs rapidly and efficiently (Morgan, 1991). Furthermore, a fundamental understanding of biodegradation kinetics and the factors controlling the catabolic rate can provide an insight of the optima and ranges of specific environmental parameters for microbiological activity and, thus, contaminant biodegradation (Tabak *et al.*, 1995).

If laboratory studies are to be used to predict biodegradation rates *in situ*, it is important that the rate-determining factors are identified and understood (Spain and van Veld, 1983). Madsen (1991) stated that the majority of biodegradation investigations which used environmental samples (water, soil and sediment from contaminated and uncontaminated sites) in laboratory flask assays demonstrated biodegradative potential but not necessarily *in situ* bioremediation. However, once microbial metabolism of the contaminant compound has been established, a variety of influential environmental and ecological parameters such as pH, redox potential, water activity, nutrients, electron acceptors, inocula and contaminant concentrations may be investigated.

1.5.3 Use of Laboratory Bioreactors

As introduced earlier (1.4.1), bioreactors have been adopted for the *ex situ* remediation of contaminated soils and groundwater (Eweis *et al.*, 1998; Alexander, 1999; Campos *et al.*, 2000). They provide the physical environment in which biochemical transformations, mediated by the catalytic effects of intact, free-living microbial cells or enzymes, can occur (Senior *et al.*, 1976; Katz *et al.*, 2000).

Retentostats are laboratory bioreactors combined with filtration devices to retain the biomass and, thus, in comparison with chemostats, they offer a more realistic approach to study the physiological properties of microorganisms with respect to substrate provision and availability (van Verseveld *et al.*, 1984; Tappe *et al.*, 1999). Some parameters which are central to effective bioremediation, such as maintenance energy requirements, have been investigated with retentostat cultures (Muller and Babel, 1996; Tappe *et al.*, 1999). Through laboratory cultivation study,

conditions which promote the proliferation of specific association species which have increased catabolic capacities, for use as inocula for *in situ* and *ex situ* bioaugmented remediation, may be identified (Newcombe and Crowley, 1999).

1.5.4 Molecular Characterization

Bacterial communities are a vital part of environments. Therefore, an understanding of the compositions of the relevant associations and the factors which contribute to their survival and change is important for the development of methods to diagnose problems, predict behaviour, optimize productivity and prepare bioremediation strategies (Thomas, 1996). Although intrinsic bioremediation of compounds such as atrazine and petroleum hydrocarbons by indigenous microorganisms has motivated extensive research, the molecular microbial ecology of the key catabolizing associations has received little attention (de Souza *et al.*, 1996). Ames and Hoyle (1999) also highlighted that chemical parameters, such as (sediment) N content or atrazine residues, could not be used alone as good primary predictors of biodegradative potential. Therefore, understanding the distribution of contaminant-catabolizing microorganisms, genes or enzymes is of paramount importance.

Furthermore, the successful implementation of *in situ* bioremediation, in particular, requires a detailed characterization of the contaminated site in relation to the pollutant, the hydrogeochemistry and the microbiology (Mason *et al.*, 1998; Haack and Bekins, 2000). These researchers suggested that molecular methods could help provide answers to key questions which must be addressed, namely:

1. Is the biological potential for degradation present (Guo *et al.*, 1997);
2. What is the expressed level of biodegradative activity *in situ* (Chandler and Brockman, 1996; Shapir *et al.*, 2000);
3. What factors limit the *in situ* rate of pollutant degradation; and
4. Can the degradative activity be stimulated?

The need to explore rapidly and comprehensively the diversity and analyze the structure of catabolic microbial associations, while complementing the traditional, time-honoured microbiological procedures, has since been realized (Muyzer *et al.*, 1993; de Souza *et al.*, 1998c; Head *et al.*, 1998; Green and Scow, 2000). A number of researchers (Brockman, 1995; Garland, 1997; Heuer and Smalla, 1997a) have observed that studies of the diversity of natural populations have been restricted to

culturable microorganisms. Hence, the recognition that most microorganisms in environmental samples can be neither cultured yet in the laboratory nor studied by traditional methods involving cultivation commends the use of various molecular techniques to analyze and study microbial communities (Wagner *et al.*, 1993; Amann *et al.*, 1995; Sekiguchi *et al.*, 1999; Timmis and Stahl, 1999; Shapir *et al.*, 2000). Conventional culture-dependent approaches are also limited since any departure from the original environmental parameters during cultivation can alter the community structure through the imposition of new selection pressures (Liu *et al.*, 1997).

Some molecular techniques have been used alone or as part of a more comprehensive protocol. The study of nucleic acids extracted from environmental samples allowed analysis independent of the artefacts that can arise from laboratory degradative potential assays, laboratory culture-based enumerations and the inability to culture a large proportion of the microorganisms present in the environment (Brockman, 1995). Some workers (Kuske *et al.*, 1998; Muyzer and Smalla, 1998) highlighted the need for broad-based, non-selective DNA extraction procedures or molecular techniques which explore the complexities of microbial associations and their dynamics. Therefore, unbiased representation of community members is obtained rather than a focus on certain microorganisms for which probes have been developed. Consequently, DNA extraction from environmental samples and its subsequent purification from any co-extracted contaminants such as humic acids, must be sufficiently efficient for successful detection and characterization (Tebbe and Vahjen, 1993; Kuske *et al.*, 1998). It must be also of a sufficient quality to permit optimal enzyme activity in subsequent procedures (Head *et al.*, 1998).

Techniques which are dependent on DNA melting behaviour have been employed (Stefan and Atlas, 1991; Head *et al.*, 1998; Madsen, 2000) to assess community structure and provide relatively qualitative estimates of species or microbial diversity. Some researchers (Ramsing *et al.*, 1996; Liu *et al.*, 1997; Stapleton and Sayler, 1998; Tani *et al.*, 1998) adapted these to provide more quantitative information. The polymerase chain reaction (PCR) and other PCR-based methods (*e.g.* denaturing- or temperature-gradient gel electrophoresis (DGGE / TGGE) and terminal-restriction fragment length polymorphism (T-RFLP)) have been used to detect catabolic genotypes in soil and have also been reported to provide information on *in situ* conditions without requiring cultivation or activation of the indigenous microbial populations (Hallier-Soulier *et al.*, 1996; Tani *et al.*, 1998;

Osborn *et al.*, 2000; Weber *et al.*, 2001). Specifically, PCR product analysis provides a sensitive and specific means to detect and monitor microorganisms in complex environmental samples (Kuske *et al.*, 1998). Salyers (1999) concluded that a sweep of the molecular revolution through environmental microbiology has sensitized an appreciation for the diversity of microorganisms and has further posed an irrefutable challenge to cultivate and/or study all the microorganisms which might have been “missed” to date. Furthermore, a combination of classical culture-based microbiological techniques and molecular approaches should help molecular microbial ecologists/molecular environmental microbiologists to address these challenges. Although indisputably effective, the uses of molecular techniques in microbial ecology have some limitations and these have been collated in several reviews (Amann *et al.*, 1995; Wilson, 1997; Head *et al.*, 1998; Muyzer and Smalla, 1998).

Denaturing-gradient gel electrophoresis has been identified as one of the molecular techniques available to environmental microbiologists and has revolutionized the monitoring of diverse bacterial communities (Thomas, 1996) thus facilitating the study of microbial population dynamics. Kowalchuk *et al.* (1997) described it as a powerful and convenient tool to analyze the sequence diversity of complex natural microbial populations. The technique is further commended by its specificity and sensitivity since it is able to detect rapidly as little as a single base change in a given sequence without the need for sequence information (Fodde and Losekoot, 1994; Muyzer and Smalla, 1998). Denaturing-gradient gel electrophoresis provides information about sequence variation in a mixture of polymerase chain reaction (PCR) fragments of identical length and is, thus, used to identify differences in the compositions of microbial associations. The denaturing gradient is achieved chemically, with urea and formamide, or physically, with temperature in TGGE (Reisner *et al.*, 1989). Heuer and Smalla (1997a) reported that the two techniques are interchangeable since they give comparable fingerprints of the same microbial association. When coupled with sequencing, DGGE/TGGE facilitates the detection of specific pollutant-catabolizing members in relation to key environmental factors (Kowalchuk *et al.*, 1997).

1.5.5 Community-Level Physiological Profiling (CLPP)

Garland (1997) postulated that microbial associations offered a potentially powerful tool to advance our understanding of how community processes affect ecosystem processes; a central challenge in ecology. A community-level approach (the Biolog EcoPlate assay) to assess patterns of sole carbon source utilization by microbial associations has been used increasingly to counter the lack of effective methods to describe microbial associations which has limited the elucidation of microbial community dynamics (Garland, 1997; Konopka *et al.*, 1998). The assay has been employed for investigations of soil microbial communities to: distinguish spatial and temporal differences (Hitzl *et al.*, 1997; Bossio and Scow, 1998; Laverman, 2000); determine the effects of herbicides and organic pollutants (El Fantroussi *et al.*, 1999; Thompson *et al.*, 1999); and explore the implications of inoculation with non-indigenous or genetically-modified microorganisms (England *et al.*, 1995; Vahjen *et al.*, 1995; van Elsas *et al.*, 1998).

Some sources of error have, however, been encountered by researchers in the use of the Biolog system to profile microbial associations. For example, inoculum density, whether high or low, allows for shifts in microbial community structure as a result of competition for the carbon source(s). Thus, the obtained physiological profile may not be representative of the original association but may simply reflect the more versatile members which can adapt and grow under the assay conditions (Haack *et al.*, 1995; Garland 1997; Heuer and Smalla, 1997b; Smalla *et al.*, 1998).

The physiological state of some association members may influence the results with dormant, starved and “viable but non-culturable” species, particularly in environmental samples, (Roszak and Colwell, 1987) giving negative results or contributing little or nothing to the physiological profile of the microbial association (Konopka *et al.*, 1998).

Incubation time is another significant variable. In contrast to short incubation periods which discriminate fast-growing microorganisms and readily-catabolized substrates, and often give few positive wells, long incubation periods result in more positive wells but less resolution between inoculated samples (Konopka *et al.*, 1998). Thus, substrates which allow the greatest discrimination between samples may change with time (Kersters *et al.*, 1997).

Although conflicting, two recommendations of a high inoculum density with a short incubation time (Garland, 1997) or a prolonged incubation time to overcome

inoculation density problems (Wunsche *et al.*, 1995), have been made to optimize the impacts of these two parameters on the resulting physiological profiles. The use of: inocula of approximately equal density (Haack *et al.*, 1995); calculation of average well colour development for each microtitre plate (Garland and Mills, 1991); readings after different incubation times (Garland, 1996); choice and standardization of data analysis (Zak *et al.*, 1994; Hackett and Griffiths, 1997; Howard, 1997; Karthikeyan *et al.*, 1999); and cautious interpretation of the results, should address adequately the possible concerns.

Community-level physiological profiling has been identified as ideal to elucidate carbon source utilization patterns since molecular techniques based on PCR and 16S rDNA, for example, do not give information on physiological capabilities (Röling *et al.*, 2000a; b). Substrate utilization profiles, however, do not assess directly microbial community diversity or changes at the genetic level owing, particularly, to the genetic redundancy inherent in microbial communities (Konopka *et al.*, 1998). Thus, microbial diversities can be examined best by a combination of physiological, molecular and cultivation techniques.

1.6 Conclusions

Catallo and Portier (1992) identified the need to develop waste reduction and remediation technologies which are efficient, economic and rapidly deployable in a wide range of settings. Remediation processes must reduce effectively chemical mobility, soil toxicity and chemical concentration (Loehr and Webster, 1997). Biostimulation of indigenous microbial populations with nutrient supplements and bioaugmentation with degradative inocula are attractive bioremediation strategies because they increase the pollutant degradation rates (Whyte *et al.*, 1999). There is an increasing urgency to protect groundwater in South Africa where desertification continues. This, in turn, motivates the development of appropriate biological technologies to remediate contaminated soils and policies or codes of practice to protect surface- and ground-water (Pick *et al.*, 1992; Pearce *et al.*, 1995).

An understanding of the dynamics of microbial communities has remained limited because only a small fraction of all cells in natural ecosystems is, as yet, amenable to cultivation techniques. The incorporation of molecular protocols in

environmental microbiology is, therefore, imperative to address a specific problem and develop methods for implementation under specific (South African) conditions.

1.7 Research Objectives

Against the above background, this research programme was developed with five central objectives:

1. Enrichment/isolation of indigenous aerobic microbial associations which catabolized atrazine and selected petroleum hydrocarbons (benzene, toluene and *o*-, *m* and *p*-xylene) for, subsequent, bioremediation of soils contaminated with these molecules;
2. Optimization of the efficacies of the associations through manipulation of key physiological determinants;
3. Elucidation of atrazine-catabolizing association dynamics under C- and N-limited conditions in retentostats (and so model *ex situ* bioremediation);
4. Molecular and physiological characterization of the interacting microbial associations and determination of their catabolic potentials; and
5. Identification of selected cloned association members by sequencing of 16S rDNA.

Chapter 2: Materials and Methods

2.1 Soil Collection, Storage and Analysis

Ten kg of a loamy atrazine-exposed soil were obtained from the University of Natal Farm at Ukulinga in KwaZulu-Natal Province, South Africa. Atrazine ($5 \text{ ml } 5\text{l}^{-1}$) had been applied as annual spray applications of $4.5 - 5 \text{ l ha}^{-1}$ for almost 20 years in maize trial plots. Pristine soil (10 kg) was also collected from the same area from an adjacent plot.

Similarly, 10 kg of petroleum hydrocarbon-contaminated sandy soil were collected from several areas near a leaking ($>2 \text{ yr}$) oil storage tank on a construction site in a Pietermaritzburg industrial area, also in KwaZulu-Natal.

The different soils were homogenized by hand and stored in sealed plastic bags in the dark at 4°C until needed. Some pristine soil (1 kg, dry weight) was stored under the same conditions after air-drying, sieving (0.2 - 0.4 mm) and gamma irradiation (Gamwave, Durban, South Africa).

Samples of the chemically-compromised soils were classified and analysed for density, P, K, Ca, Zn, Mn and Mg contents, exchange acidity, acid saturation, pH (KCl), total organic carbon and clay content (Soil Fertility and Analytical Services, KwaZulu-Natal Department of Agriculture, Pietermaritzburg).

2.2 Preparation of Media

Three basic mineral salts solutions and a soil extract solution were prepared for use in this study.

2.2.1 Mineral Salts Solutions

The first salts solution, designated M-I, contained (g l^{-1} distilled water): K_2HPO_4 , 1.5; KH_2PO_4 , 0.5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2. The second solution (M-II) was a modification of M-I and contained (g l^{-1} distilled water): K_2HPO_4 , 10.1; KH_2PO_4 , 1.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6; NH_4Cl , 0.25; Titriplex I, 0.1; and trace element solution, 1 ml. The trace element solution contained (g l^{-1} distilled water): $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 14.7; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 24.32; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 9.89; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 7.19; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.38; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.85; H_3BO_3 , 0.31; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.42; and concentrated H_2SO_4 , 5 ml. The third mineral salts solution (M-III) was prepared as described by

Mandelbaum *et al.* (1993; 1995). Sterilization was made by autoclaving at 15 lb psi-121⁰C for 15 min.

2.2.2 Soil Extract Solution

Pristine soil (1 kg, fresh weight) was mixed with 1 l of distilled water and stirred vigorously for 1 h. The slurry was then passed through a series of three #2 Whatman filter papers into clean flasks. The soil extract solution was then filter-sterilized (Millipore, 0.22 μ m) and stored at 4⁰C in a sealed autoclaved (15 lb psi-121⁰C, 15 min) bottle until needed.

2.3 Enrichment/Isolation of Aerobic Catabolic Microbial Associations

The enrichment/isolation of catabolic microbial associations was initiated soon after collection of the contaminated soils.

2.3.1 Atrazine-Catabolizing Microbial Associations

Twenty enrichment cultures were made in 250 ml cotton wool-closed Erlenmeyer flasks which contained 10 g (fresh weight) of atrazine-exposed soil and 100 ml of basic mineral salts solution (M-I) (2.2.1). The flasks were divided into two groups based on pH which was poised initially at 5.5 or 7.5 with 1 N HCl or 1 N NaOH, respectively.

Four atrazine concentrations: 10, 20, 30 and 33mg l⁻¹ (0.046, 0.092, 0.138 and 0.152 mM), together with a control, were used each in duplicate. Two additional sets of soil-inoculated controls, poised at pH 5.5 and 7.5, were autoclaved (15 lb psi-121⁰C, 15 min) and used to confirm that any atrazine catabolism observed was due to microbial activity. The flasks were agitated on a rotary shaker (New Brunswick Scientific) at 150 rpm and incubated at 30⁰C in the dark for 25 weeks. Subculturing (10% v/v) was made every 4 weeks. Analyses of residual atrazine (2.9.5) and pH (2.9.1) were made every week. One atrazine-catabolizing microbial association for each concentration and each pH was selected and the eight cultures (Table 2.1) were then stored in 20% (v/v) glycerol at 4⁰C.

2.3.2 BTX-Catabolizing Microbial Associations

A similar protocol of two pH values and four concentrations for each molecule was adopted for the enrichment/isolation of forty benzene-, toluene-, *o*-, *m*- and *p*-xylene-catabolizing microbial associations. The petroleum-contaminated soil (500 g, fresh weight) was mixed with 1 l of distilled water and shaken vigorously by hand to displace the microorganisms. The suspension was then filtered through a series of three #2 Whatman filter papers into clean flasks.

Fifty ml volumes of basic mineral salts solution M-I were then each inoculated with 5 ml of the filtrate in 250 ml Erlenmeyer flasks which each contained a small bottle with 10 ml of the molecule (100%) of interest inserted. Thus, the molecule was made available to the microbial populations via volatilization. The flasks were also closed with cotton wool and teflon tape and incubated (30°C) in the dark with shaking (150 rpm) for eight weeks. The progress of each enrichment was monitored every week by pH (2.9.1) and optical density measurement (2.9.2).

Subculturing (10% v/v) was then made every week, for eight weeks, into sterile mineral salts solution M-I to which the BTX compounds were added separately and directly to final concentrations of 5, 50, 500 and 5 000 mg l⁻¹. The final microbial associations (Table 2.2) were stored in 20% (v/v) glycerol at 4°C.

Table 2.1: Aerobic microbial associations enriched/isolated from soil in the presence of four atrazine concentrations and two initial pH values.

Initial pH Value	[Atrazine] (mg l ⁻¹)	Association Designation
7.5	33	KRA01
	30	KRA02
	20	KRA03
	10	KRA04
5.5	33	KRA05
	30	KRA06
	20	KRA07
	10	KRA08

Table 2.2: Aerobic microbial associations enriched/isolated from soil in the presence of four different concentrations of the individual BTX components and two initial pH values.

Initial pH Value	[BTX] (mg l ⁻¹)	Association Designation				
		Benzene	Toluene	<i>o</i> -Xylene	<i>m</i> -Xylene	<i>p</i> -Xylene
7.5	5 000	KRB01	KRT01	KRO01	KRM01	KRP01
	500	KRB02	KRT02	KRO02	KRM02	KRP02
	50	KRB03	KRT03	KRO03	KRM03	KRP03
	5	KRB04	KRT04	KRO04	KRM04	KRP04
5.5	5 000	KRB05	KRT05	KRO05	KRM05	KRP05
	500	KRB06	KRT06	KRO06	KRM06	KRP06
	50	KRB07	KRT07	KRO07	KRM07	KRP07
	5	KRB08	KRT08	KRO08	KRM08	KRP08

2.4 Isolation of Culturable Component Monocultures

For each isolated microbial association, a dilution series (10^{-2} to 10^{-8}) with 1/4-strength Ringer's solution was made to prepare inocula (each 0.1 ml) for spread plates. The media used were nutrient agar, and soil extract solution (2.2.2) and basic mineral salts solution (M-I) (2.2.1) supplemented with atrazine or individual BTX component and set with agar (1.5% m/v). The plates were incubated at 30°C until growth was observed. Individual colonies were subcultured onto fresh medium and reincubated prior to morphological characterization by light and scanning electron microscopy. Plate cultures of the isolates were then stored at 4°C in sealed plastic bags.

2.5 Physiological Optimization

2.5.1 Inoculum Preparation

After storage at 4°C in the dark for approximately three months, the isolated aerobic atrazine- and BTX-catabolizing microbial associations were inoculated (50% v/v) into sterile mineral salts solution M-I (2.2.1) poised at the respective pH values (5.5 or 7.5) and supplemented with appropriate concentrations of the compounds. The flasks were closed with cotton wool bungs and incubated (30°C) in the dark on a rotary shaker (150 rpm) for two weeks. The pH 5.5- and 7.5-poised atrazine (30 mg l⁻¹)-catabolizing

cultures were then mixed to ensure that all the components were present. This mixing step was then repeated for the respective BTX (50 mg l^{-1})-catabolizing microbial associations.

The resulting cultures (Table 2.3) were then inoculated (5 ml) individually into 200 ml of sterile mineral salts solution M-I (2.2.1) supplemented with the appropriate concentration of atrazine (30 mg l^{-1}) or BTX molecule (50 mg l^{-1}). After incubation (30 $^{\circ}$ C with shaking on a rotary shaker at 150 rpm) in the dark overnight until the log phases were reached, the cultures were centrifuged (J2-HS, Beckman) at 10 000 rpm x g for 10 min. The pellets were rinsed with and resuspended in fresh sterile mineral salts solution M-I prior to reinoculation (10 ml) into 40 ml of the appropriate culture medium.

2.5.2 *Experimental Protocol*

A total of twenty-five and twenty 250 ml Erlenmeyer flasks were inoculated for five replicates for each pH (4, 5, 6, 7 and 8) and each temperature (15, 20, 25 and 30 $^{\circ}$ C), respectively, for atrazine (30 mg l^{-1}) and each of the BTX components (50 mg l^{-1}). The flasks were incubated in water baths in the dark and shaken (150 rpm) to facilitate aerobiosis. Hourly optical density readings (2.9.2) were made on 1 ml aliquots of the cultures until the late logarithmic phases were reached. The samples were placed on ice in plastic cuvettes to slow the growth and thus minimize sampling/analysis time variability. The cuvettes were wiped thoroughly to avoid inaccurate readings from condensation. At each sampling time, atrazine residual substrate concentration determinations (2.9.5) were also made.

Table 2.3: pH 7.5 and 5.5 and combined aerobic cultures of atrazine (30 mg l^{-1})- and BTX (50 mg l^{-1})-catabolizing microbial associations.

pH 7.5 Association	pH 5.5 Association	Combined Association
KRA02	KRA06	KRA30
KRB03	KRB07	KRB50
KRT03	KRT07	KRT50
KRO03	KRO07	KRO50
KRM03	KRM07	KRM50
KRP03	KRP07	KRP50

pH Optimization

To identify the optimum pH for growth of the combined microbial associations, the medium was poised with citrate-phosphate buffer at five different pH values (Table 2.4) on the basis of the pH range of South African soils. The pH of each culture was measured (2.9.1) initially and then at the end of the log phase.

Table 2.4: Constituents (m l) of citrate-phosphate buffer used for pH control of batch cultures (adapted from Merck Tables, 1999).

pH	tri-Sodium citrate (0.1 mM)	di-Sodium hydrogen phosphate (0.2 mM)
4	62.0	38.0
5	49.0	51.0
6	37.4	62.6
7	19.0	81.0
8	9	91.0

Temperature Optimization

The four temperatures were selected according to the annual soil temperatures of KwaZulu-Natal. The pH value for which the highest specific growth rate was recorded for each combined microbial association was used and was poised as described above.

2.6 The Catabolic Potential of Association KRA30

2.6.1 Media

Continuous cultures, set in retentostat mode, were made under C- and N-limited conditions with three mineral salts solutions: M-I; M-II in the presence/absence of NH₄Cl; and M-III (2.2). Where atrazine was added the concentration used was 100 mg l^{-1} and the herbicide was solubilized by autoclaving with the salts solution. High-performance liquid chromatography analysis (2.9.5) was made before and after autoclaving to ensure that the structure and concentration of the molecule were maintained. Where citric acid or succinic acid was added as a primary or supplementary carbon source then the concentration was 3 mM. Details of the different carbon and nitrogen source additions are given in Table 2.5.

Table 2.5: Retentostat cultivations of microbial association KRA30 and *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995) under C- and N-limited conditions.

Retentostat	Association/ Monoculture	C/N Source			Medium			
		Atrazine	Citric acid/tri- Sodium citrate	Succinic acid	NH ₄ ⁺	M-I	M-II	M-III
1	KRA30	+	-	-	+	+	-	-
2	+	+	+	-	+	+	-	-
3	+	-	+	-	+	+	-	-
4	+	+	-	-	+	-	+	-
5	+	+	-	-	-	-	+	-
6	+	+	+	-	-	-	+	-
7	+	+	-	+	-	-	+	-
8	+	+	+	-	-	-	-	+
9	+	+	-	-	-	-	-	+
10	+	-	+	-	-	-	-	+
11	-	+	-	-	+	+	-	-
12	-	+	+	-	-	-	-	+
13	<i>Pseudomonas</i> sp. strain ADP	+	-	-	+	+	-	-
14	+	+	+	-	+	+	-	-
15	+	+	+	-	-	-	-	+
16	+	-	+	-	-	-	-	+

2.6.2 Inoculum Preparation

Following physiological optimization, the combined atrazine-catabolizing association KRA30 was used as the inoculum in this study. Aerobic shake flasks of the different media (100 ml) (Table 2.5) were inoculated (10% v/v) with this association and the cultures were incubated at 30⁰C until the logarithmic phase was reached. The cultures were then transferred aseptically to the bioreactors to give initial optical density readings of 0.02 (A₆₀₀). This was repeated for *Pseudomonas* sp. strain ADP (Mandelbaum *et al.*, 1995).

2.6.3 Experimental Protocol

Twelve 1 l retentostats (working volume 400 ml) (Figure 2.1) with M-I or M-III were used to examine the growth of association KRA30 and *Pseudomonas* sp. strain ADP under C- and N-limited conditions (Table 2.5) while four 5 l bioreactors (working volume 4.5 l) with M-II supplemented with atrazine (100 mg l⁻¹), in the presence or absence of NH₄Cl, citric acid and succinic acid, were also used to examine growth of the association under elemental limitations. Two uninoculated retentostats, M-I plus atrazine and M-III plus atrazine, were included as controls.

Medium was pumped into each culture vessel at a rate of 1 l h⁻¹ (Watson-Marlow, Falmouth, UK). The individual culture volumes were maintained with a liquid level sensor (Biology Faculty Mechanical Department, Vrije Universiteit, Amsterdam, The Netherlands) which activated a peristaltic pump to remove the filtrate at a rate of 1 l h⁻¹. The bioreactors were aerated at a rate of 20 l h⁻¹ and stirred at 250 – 300 rpm. For each vessel, the speed of the stirrer was modified to reduce foaming. Mass spectrometry connections were made to measure the oxygen consumption and carbon dioxide production rates. The outlet gas was passed through a cooled condensing flask (250 or 500 ml) before it entered the mass spectrometer (MM8-80F, VG Gas Analysis Systems). The gas inlet and outlet tubes were stainless steel and each retentostat was connected to a thermocirculator (Jubalo Poratherm U5, Juchheim Labortechnik, Germany) to maintain the temperature at 30°C.

Prior to sampling, the sample ports were flushed with the culture to eliminate dead spaces. Samples (5 ml) were taken every 24 h with sterile syringes fitted with sterile needles and dispensed into sterile 1.5 ml Eppendorf tubes. Analyses of optical density (2.9.2), residual substrate concentration (2.9.5), total organic carbon (2.9.6), cell counts and size distribution (2.9.7), and molecular fingerprinting (2.10) were made immediately or after storage at 4°C or -20°C.

The combined association KRA30 was used also to study the effects of different citrate/citric acid concentrations on nitrogen-limited atrazine catabolism in aerobic batch cultures. Thus, KRA30 was cultured (30°C) in the presence of atrazine (30 mg l⁻¹)-supplemented M-III in the presence and absence of supplementary citric acid (3 mM). Two controls were included, one which contained M-III plus supplementary citric acid but no atrazine and one which contained atrazine-supplemented M-III but was not inoculated. The cultures were sampled at regular

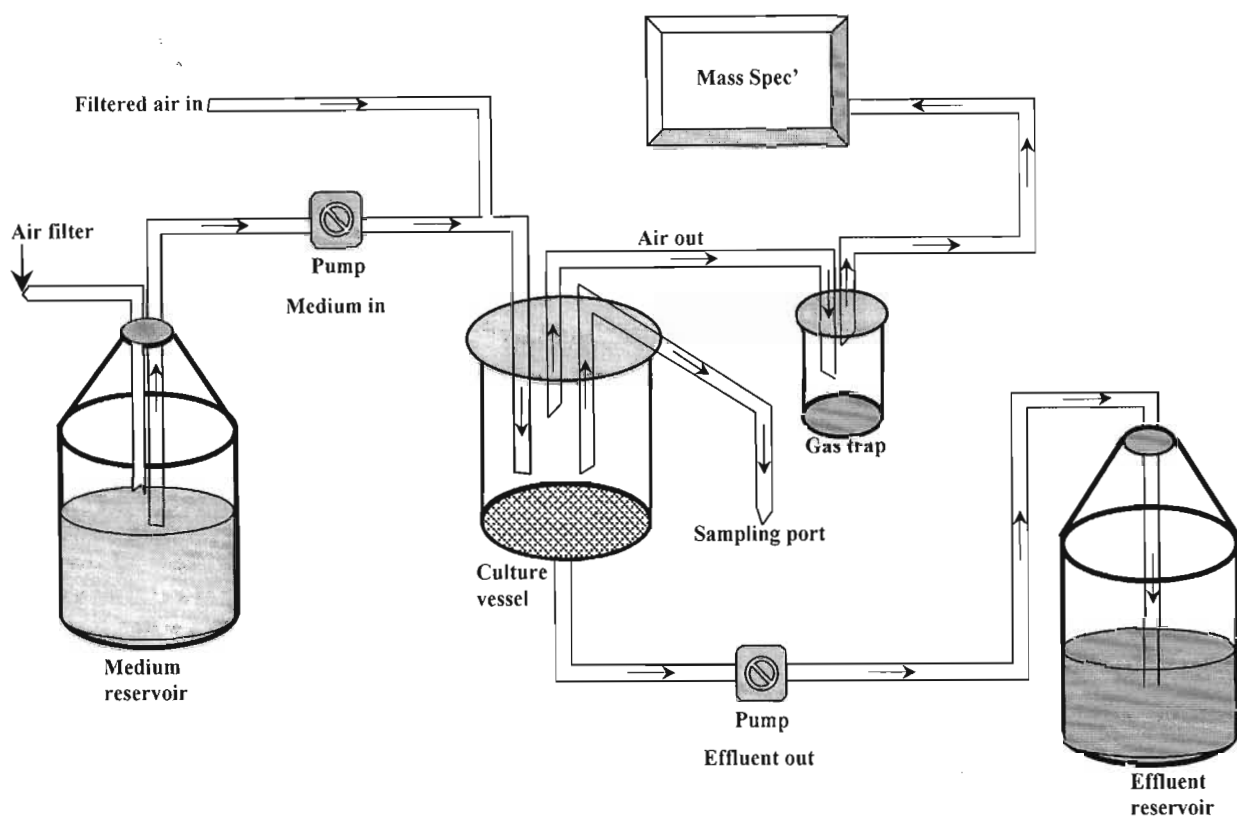


Figure 2.1: Retentostat configuration (Stouthamer *et al.*, 1990) used to culture the combined atrazine-catabolizing microbial association KRA30 and *Pseudomonas* sp. strain ADP under C- and N-limited conditions.

intervals for optical density (2.9.2) and residual atrazine concentration (2.9.5) determinations.

2.7 Bacterial Adsorption and Biodegradation of Atrazine

2.7.1 *Inoculum Preparation*

Microbial association KRA30 was grown to mid-logarithmic phase ($A_{600} = 0.2$) in M-III supplemented with atrazine to a final concentration of 100 mg l^{-1} . *Pseudomonas* sp. strain ADP was cultured in the same medium and was used as a positive control. Both cultures were centrifuged (J2-HS, Beckman) at $10\,000 \text{ rpm} \times g$ for 10 min and the pellets rinsed with sterile phosphate buffer (pH 7) prior to recentrifugation under the same conditions. The pellets were then resuspended in phosphate buffer to reinstate the optical density of 0.2. A second log phase culture of KRA30 was treated in the same way, after the O.D. was adjusted to 0.2 with M-III, prior to autoclaving (15 lb psi – 121°C , 15 min) and use as the inoculum for the negative control.

2.7.2 *Experimental Protocol*

Cultures and controls (50 ml M-III, 100 mg l^{-1} atrazine) with 10% (v/v) inocula in 250 ml Erlenmeyer flasks were initiated together with uninoculated blanks. All the flasks were incubated (30°C) in the dark with shaking at 150 rpm. Samples were taken at regular intervals for optical density (2.9.2) and residual atrazine concentration (2.9.5) determinations.

2.7.3 *Adsorption to Biomass in Retentostat Cultures*

Samples (1 ml) of the atrazine-catabolizing association KRA30 cultured (30°C) in 5 l retentostats (working volume 4.5 l) with mineral salts solution M-II supplemented with atrazine (100 mg l^{-1}) plus citric acid (3 mM) were taken and centrifuged (Eppendorf Centrifuge 5410) at $14\,000 \text{ rpm} \times g$ for 5 min. The pellets were then rinsed with HPLC-grade water prior to recentrifugation under the same conditions. The pellets were then resuspended with moderate shaking by hand in 1 ml HPLC-grade methanol. Following centrifugation (Eppendorf Centrifuge 5410) at $14\,000 \text{ rpm} \times g$ for 5 min, the supernatants were analyzed by HPLC to determine the concentration of atrazine (2.9.5) adsorbed to the biomass.

2.8 Sorption of Atrazine in Soil

2.8.1 Effect of Atrazine Concentration on Adsorption/Desorption

Some modifications were made to the protocol described by Gao *et al.* (1998). Sterile sieved pristine soil samples (10 g, dry weight) (2.1) were mixed with 25 ml of 0.01 M CaCl_2 in 100 ml Erlenmeyer flasks. Blanks without soil were used as controls. The final atrazine concentrations used were 30, 50, 100, 500 and 1 000 mg l^{-1} (0.138, 0.23, 0.46, 2.3, 4.6 mM). Triplicate flasks for each concentration were shaken (150 rpm) at room temperature for 24 h. The soil slurries were then centrifuged (J2-HS, Beckman) at 10 000 rpm x g for 10 min. Each supernatant (15 ml) was extracted with a C18 solid-phase column (1 g, Baker, Phillipsburg, N.J., USA) which was then eluted with 4 ml of ethyl acetate. Each sample was reduced to about 1 ml with a rotovapour (Heidolph, Germany) at 45°C and 140 rpm, and dried under a slow stream of nitrogen. The resulting residue was dissolved in 1 ml of 80:20 (v/v) methanol:water and the atrazine concentration determined by GC analysis (2.9.5). The atrazine adsorbed was calculated by subtracting the supernatant concentration from the original.

For each soil sample three consecutive desorption measurements were made. After centrifugation, the supernatants were replaced by an equal volume of 0.01 M CaCl_2 and the pellets resuspend by agitation. pH measurements were made at each resuspension to ensure pH maintenance. Centrifugation, extraction and analysis were then made as above.

2.9 Analyses

2.9.1 pH

Culture supernatant pH values were measured with a Crison micro pH 2000 meter fitted with a Crison electrode.

2.9.2 Optical Density

A Milton Roy Spectronic 301 spectrophotometer was used to measure optical densities at A_{600} . When necessary, the optical density measurements were corrected by dilution for linearity.

2.9.3 Gram Characterization

Gram-stained preparations were examined with a light microscope (Zeiss, x100 lens). *Escherichia coli* B was used as a Gram-negative control.

2.9.4 Electron Microscopy

A Hitachi 570-S Scanning Electron Microscope was used to view both the atrazine- and BTX-catabolizing associations/component monocultures (2.4). Aliquots (2 ml) of liquid cultures were filtered (Nucleopore Track-Etch Membrane, 0.4 μm x 47 mm) and fixed with 3% (v/v) glutaraldehyde. Sample fixation was repeated for colonies of the isolated microbial associations/component monocultures. Each sample was subjected to an incrementally-increased ethanol concentration series (30 to 100% v/v) for 10 min per wash with the last wash repeated three times. A final wash in 0.05 M cacodylate buffer (2 x 30 min) was then made. Critical point drying (CPD) was made in a Hitachi Critical Point Dryer (HCP-2) at 30°C and a pressure of 80 kgfcm⁻² for 1 h. The dry samples were then carbon coated and viewed.

2.9.5 Residual Substrate Concentration

Residual atrazine concentrations were quantified by GC and HPLC. Samples (1 ml) of culture were centrifuged (Eppendorf Centrifuge 5410) at 14 000 rpm x g for 15 min and filtered (Millipore, 0.45 μm). Aliquots (1 μl) were then analyzed with a GC (Varian 4000) fitted with a flame ionization detector and Megabore capillary column (Zebron ZB-5: 15 m L x 0.53 mm i.d. x 1.50 μm DF). Helium was used as the carrier gas at a flow rate of 10 ml min⁻¹. The operating conditions were: initial column temperature, 120°C for 2 min and then programmed to increase to 200°C at a ramp rate of 10°C min⁻¹; hold time, 0.5 min; injector temperature, 230°C; and detector temperature, 280°C.

Reverse-phase HPLC (LC-235, Perkin-Elmer) analyses were made by injecting 20 μl samples into a C18 solid phase (Luna 5 μ C18: 250 x 4.6 mm) column. Elution was made at 1 ml/min⁻¹ with acetonitrile and water (45:55 v/v) (Fisher Scientific, Pittsburg, USA) as the mobile phase while UV detection was made at 220 nm.

To prepare standards, analytical-grade atrazine (Riedel de Haën, Seelze, Germany) was dissolved in HPLC-grade methanol (Fisher Scientific, Pittsburg, USA).

Standard curves ($0.1 - 1\,000\text{ mg l}^{-1}$) were constructed by peak area following GC and HPLC analyses and were used to quantify the residual atrazine concentrations.

2.9.6 Total Organic Carbon (TOC)

Samples (1.5 ml) from the different retentostat cultures (2.6.3) were taken for total organic carbon content determination. Analysis was made as described by Tappe *et al.* (1999) with some modification. Triplicate injections (25 μl) were made into a TOC analyzer (Dohrmann DC-190). The remaining volumes were then centrifuged (Eppendorf Centrifuge 5410) at 14 000 rpm \times g for 5 min and the supernatants used for triplicate injections. Thus, the particulate total organic carbon content was calculated as the difference between the pre- and post-centrifugation samples.

2.9.7 Cell Counts and Size Distribution

Aliquots (20 μl) from the bioreactors (2.6.3) were mixed thoroughly with 20 ml of sterile (Millipore, 0.22 μm) isotonic solution. Cell counts and size distributions were then determined with a Coulter Counter (Type Multisizer II) with the following settings: orifice diameter, 30 μm ; analytical volume, 100 μl ; 5% coincidence; and 10^3 counts sec^{-1} .

2.10 Molecular Characterization and Community-Level Physiological Profiling

2.10.1 Bacterial and Soil Samples

The aerobic enriched and isolated atrazine- and BTX-catabolizing microbial associations (2.3) were subjected to molecular characterization and community-level physiological profiling. Molecular characterization was repeated for the combined associations after the physiological optimization studies (2.5) and for the atrazine-catabolizing association (KRA30) used in the retentostat cultures (2.6). Anaerobic atrazine-catabolizing microbial associations (PMA01 to PMA08) from a parallel study (P.T. Mokebe, personal communication) were also characterized. Genetic analyses of the specific soils (2.1) with and without atrazine and petroleum hydrocarbon contaminants were also made.

2.10.2 Solution Preparations

120 mM Phosphate Buffer (pH 8)

This solution was made by preparing 120 mM K_2HPO_4 (20.9 g l^{-1}) and 120 mM KH_2PO_4 (16.3 g l^{-1}) and mixing 947 ml of the former with 53 ml of the latter. The pH was adjusted with the appropriate solution before autoclaving (15 lb psi pressure-121°C, 15min).

TAE Buffer (pH 8)

A 50X TAE buffer solution was made by mixing 242 g of Tris-base, 57.1 ml of glacial acetic acid and 37.2 g of $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ with the final volume adjusted to 1 l with distilled water. Twenty ml of the buffer were mixed with 980 ml of distilled water to make a 1X TAE running buffer solution. These solutions were then sterilized by autoclaving (15 lb psi-121°C, 15 min).

TE Buffer (pH 8)

To prepare this solution, 0.5 M $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (2 ml) was mixed with 1 M Tris-HCl (10 ml).

TCM Buffer

The buffer was prepared with distilled water to final concentrations of 10 mM Tris-HCl (pH 7.5), 0.1 mM CsCl and 5 mM MgCl_2 .

Loading Buffer

A 6X loading buffer solution was prepared by mixing 0.05 g of bromophenol blue, 40 g of sucrose, 20 ml of 0.5 M $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 0.5 g of sodium dodecyl sulphate (SDS). The resulting solution was diluted with distilled water to a final volume of 100 ml.

2.10.3 DNA Isolation and Purification

Genomic DNA of the isolated and combined associations and soil DNA were extracted according to the protocol described by Duarte *et al.* (1998) but with the extraction potassium phosphate buffer poised at pH 8. Some modifications were made to the procedure described by Duarte *et al.* (1998) for the direct/indirect extractions of soil DNA. Soil (0.5/20 g, fresh weight) and 0.8/100 ml of 0.1% (m/v) sodium

pyrophosphate (NaPP) were used. The potassium phosphate buffer, which replaced sodium phosphate buffer, and the liquefied phenol: potassium phosphate buffer (25:1 v/v) solution were poised at pH 8 with 120 mM K_2HPO_4 . Centrifugation (20 min) after each wash of the soil sample with the phosphate buffer was made at 4°C and 2 000 rpm x g in a Europa 24M centrifuge (Bovenkamp, Germany) fitted with a 6 x 500 ml rotor. The purification protocol adopted was the same as that outlined by van Elsas and Smalla (1995) and included the Wizard (Promega Benelux, Leiden, The Netherlands) DNA cleanup system.

2.10.4 RNA Isolation and Purification

Some modifications were made to the protocol for genomic DNA isolation (Duarte *et al.*, 1998) (2.10.3) with all the extraction solutions poised at pH 5 with 120 mM KH_2PO_4 . The isolated RNA was resuspended in TCM buffer (200 µl) instead of TE buffer. Purification of the RNA was made with an RNeasy Total Kit (Qiagen, Germany).

2.10.5 Polymerase Chain Reaction (PCR) Conditions

The reaction mixtures for the PCR contained: the Muyzer primers 357-GCf and 518r (each 1 µl, 0.01 mM); 1 µl of deoxynucleotide triphosphates (dNTPs) (0.2 mM); 1 µl of bovine serum albumin (BSA) (10 mg ml^{-1}); 2.5 µl of expand buffer (500U) (Bohringer, Mannheim, Germany); 16.75 µl of MilliQ water; and 0.75 µl of expand enzyme (250U) (Bohringer, Mannheim, Germany) to give a final volume of 24 µl. Sterile mineral oil (25 µl) was added as a top layer through which 1 µl of template (*e.g.* microbial association DNA) DNA was added. The control PCR mixtures included *Escherichia coli* DNA as a positive control together with a negative control with no template DNA. Deoxyribonucleic acid amplification was made with a Model 9600 Thermal Cycler (Perkin-Elmer) with the following programme: a 4 min initial denaturation at 94°C followed by 35 cycles with 1 cycle consisting of denaturation (30 sec at 94°C), annealing (1 min at 54°C), extension (1 min at 72°C) and final extension at 72°C for 5 min. Amplified PCR products (5 µl of each 25 µl reaction mixture) were visualized by electrophoresis in 1.5% (m/v) agarose stained with ethidium bromide and placed in 1X TAE buffer.

2.10.6 Reverse Transcription-PCR Conditions

Reverse Transcription-PCR was made on part of the 16S rRNA. The reaction mixture contained the Muyzer primer 518r (1.5 μ l, 0.01 mM); 1 μ l of deoxynucleotide triphosphates (dNTPs) (10 mM); 2.5 μ l of DMSO; 5 μ l of Mn(OAc)₂ (25 mM); 10 μ l of buffer; 28 μ l of RNAase-free MilliQ water (Bohringer, Mannheim, Germany); and 2 μ l of Reverse Transcriptase (rTth DNA polymerase) enzyme (250U) (Promega, Madison, Wis., USA) to give a final volume of 50 μ l. The control PCR mixtures included *S-503* RNA as a positive control together with a negative control with no template DNA. The amplification programme was made with a Model 9600 Thermal Cycler (Perkin-Elmer) and consisted of 1 cycle of 10 min annealing at 60°C and 20 min extension at 62°C. The RT-PCR products were then amplified with the Muyzer primer set (2.10.5) but with five controls included. The two positive controls were *Escherichia coli* DNA and the positive RT-PCR product of *S-503* RNA while the three negative controls were the negative control from the RT-PCR programme, RNA not amplified with RT-PCR and no template RNA/DNA.

2.10.7 Denaturing-Gradient Gel Electrophoresis (DGGE)

The 8% (v/v) polyacrilamide gels with the stated gradients were prepared as described by Muyzer *et al.* (1993) and used for DGGE with a Bio-Rad Dcode™ System. The operating conditions were the same as those described by Röling *et al.* (2000a) with the exception that a 50 - 65% (v/v) denaturing-gradient was used. Analysis of the gels was made by GelCompar 4.0 as described by the same researchers.

2.10.8 Colony Selection, Media and Growth Conditions

Shake flask cultures of the enriched and isolated aerobic microbial associations (2.3) were diluted (10^{-1} - 10^{-3}) with 0.85% (m/v) NaCl. Aliquots (150 μ l) of each dilution were inoculated onto 1% (m/v) tryptic soy broth (TSB) and sterile mineral salts M-I supplemented with the respective BTX molecules or atrazine at the selected concentrations (2.3) and set with agar (1.5% m/v). Duplicate plates were incubated at 30°C in the dark until colony development was evident. The colonies were categorized according to morphological, colour and size differences and DNA was extracted from single colonies and a combination of all the colonies from each plate.

The PCR and DGGE were made on the extracted DNA and the banding patterns were compared.

2.10.9 Characterization of Dominant Bands

High density (dominant) bands were excised from the DGGE gels under UV light, resuspended in 200 μ l of 1X TE buffer, mixed with 0.2 g of glass beads, shaken with a mini-beadbeater (Biospec Products) at 4 200 rpm for 30 sec and maintained at 4⁰C overnight. The supernatant was removed carefully after centrifugation (14 000 rpm x g for 5 sec) in a microcentrifuge (Eppendorf Centrifuge 5410). A 1 μ l aliquot of each supernatant was amplified by PCR and processed by DGGE as described above.

2.10.10 Clone Bank

The genomic DNA extracted from one anaerobic atrazine-catabolizing association (PMA03) and two of the aerobic toluene-catabolizing associations (KRT04 and KRT07) was used to initiate a clone bank according to the method described by Felske *et al.* (1998). Thus, polymerase chain reaction primers 8f and 1512r were used to amplify 16S rDNA sequences. The products were then cleaned with the Qiaquick Rep Purification Kit (Qiagen, Hilden, Germany) and cloned in pGEM-T linear plasmid vector and *Escherichia coli* JM109 competent cells as specified by the manufacturer (Promega, Madison, Wis., USA). Randomly-selected recombinants/clones were reamplified with the primer set F357-GC/R518 (Isogen Bioscience BV, Maarsen, The Netherlands) and the products were compared on DGGE gels with the initial association profiles. Some of the recombinants were then selected for sequencing.

2.10.11 Potential Impacts of Methanol as a Solvent for Atrazine

Microbial association KRA30 was selected to examine the effects of using methanol to solubilize atrazine in growth media. Thus, a comparison of the DGGE profiles of this association cultured in 100 mg l⁻¹ atrazine-supplemented mineral salts solution M-I (50 ml) in the presence and absence of the solvent (\leq 5 ml, 100% v/v) was made. Also, genomic DNA from the association cultured under both conditions was cloned (Felske *et al.*, 1998). In total, 70 recombinants were selected randomly from each group and analyzed by PCR-DGGE. Only the recombinants with different DGGE banding locations were chosen for sequencing.

Aliquots (10 μ l) of clean (Qiaquick Rep Purification Kit, Qiagen, Hilden, Germany) diluted (10^{-2} , TE buffer) PCR products of the individual clones were mixed to create clone profiles of association KRA30 in the presence and absence of methanol. The mixtures were used as templates for subsequent PCR-DGGE analysis to correlate the profiles to the atrazine-compromised source soil and its pristine equivalent.

2.10.12 Sequencing of Randomly-Selected Clones

To obtain the complete sequences of the 16S rDNA, reamplification of selected 8f-1512r clones was made with the T7/sP6 primer set (Isogen Bioscience BV, Maarsen, The Netherlands). Sequencing PCR was made with an ABI PRISM™ Dye Terminator Cycle Sequencing Core Kit (Perkin-Elmer) and the purified products were run in SEQUAGEL-6 sequence gel (National Diagnostics, USA) in a 373A/DNA Sequencer (Applied Biosystems, USA). Both strands of the 16S rDNA gene were sequenced. Basic Logical Alignment Search Tool (BLAST) Network Service (15-vi-99 and 12-iv-00) was used to compare the sequences with available databases to determine their approximate phylogenetic affiliations. The sequences of these clones were then submitted to GenBank.

2.10.13 Environmental Biolog Analysis

The isolated aerobic atrazine-catabolizing microbial associations were diluted (10^{-1} to 10^{-6}) with 0.85% (m/v) NaCl and inoculated into Biolog EcoPlates (van Verseveld *et al.*, 1999; Röling *et al.*, 2000b) for community-level physiological profiling (CLPP). The plates were incubated at 30⁰C in the dark. Changes in optical density were measured with a Plate Microreader (Titertek Multiscan MCC/340) after 1, 4 and 8 days. The data were then analyzed by Pearson Correlation on Gel Compar (Applied Maths, Kortijk, Belgium).

Chapter 3: Enrichment/Isolation and Preliminary Characterization of Catabolic Soil Microbial Associations

3.1 Introduction

Atrazine is a relatively new molecule in the environment. Thus, it is possible that the occurrence of microbial species with catabolic enzymes and pathways for this pesticide is limited. The herbicide is used worldwide, however, so the existence of known microorganisms, with newly-induced catabolic enzymes, or novel microorganisms, with atrazine-catabolic capacities, may, likewise, be distributed globally. Some indigenous soil microbial associations and monocultures with atrazine-catabolizing capacities have been enriched/isolated from compromised sites in different geographical regions (Radosevich *et al.*, 1996; Bouquard *et al.*, 1997; Sparling *et al.*, 1998; Topp *et al.*, 2000a).

Since petroleum is a fossil fuel, the occurrence of microorganisms which degrade its components, such as the BTX molecules, is common. As a result, BTX molecule catabolisms have been well researched and elucidated with numerous microorganisms (Kappeler and Wuhrmann, 1978; Atlas, 1981; Leahy and Colwell, 1990).

As a xenobiotic molecule with no natural analogues, atrazine may be regarded as a greater challenge for microbial catabolism than BTX molecules. Although a more limited energy source with the ring carbons fully oxidized (Radosevich *et al.*, 1996), it may, however, benefit from the presence of both nitrogen and carbon in comparison with BTX molecules which do not contain nitrogen. Radosevich *et al.* (1996) identified biodegradation as one of the most important processes which govern the environmental fates of atrazine.

Bioremediation, which was developed by the petroleum industry, has, therefore, been espoused largely as the technology of choice for BTX- and atrazine-compromised sites despite the availability of various alternative treatment and containment methods such as: ozonation (Ma and Graham, 2000); powdered activated carbon adsorption (Campos *et al.*, 2000; Martin-Gullon and Font, 2001); diatomaceous earth remediation (Agdi *et al.*, 2000); photochemical degradation

(Konstantinou *et al.*, 2001); ultrasonication (Feng and Aldrich, 2000); and clay sorption (Jaynes and Vance, 1995; 1999; Sharmasarkar *et al.*, 2000).

Although a monoculture approach may be adopted, the focus of this research was to investigate molecule catabolism by interacting microbial associations as a prerequisite for site amelioration. Several researchers (Catallo and Portier, 1992; Otte *et al.*, 1994; Atlas, 1995; Corseuil and Alvarez, 1996; Whyte *et al.*, 1999) have reported that the enrichment of key indigenous catabolic associations, which are acclimated, resistant and active, may be considered to facilitate rapid and efficient bioremediation of a polluted site, by bioaugmentation in particular.

Since many soils are electron donor poor and electron acceptor variable, continuous culture protocols are adopted to enrich/isolate slow-growing indigenous catabolic species. Once chemically compromised, however, soils are electron donor rich and electron acceptor variable. Thus, competition is the major selection determinant so enrichment/isolation is best achieved with batch cultures. These cultures were, therefore, used to enrich/isolate aerobic catabolic microbial associations from atrazine- and petroleum hydrocarbon-contaminated loamy and sandy KwaZulu-Natal (South Africa) soils, respectively.

An extensive enrichment/isolation programme was made as detailed in 2.3 and was undertaken to minimize the limitations of liquid enrichment cultures (Dunbar *et al.*, 1997) and so validate the conclusions of the subsequent studies.

3.2 Results and Discussion

3.2.1 Soil Analyses

Contaminated soil samples (2.1) were assayed for density, P, K, Ca, Zn, Mn and Mg contents, exchange acidity, total cations, acid saturation, pH (KCl), total organic carbon and clay content (Table 3.1). The loamy and sandy soils recorded clay contents of 33 and 34% (m/m), respectively and thus provided sorption surfaces for the molecules and chemical intermediates and the catabolic species. Although reduced molecule bioavailability may have resulted, concomitant microbial adhesion to the soil surfaces could have counteracted this. It is possible, however, that some species were excluded from the final enriched/isolated microbial associations due to soil surface adsorption.

Since the loamy soil contained phosphorus (19 mg kg^{-1}) it is possible that atrazine adsorption was reduced due to competitive binding. Smit *et al.* (1981), for example, reported that atrazine adsorption decreased in response to a KH_2PO_4 addition to soil. Similar increased mobility may be effected by binding to sulphates or added nutrients.

Together with the above, clay components play a further role since they can effect non-biological atrazine degradation to hydroxyatrazine (Armstrong and Chesters, 1968; Skipper and Volk, 1972; Alvey and Crowley, 1996) which is a labile intermediate. Similarly, although not determined, the presence of iron and aluminium could facilitate chemical degradation of atrazine.

The total cation contents of 9.58 and 13.8 cmol l^{-1} of the soils were relatively low and, thus, reduction of the target molecule concentration and catabolic enzyme inhibition through chelation was assumed to be minimal. The trace element concentrations did not identify possible elemental limitations. Since the provision of trace elements for large-scale inoculum preparation for bioaugmented *ex situ/in situ* bioremediation (1.4.1) could represent a significant cost factor, the site soil, through soil extracts, could be considered as a source for these.

Table 3.1: Analyses of selected physico-chemical variables of the atrazine (loamy)- and petroleum hydrocarbon (sandy)-contaminated soils.

Soil	Sample Density (g m^{-3})	P	K	Ca	Zn	Mn	Mg	Exchange Acidity	Total cations (cmol l^{-1})	Acid Saturation %	pH (KCl)	NIRS Organic Carbon % (m/m)	Clay % (m/m)
				(mg kg^{-1})									
Loamy	1.08	19	118	1 270	4.3	36	337	0.17	9.58	2	4.24	1.6	33
Sandy	1.14	2	35	1 693	0.7	2	639	0.00	13.80	0	7.14	3.2	34

The atrazine and BTX component molecule concentrations were not determined due to the, often, severe limitations of existing extraction methods (F. Khan, personal communication). Thus, the molecule concentrations in the primary enrichment/isolation cultures were probably increased by the residual concentrations

although molecule solubility was assumed to be the primary determinant of availability to the catabolic species.

3.2.2 Atrazine Sorption to Soil

Although Radosevich *et al.* (1996) reported that sorption of atrazine influenced its mineralization rate, marked differences between soils prevent generalizations on the rate and extent of sequestration and, thus, the bioavailability of the pesticide and organic compounds in general (Chung and Alexander, 1998; Friedrich *et al.*, 2000). Friedrich *et al.* (2000), for example, observed that different microbial populations were selected under different phenanthrene bioavailability conditions. It is essential, therefore, to elucidate the adsorption/desorption characteristics of the target contaminant molecule in the specific soil which is to be remediated so that the enrichment/isolation protocol models the *in situ* conditions.

Pristine sterile loamy soil (2.1) was used to determine the influence of possible sorption on molecule bioavailability and, thus, enrichment/isolation of atrazine-catabolizing microbial associations. Although some differences might have existed, the pristine soil was assumed to have very similar characteristics to those of its chemically-compromised equivalent. To eliminate microbial catabolism, the pristine soil, with no known history of atrazine application, was sterilized by gamma irradiation. It must be recognized, however, that this soil sterilization method can effect soil structure changes (McLaren, 1969; Sims, 1986; Wolf *et al.*, 1989). Stroetmann *et al.* (1995), for example, reported that gamma irradiation influenced slightly the cation exchange capacity of soil but concluded that it was the best sterilization method. To ensure efficacy, the soil was sterilized in dry-weight batches of 1 kg. After sterilization, atrazine adsorption/desorption was tested (2.8) and the results are shown in Figures 3.1 and 3.2.

In general, the percentage of atrazine adsorbed increased with the initial concentration with 32.7, 35.2, 51.5, 66 and 61.5% adsorption recorded for the 30, 50, 100, 500 and 1 000 mg l⁻¹ concentrations, respectively (Figure 3.1). These results were, perhaps, surprising since with a clay content of 33% (m/m) the percentage of atrazine adsorbed would have been expected to decrease as the atrazine load increased. Although, the rate/extent of desorption is dependent on the concentration of herbicide added and adsorbed to the soil, the results indicated a trend towards complete recovery (Figure 3.2). For the three lower concentrations 100% recovery was effected by three

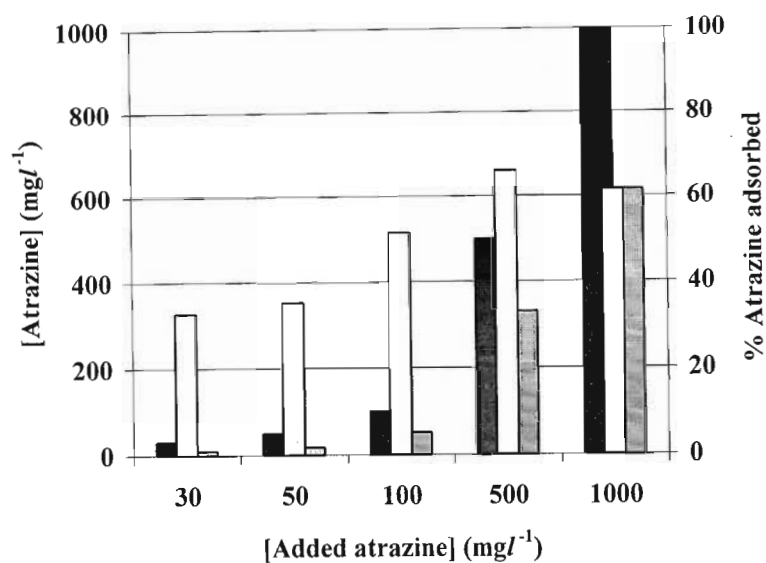


Figure 3.1: Changes in concentrations of added (■), adsorbed (■) and percentage adsorbed (□) atrazine solutions exposed to gamma-irradiated pristine loamy soil.

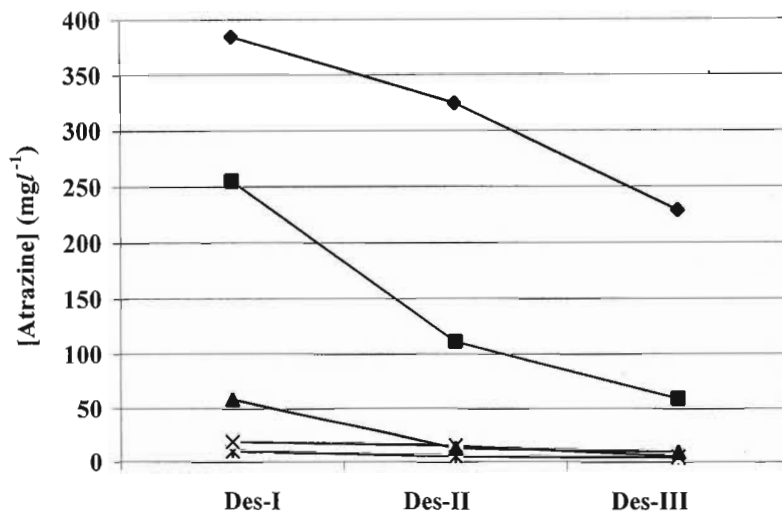


Figure 3.2: Atrazine concentrations of supernatants after consecutive desorptions (Des-I, II and III) from gamma-irradiated pristine loamy soil following initial suspension in 30 (*), 50 (x), 100 (▲), 500 (■) and 1 000 (◆) mg l⁻¹ atrazine.

extractions while the 500 and 1 000 mg l⁻¹ initial concentrations required further extractions. For all the concentrations the results suggested that minimal irreversible adsorption had occurred during the 24 h exposure.

Previous work by Tyess *et al.* (1995) showed that in inoculated soils the degradation/mineralization of the pesticide correlated with both its adsorption coefficient (K_d) and soil properties such as NO₃-N, P and organic C concentrations and pH. The low atrazine adsorption coefficient of the soil under investigation was, probably, accounted for by the phosphorus (19 mg kg⁻¹) and organic carbon (1.6% m/m) concentrations (3.2.1) which, in turn, should have facilitated increased herbicide bioavailability and, thus, successful enrichment/isolation of catabolic species. It must be noted, however, that “ageing” due to extended exposure and incubation periods could have resulted in low desorptions and, thus, reduced bioavailabilities.

3.2.3 *Enrichment/Isolation of Catabolic Microbial Associations*

Batch culture enrichments were used to isolate aerobic atrazine- and BTX molecule-catabolizing microbial associations. The low volume cultures (2.3) were selected to facilitate: enrichment/isolation replication; culture conditions control with respect to temperature, pH and mineral salts additions; and ease of representative sampling. The possible limitations introduced as a result of the small scale in comparison with the contaminated sites were, however, recognized. Two pH values (5.5 and 7.5) were chosen to represent acidic and near neutral soil conditions, respectively.

A simple medium M-I (2.2.1) was used to complement the trace elements/nutrients present in the nutrient-limited source soils. The target molecules were supplied as carbon and energy sources. Following primary enrichment, the principal selection pressures were pH and electron donor concentration.

For each molecule and each concentration, and each pH value, catabolic associations were enriched/isolated after 25 (atrazine) and 13 (BTX) weeks of primary enrichment and four consecutive subcultures (Tables 2.1 and 2.2).

During the primary enrichments, pH decreases to 3.5 in the pH 7.5-poised atrazine-supplemented cultures were recorded and seemed to be independent of the pesticide concentration (Figure 3.3A). These results contrasted the control where, after the first day, the pH values remained within the range of 6.5 to 6.9. Initial pH reductions were recorded also for the enrichment cultures poised initially at pH 5.5

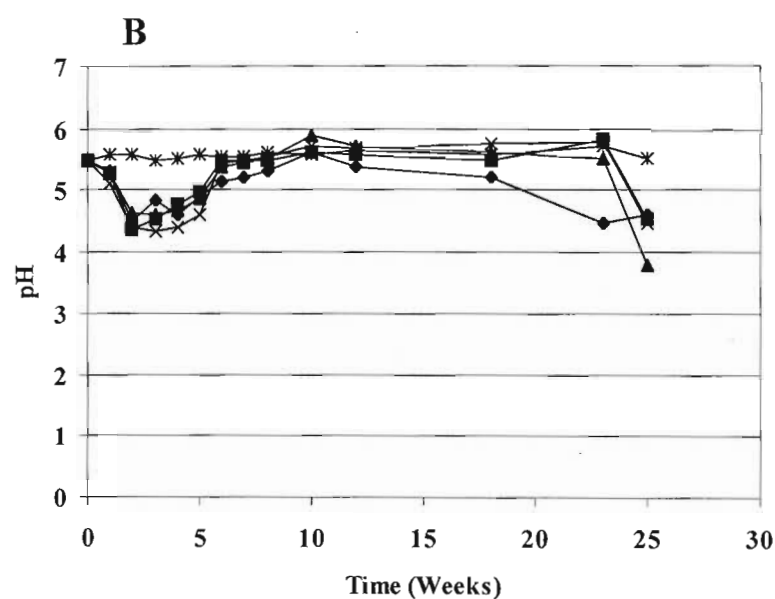
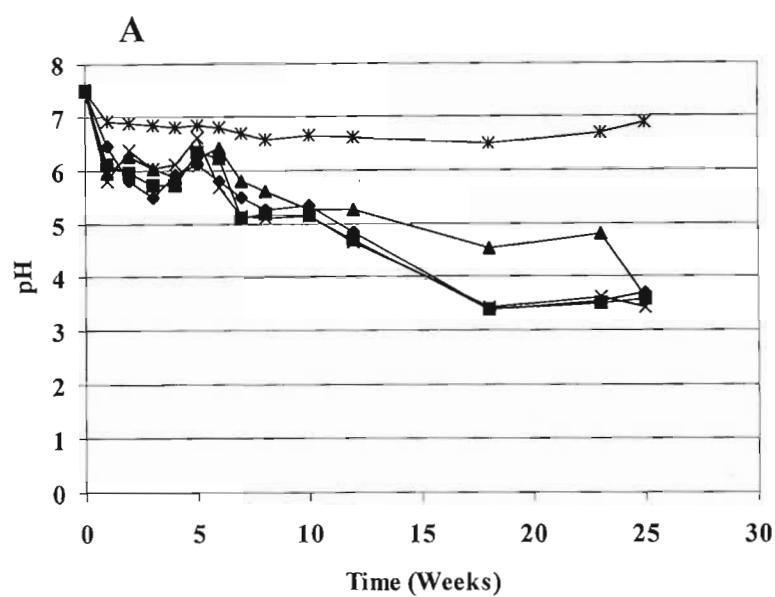


Figure 3.3: Changes in pH values with time of aerobic primary enrichment cultures poised at pH 7.5 (A) and pH 5.5 (B) and supplemented to final atrazine concentrations of 0 (*), 10 (x), 20 (▲), 30 (■) and 33 (◆) mg l^{-1} .

although these were followed by increases and then decreases after 23 weeks of incubation (Figure 3.3B). Once again, the pH of the control fluctuated little during the incubation period.

Although abiotic atrazine hydrolysis to hydroxyatrazine possibly accounted for the recorded initial pH decrease of the pH 7.5-poised control, the pH reductions in the enrichment cultures were probably indicative of dechlorination which has been reported to be the first step of microbial atrazine catabolism (Bouquard *et al.*, 1997). In this event, pH change could be used to monitor the progress of atrazine enrichment cultures.

For each of the BTX molecule enrichment cultures poised at pH 7.5, a pH decrease of ± 0.5 units was effected during the 13-week incubation period (Figure 3.4A). In contrast, pH increases of ± 1.0 units were recorded for the pH 5.5-poised enrichments (Figure 3.4B).

In general, for all the BTX molecule enrichment/isolation cultures, optical density measurements indicated only limited biomass increases during 13 weeks of incubation (Figures 3.5-3.9). For *m*- and *p*-xylene ($5\ 000\ \text{mg l}^{-1}$), however, both the pH 5.5- and 7.5-poised cultures recorded marked increases during the first week of incubation but these decreased progressively (Figures 3.8 and 3.9). From the results it appeared that chemical intermediate- and CO_2 production accounted for the majority of molecule catabolism.

To complement the aerobic enrichment/isolation programme for atrazine-catabolizing microbial associations, a parallel anaerobic study was made (P.T. Mokebe, unpublished results). In total, eight atrazine-catabolizing associations were isolated (Table 3.2).

3.2.4 Isolation of Association Components

To initiate characterization of the microbial associations the culturable component species were isolated (2.4). Mineral salts solution M-I (2.2.1) and soil extract (2.2.2) supplemented individually with the different target compounds and set with agar were used to isolate the primary (target molecule) catabolic species while nutrient agar was used to isolate all the culturable component species. Soil extract, which is usually used in conjunction with basic mineral salts solution to maximize the chances of isolation during the initial subculturing steps prior to use of defined medium, was used in this study to isolate catabolic association members under nutrient-limited

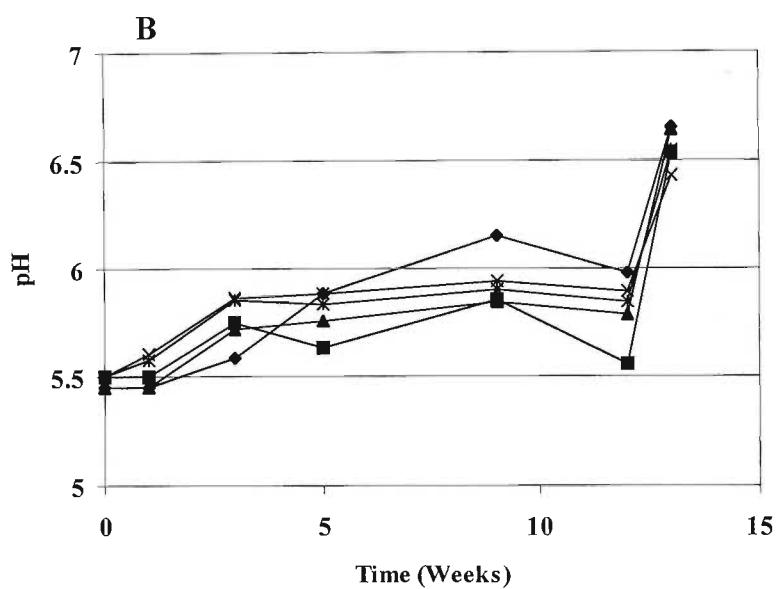
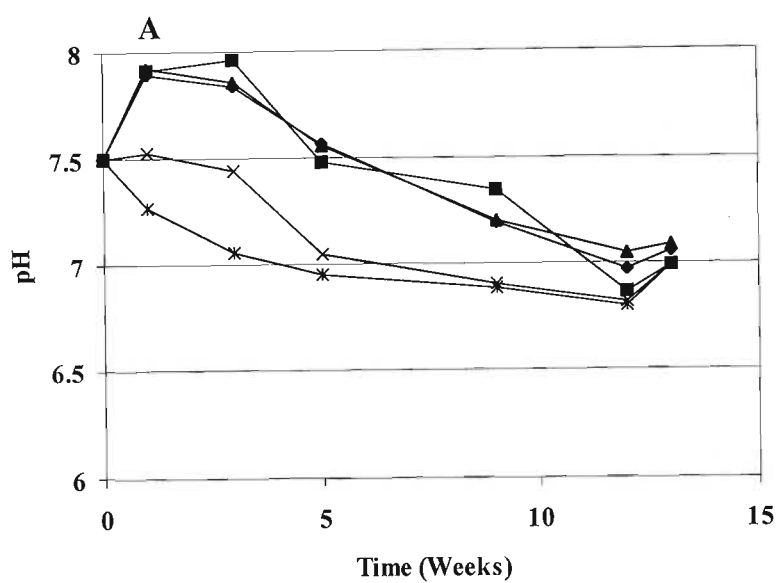


Figure 3.4: Changes in pH values with time of aerobic primary enrichment cultures poised at pH 7.5 (A) and pH 5.5 (B) in the presence of volatilized benzene (◆), toluene (■), *o*-xylene (▲), *m*-xylene (×) and *p*-xylene (*).

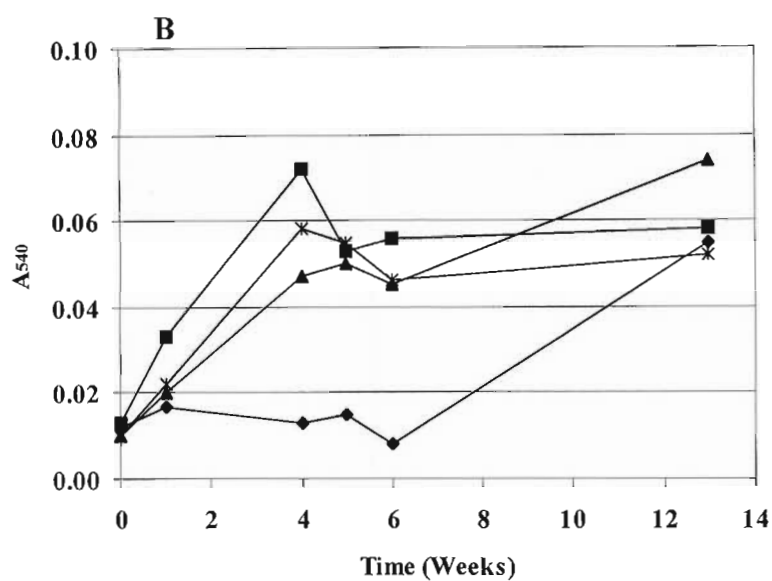
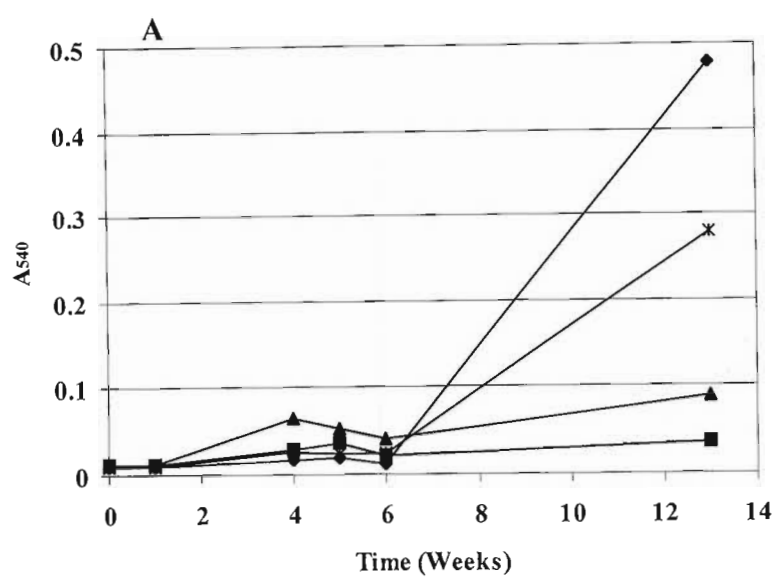


Figure 3.5: Changes in optical densities with time of aerobic primary enrichment cultures poised at pH 5.5 (A) and 7.5 (B) and supplemented to final benzene concentrations of 5 (*), 50 (▲), 500 (■) and 5 000 (◆) mg l⁻¹.

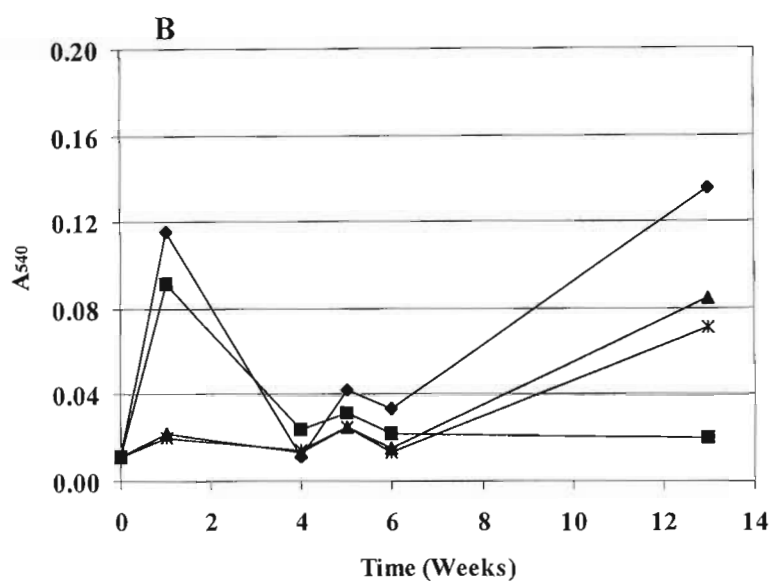
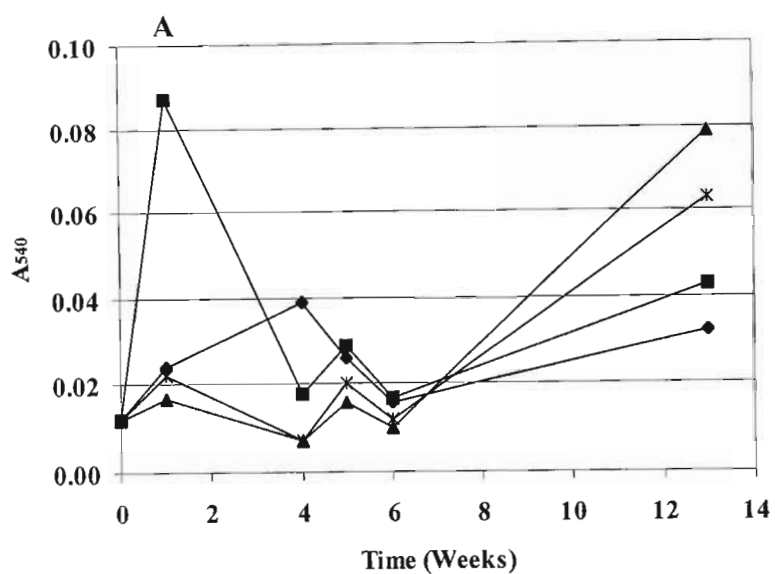


Figure 3.6: Changes in optical densities with time of aerobic primary enrichment cultures poised at pH 5.5 (A) and 7.5 (B) and supplemented to final toluene concentrations of 5 (*), 50 (▲), 500 (■) and 5 000 (◆) mg l⁻¹.

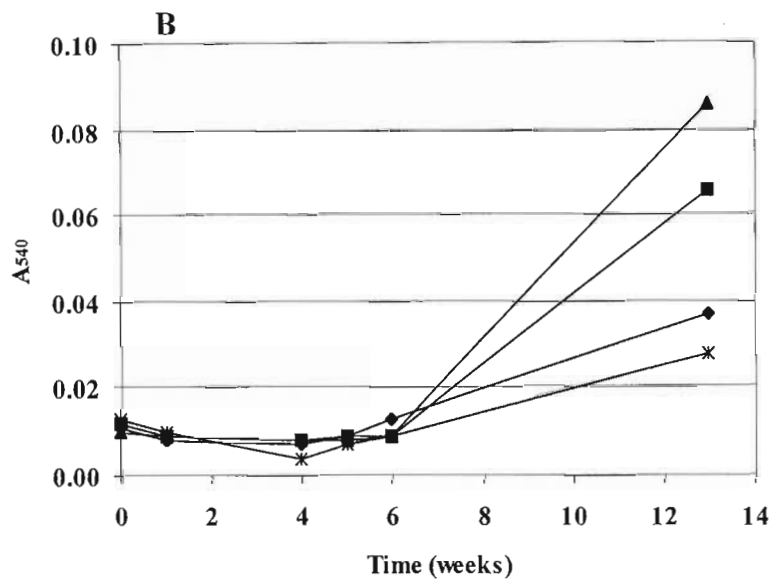
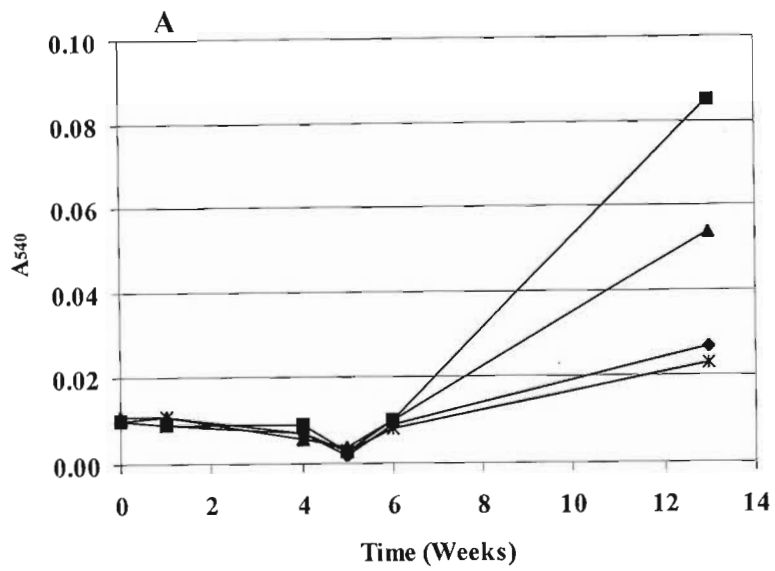


Figure 3.7: Changes in optical densities with time of aerobic primary enrichment cultures poised at pH 5.5 (A) and 7.5 (B) and supplemented to final *o*-xylene concentrations of 5 (*), 50 (▲), 500 (■) and 5 000 (◆) mg l⁻¹.

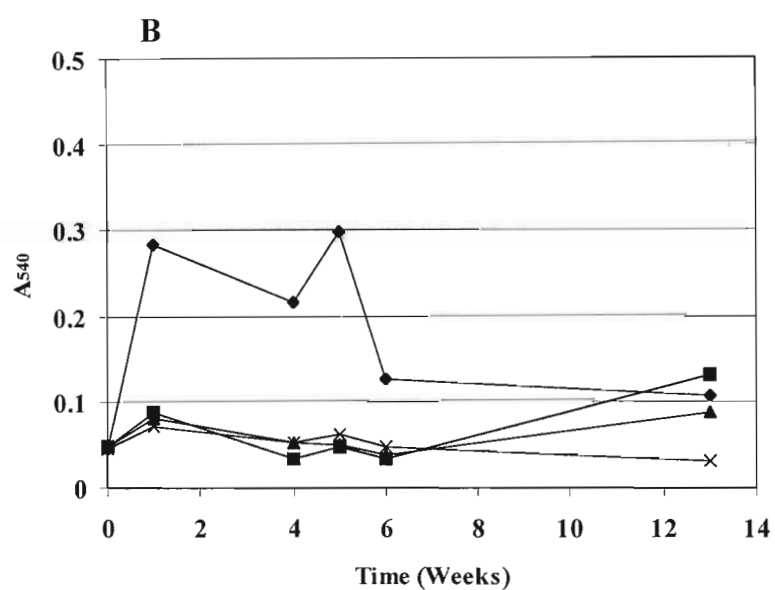
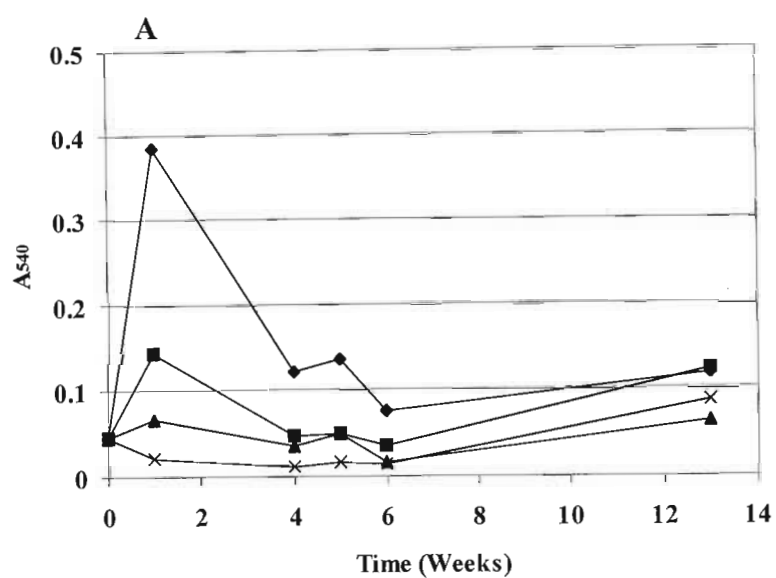


Figure 3.8: Changes in optical densities with time of aerobic primary enrichment cultures poised at pH 5.5 (A) and 7.5 (B) and supplemented to final *m*-xylene concentrations of 5 (*), 50 (▲), 500 (■) and 5 000 (◆) mg l⁻¹.

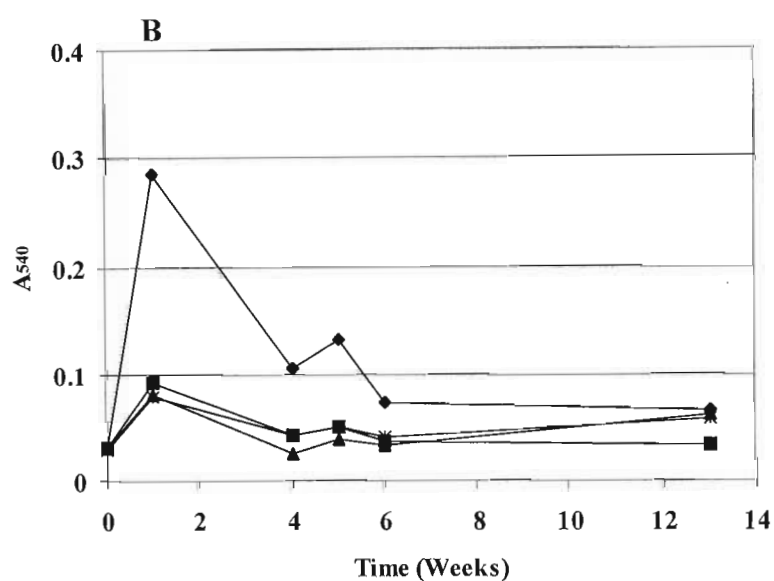
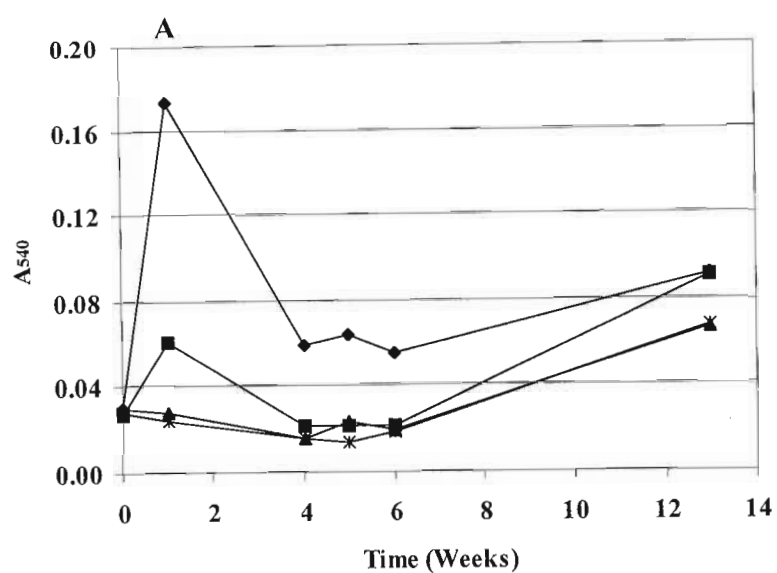


Figure 3.9: Changes in optical densities with time of aerobic primary enrichment cultures poised at pH 5.5 (A) and 7.5 (B) and supplemented to final *p*-xylene concentrations of 5 (*), 50 (▲), 500 (■) and 5 000 (◆) mg l⁻¹.

Table 3.2: Anaerobic microbial associations enriched/isolated in the presence of four atrazine concentrations and two pH values.

pH Value	[Atrazine] (mg l ⁻¹)	Association Designation
7.5	33	PMA01
	30	PMA02
	20	PMA03
	10	PMA04
5.5	33	PMA05
	30	PMA06
	20	PMA07
	10	PMA08

conditions typical of soil. The resulting colonies were then subjected to preliminary characterization on the basis of colony morphology, colour and size.

Colonies of different size, shape and colour were apparent on nutrient agar while the target molecule-supplemented M-I and soil extract media supported colony growth and differentiation only on the basis of shape and size. Representative results of each of these are shown in Plates 3.1A and B. The electron donor concentration and the enrichment pH seemed to effect some differences in the types and predominance of the isolated colonies.

Following monoculture isolation, examination by light microscopy of the cells of the different colonies revealed that they were constituted by mixtures of Gram-positive and Gram-negative rods and cocci irrespective of the molecule type, concentration and enrichment pH. For all the target molecules, Gram-negative rods were dominant numerically. This was as expected since rod-shaped species have been reported for atrazine-catabolizing bacteria (*Pseudomonas* spp., Behki and Khan, 1986; Yanze-Kontchou and Gschwind, 1994; Mandelbaum *et al.*, 1995; *Rhizobium* sp., Bouquard *et al.*, 1997; *Agrobacterium radiobacter* J14A, Struthers *et al.*, 1998; and *Pseudaminobacter* sp., Topp *et al.*, 2000b) and petroleum hydrocarbon-catabolizing bacteria (Lees, 1996; Pfiffner *et al.*, 1997; Shen *et al.*, 1998). For microbial associations, Mallakin and Ward (1996) reported the presence of *Pseudomonas maltophilia*, *P. testosteroni* and *P. putida* biotype A in a culture which degraded individual and combined BTEX components.

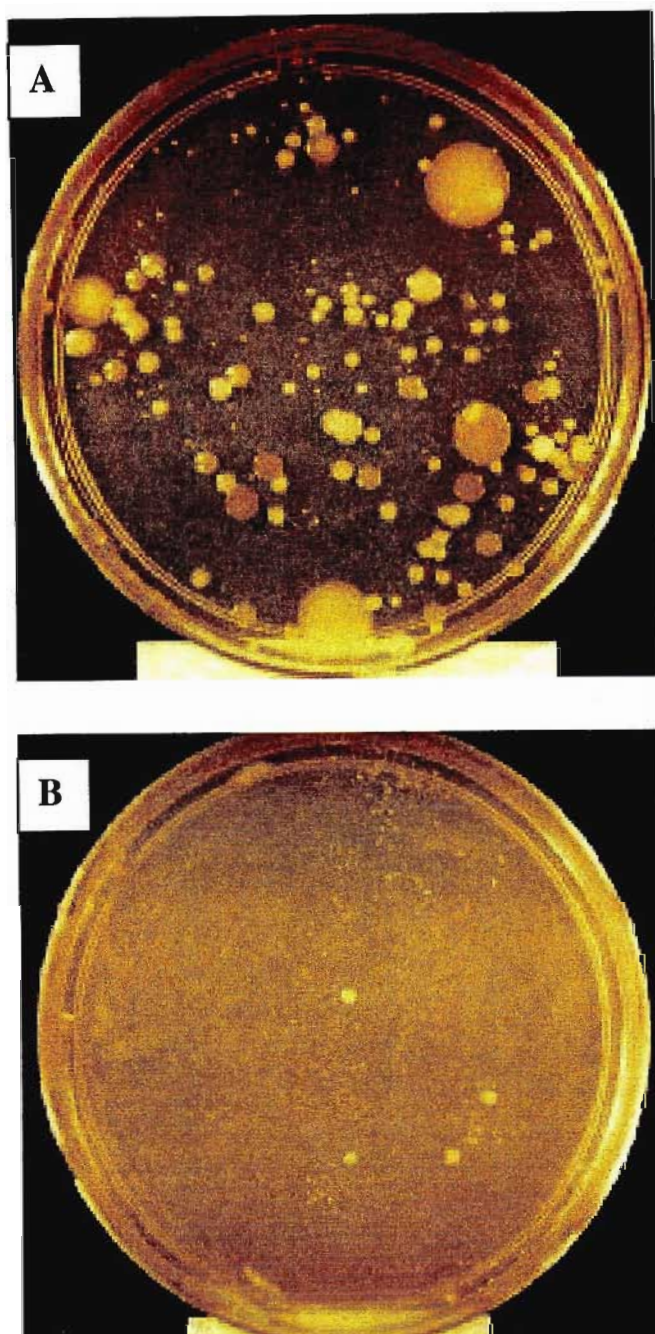


Plate 3.1: Spread plates (10^{-4} dilution) of the aerobic atrazine (33 mg l^{-1} , pH 5.5) catabolic association (KRA05) cultured on nutrient agar (A) and atrazine-supplemented (33 mg l^{-1}) mineral salts agar (B).

Some coccoid bacteria have also been reported to catabolize atrazine. These include: *Nocardioides* sp. (Topp *et al.*, 2000a); and *Rhodococcus* spp (Behki *et al.*, 1993; Behki and Kahn, 1994). For BTEX components the coccoid bacterium *Rhodococcus rhodochrous* was isolated by Deeb and Alvarez-Cohen (1999).

In one study which examined the enrichment of catabolic species on a mixture of aromatic hydrocarbons which included benzene, dicyclopentadiene, toluene, styrene, xylenes and naphthalene (Greene *et al.*, 2000), a time-dependent succession of *Pseudomonas* (rod), *Rhodococcus* (coccus) and *Alcaligenes* (rod) species resulted.

Individual colonies selected from the solid media were assumed to be distinct component monocultures of the different associations. For all substrates, however, Gram-stained single colonies showed mixtures of both Gram-positive and Gram-negative rods and cocci. This lack of isolation of association members highlighted, perhaps, their overlapping activity domains (Senior *et al.*, 1976; de Souza *et al.*, 1998a).

Attempts to isolate the component species for further association analysis, although protractive, were deemed necessary to identify the catabolic capabilities of each. For atrazine, additional subculturing led, ultimately, to the isolation of monocultures of three distinct colony types but only when the pesticide was dissolved in methanol. Subsequent attempts to culture these isolates in shake flasks in the absence of methanol proved unsuccessful. Other researchers (Mandelbaum *et al.*, 1993; Assaf and Turco, 1994a) reported similar results where individual constituent species could not be isolated (under nitrogen-limited conditions) from enriched/isolated microbial associations with proven and effective pollutant catabolic capabilities.

Examination by scanning electron microscopy confirmed that many colonies were constituted by two to four morphologically-distinct microbial types and, numerically, rods dominated the atrazine and BTX molecule catabolic associations independent of the dilution plate medium. Some structures which might have been bacterial endospores or fungal reproductive bodies were also observed, particularly in the atrazine-catabolizing associations. Plate 3.2A shows the rods (1.5 to 2.5 μm long) of an atrazine (33 mg l^{-1})-catabolizing colony isolated from microbial association KRA01 while Plate 3.2B shows the occurrence of some longer ($\leq 6.5 \mu\text{m}$) rods in a colony isolated from KRA05.

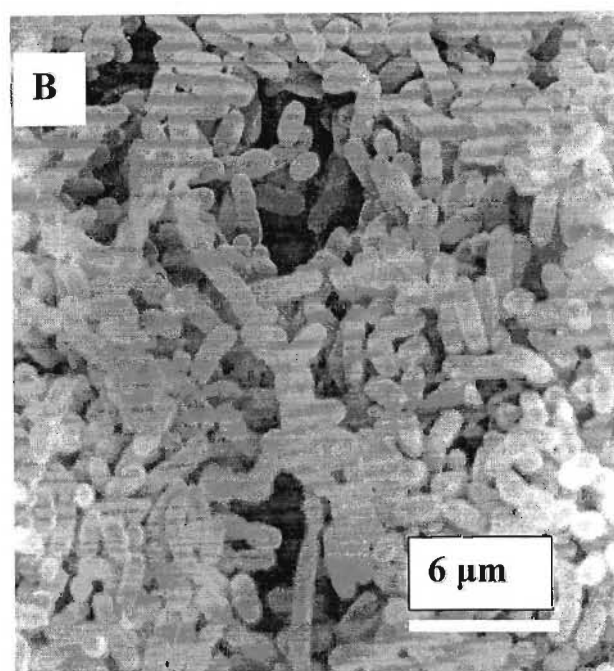
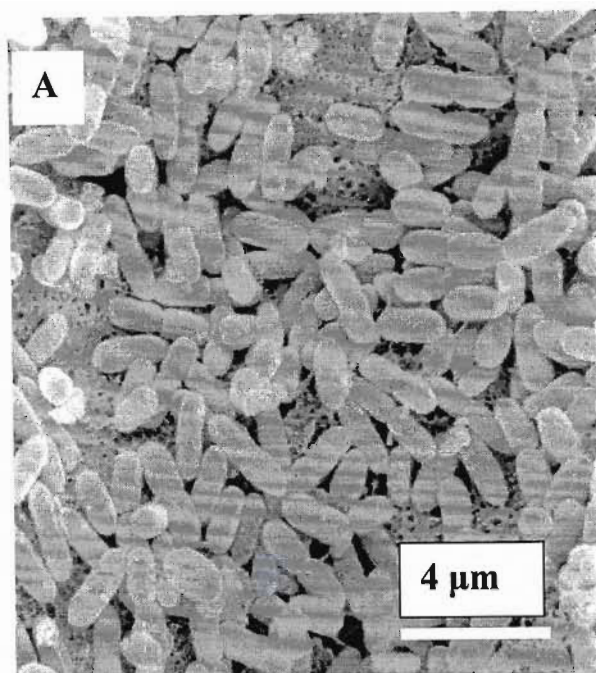


Plate 3.2: Scanning electron micrographs of bacterial cells of atrazine-catabolizing colonies, isolated on atrazine (33 mg l^{-1})-supplemented mineral salts M-I agar, from microbial associations KRA01 (A) and KRA05 (B).

Rods of various sizes characterized the colonies from the BTX molecule enrichments/isolations. For example, colonies isolated from the benzene-catabolizing (500 mg l^{-1} , both pH values) associations on benzene (500 mg l^{-1})-supplemented mineral salts M-I agar were characterized by rods which ranged in length from 1 to 4 μm (Plates 3.3A and B). Similarly, colonies isolated from the toluene-catabolizing (500 mg l^{-1} , pH 7.5) association on toluene (500 mg l^{-1})-supplemented mineral salts M-I agar were characterized by rods (Plate 3.4). Plate 3.5 shows a mixture of rods (2-3 μm long and 0.5 μm wide) and coccoid- (2 μm long and 1.5 μm wide) shaped cells which constituted a *p*-xylene-catabolizing association (KRP02) cultured on 500 mg l^{-1} -supplemented mineral salts M-I agar. Extracellular structures which appeared to link some of the association members were also evident.

Since a primary objective of this study was to enrich/isolate and characterize complete catabolic associations, the isolation of culturable atrazine- and BTX molecule-catabolizing monocultures was not pursued further. The potential role(s) of the individual association members in molecule catabolism was later elucidated partially by a molecular approach (5.3 and 6.2).

3.3 Conclusions

Eight atrazine-catabolizing and 40 BTX molecule-catabolizing microbial associations were enriched/isolated in controlled/ideal laboratory conditions in 25 and 13 weeks, respectively. Shorter enrichment/isolation periods have been reported (Bouquard *et al.*, 1997) but those recorded in this study were, perhaps, more representative of *in situ* enrichments. The application of atrazine over 20 years had effected the presence of catabolic species or related extracellular enzymes in the agricultural soil. In contrast, the enrichment/isolation of BTX molecule-catabolizing associations from a soil with a relatively short (2 yr) history of exposure highlighted the wide dissemination and intrinsic capacity of soil microorganisms to degrade petroleum hydrocarbons.

Catabolic microbial associations were isolated in the presence of different molecule concentrations and pH values which suggested that the catabolic potentials would be retained in the presence of different electron donor concentrations and pH values. Thus, the need for soil manipulation could be minimal although enrichment/isolation

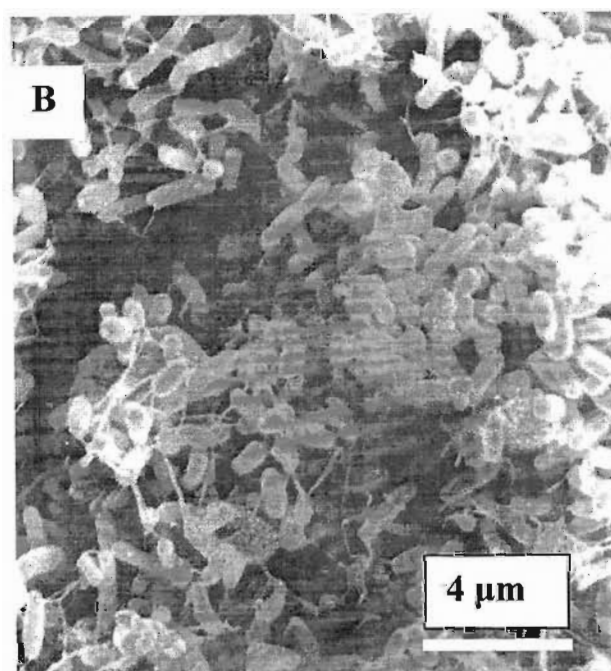
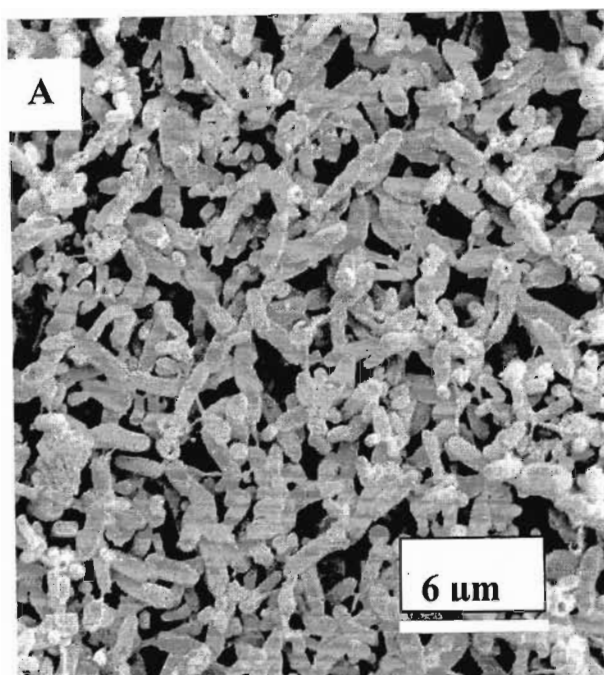


Plate 3.3: Scanning electron micrographs of bacterial cells of benzene-catabolizing colonies, isolated on benzene (500 mg l^{-1})-supplemented mineral salts M-I agar, from microbial associations KRB02 (A) and KRB06 (B).

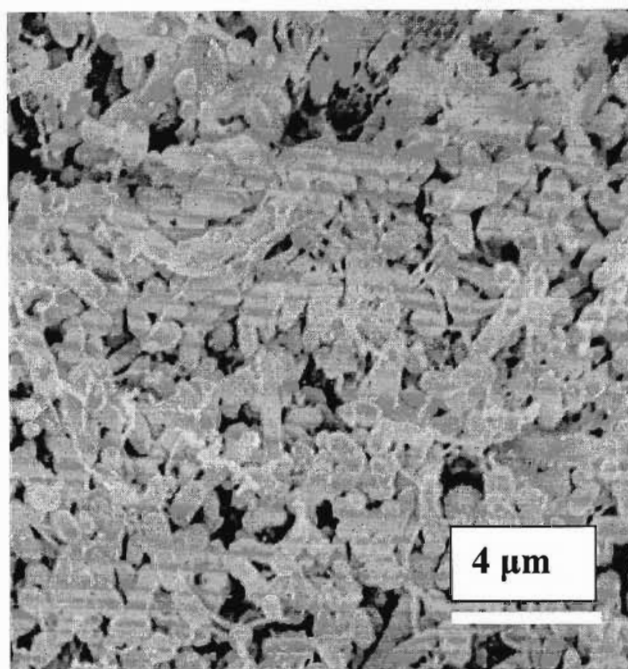


Plate 3.4: Scanning electron micrograph of bacterial cells of a toluene-catabolizing colony, isolated on toluene (500 mg l^{-1})-supplemented mineral salts M-I agar, from microbial association KRT02.

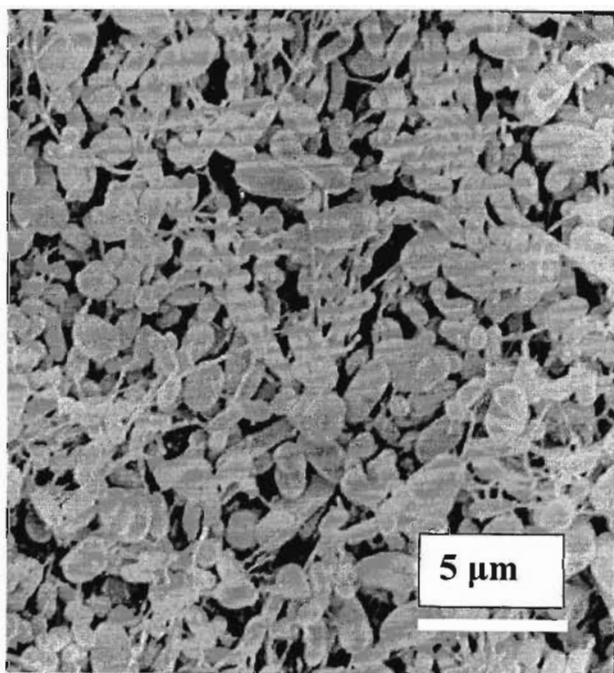


Plate 3.5: Scanning electron micrograph of bacterial cells of a *p*-xylene-catabolizing colony, isolated on *p*-xylene (500 mg l^{-1})-supplemented mineral salts M-I agar, from microbial association KRP02.

of a catabolic association (for bioaugmentation) in the presence of specific site conditions, such as pH and temperature, would be crucial.

Although not studied here, mixed BTX molecule catabolism by the enriched/isolated microbial associations should be investigated since these molecules occur together in petroleum and diesel oil.

The enrichment/isolation of atrazine- and BTX molecule-catabolizing microbial associations from local soils suggested that they could be used successfully for augmented bioremediation in South Africa. For each site, however, different biodegradation rates and remediation efficacies would be expected as they would be influenced by specific determinants such as regional soil types, seasonal changes, rainfall patterns and temperature.

Chapter 4: Determination of Catabolic Potentials and Environmental Parameters Optimization

4.1 Introduction

Concomitant with increased focus on the roles of catabolic microorganisms in the fates of environmental pollutants is interest in the rates of biodegradation and the factors which determine these. Intrinsic bioremediation is often limited by factors such as the physicochemical and environmental conditions. Thus, directed biotechnology/bioremediation offers a way to overcome these limitations and eliminate the target molecule(s). Such technology is defined mainly by the principle of environmental conditions optimization to effect rapid and efficient biodegradation (Morgan, 1991). According to Tabak *et al.* (1995), an understanding of the pertinent environmental factors provides insight of the optimal ranges of key variables for the enhancement of microbiological activity. Hence, a variety of influential environmental and ecological variables such as pH, temperature, redox potential, nutrients, inocula and electron acceptors and concentrations should be investigated.

Batch cultures of combined microbial associations (Table 2.3) were used as described in 2.5 to explore key rate-limiting factors, specifically pH and temperature, exemplary of South African soils. Since the maximum solubility of atrazine in water is approximately 30 mg l^{-1} (0.15 mM) (Erickson and Lee, 1989), this concentration was selected to both model the maximum concentration available in *in situ* bioremediation and reflect the normal dosages applied to agricultural soils. The BTX molecule concentration (50 mg l^{-1}) was chosen to be representative of recorded site and laboratory experimental concentrations (16.1-62.6 mg l^{-1}) (Yerushalmi and Guiot, 1998).

The experimental protocol adopted was to determine the optimum pH of each association and then use this as a fixed variable in the subsequent temperature study.

4.2 Results and Discussion

4.2.1 Environmental pH

pH may influence pollutant molecule catabolism since soil microorganisms are usually metabolically active between 5 and 9 with the optima often marginally above 7 (Dragun, 1988; Atlas and Cerniglia, 1995).

Combined Atrazine-Catabolizing Association (KRA30)

To ensure that all the component species were included, the pH 7.5- and 5.5-poised cultures of atrazine (30 mg l^{-1})-catabolizing microbial associations were mixed and the resulting combined association designated KRA30. Optical density readings of the association during batch cultivation at different initial pH values are shown in Figure 4.1. With the exception of the pH 8-poised culture, the lag phases were equivalent and relatively short considering the low initial optical densities. Although the batch growth curves for the pH 4-, 5- and 6-poised cultures were comparable for the first 9 hours, the exponential phase of the pH 4-poised culture continued for two hours longer than the other two and, hence, reached a much higher terminal optical density. While not examined further, the elevated optical density could have been due to more complete catabolism/mineralization.

Although liquid cultures were used in this study, the near zero specific growth rate recorded with the pH 8-poised culture supported, partly, the findings of Tyess *et al.* (1995) who reported that soils with high (unspecified) pH values did not show increased atrazine mineralization when inoculated with catabolic associations.

Figure 4.2 shows the specific growth rates calculated for the logarithmic phases. Although a decreasing trend from pH 4 to 8 was recorded, the growth rates were relatively comparable for the pH 4-, 5- and 6-poised cultures. Assuming that South African soils record, generally, low pH values, this suggested that pH adjustments of soil would probably be unjustified.

The residual atrazine concentration data recorded during the exponential growth phase of the pH 4-poised culture (Figure 4.3) showed a near linear rate of atrazine attenuation of $1.0 \text{ mg l}^{-1} \text{ h}^{-1}$ between hours 6 and 11 which suggested that catabolism was not growth linked.

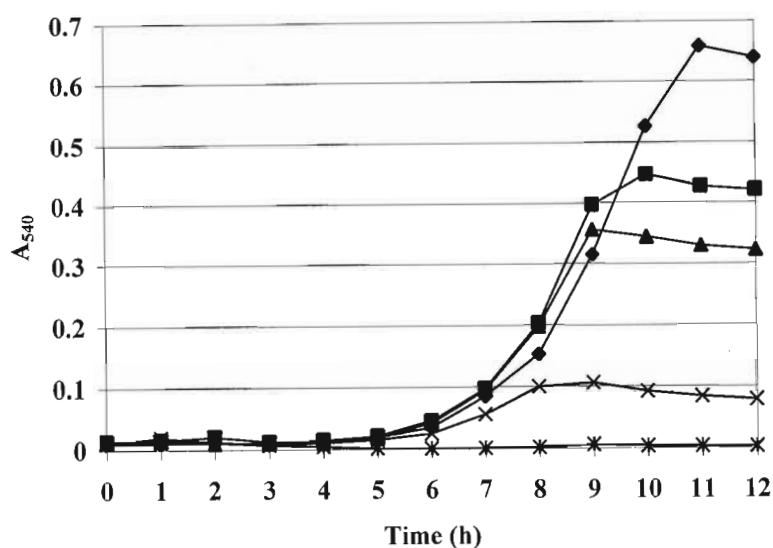


Figure 4.1: Changes in optical densities with time of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (30°C) in the presence of 30mg l^{-1} atrazine at initial pH values of 4(♦), 5 (■), 6(▲), 7(×) and 8(*).

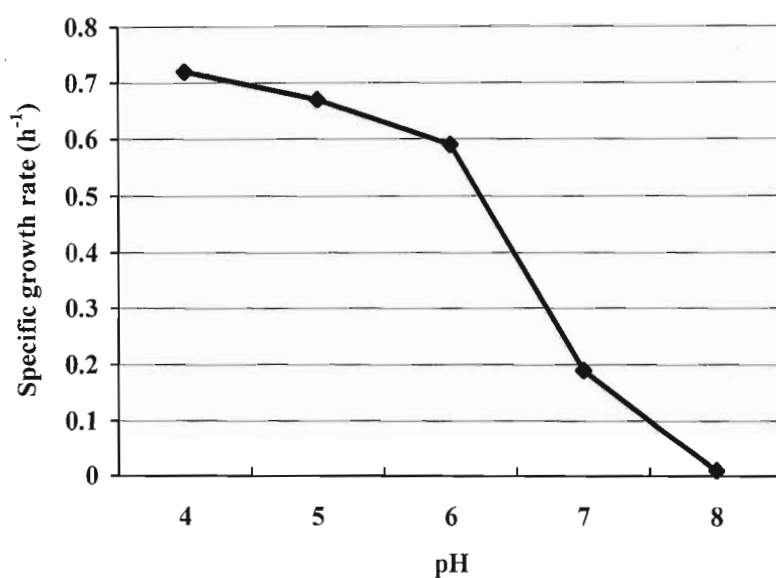


Figure 4.2: Maximum specific growth rates (h $^{-1}$) of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (30°C) in the presence of 30mg l^{-1} atrazine and five initial pH values.

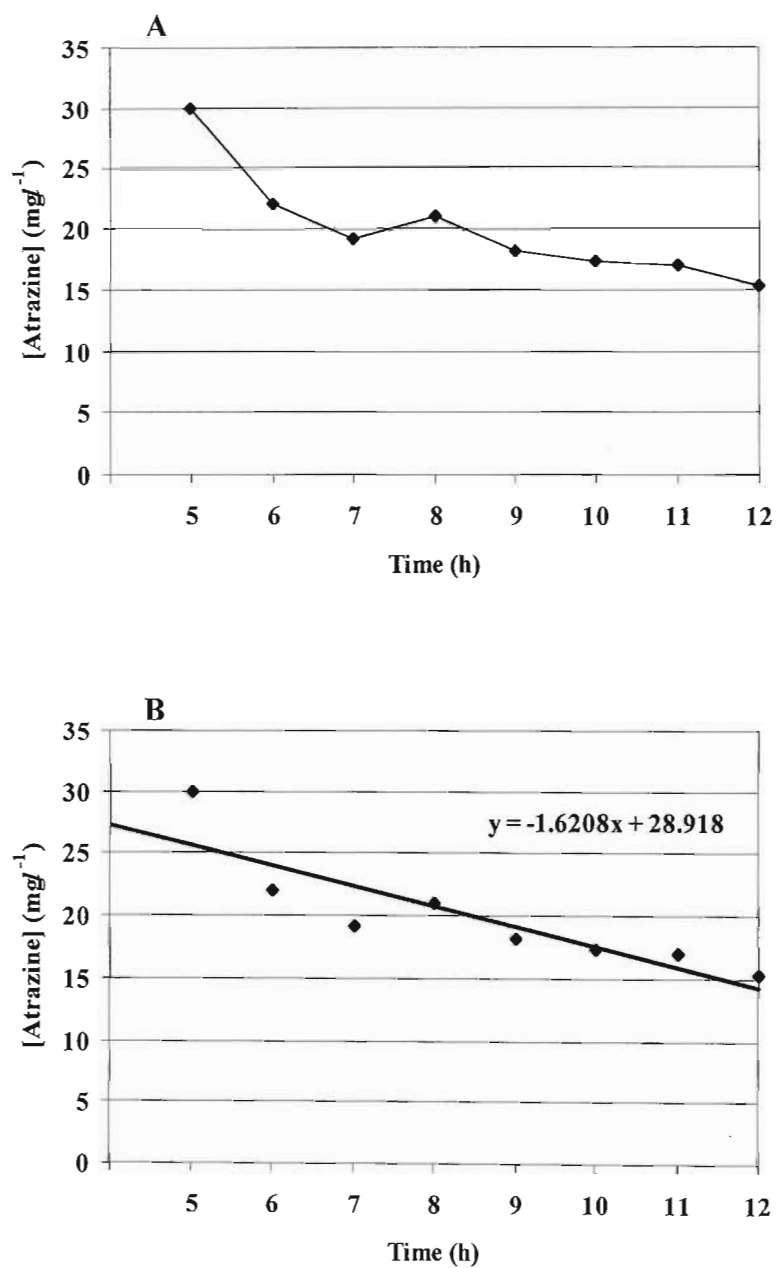


Figure 4.3: Changes in residual atrazine concentration with time (A) and regression analysis of the data (B) of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (30⁰C, pH 4) in the presence of 30 mg l⁻¹ atrazine.

Combined BTX Molecule-Catabolizing Associations (KRO50, KRM50, KRP50, KRB50 and KRT50)

As with atrazine, the pH 7.5- and 5.5-poised cultures of specific BTX molecule (50 mg l⁻¹)-catabolizing microbial associations were mixed and the resulting combined associations designated KRO50 (*o*-xylene), KRM50 (*m*-xylene), KRP50 (*p*-xylene), KRB50 (benzene) and KRT50 (toluene).

Figures 4.4–4.8 show the batch growth curves of BTX molecule-catabolizing associations cultured at 30°C and different initial pH values. Despite the protracted incubation periods, little net growth was recorded with the pH 6-, 7- and 8-poised cultures of the *o*- (Figure 4.4), *m*- (Figure 4.5) and *p*- (Figure 4.6) xylene- and benzene- (Figure 4.7) catabolizing associations due, possibly, to the initial low optical densities. For the pH 4- and 5-poised cultures, although the initial exponential phases were comparable, the former exhibited extended logarithmic phases until, for *m*-xylene, for example, the maximum optical density (0.88) was more than double (0.43) that of the pH 5-poised culture (Figure 4.5). The one exception was the *p*-xylene-catabolizing association where the highest optical density (0.62) was recorded with the pH 5-poised culture (Figure 4.6). Despite the differences in maximum optical densities, the lengths of the exponential phases were comparable for the three xylene cultures. In contrast, for the benzene- (Figure 4.7) and toluene- (Figure 4.8) catabolizing associations exponential phases were only discernable for the pH 4-poised cultures.

The maximum specific growth rates calculated for the BTX molecule-catabolizing combined associations are shown in Figure 4.9. With the exception of *p*-xylene, the optimum pH for catabolism of the BTX molecules was 4. Therefore, soil pH monitoring would be essential and the need to manipulate this variable would be required for efficient bioremediation of the South African BTX molecule-contaminated soil used in this study which recorded a pH value of 7.14 (Table 3.1).

Since this study was made with individual molecules and enriched/isolated microbial associations, further work should focus on hydrocarbon mixtures to determine the catabolic rates and potentials of the total populations as predictors of intrinsic bioremediation. Also, different concentrations must be studied to determine the optima and limits of catabolism so that, if necessary, concentration adjustments can be made by, for example, electrokinetics (1.4.1), leaching or soil amelioration.

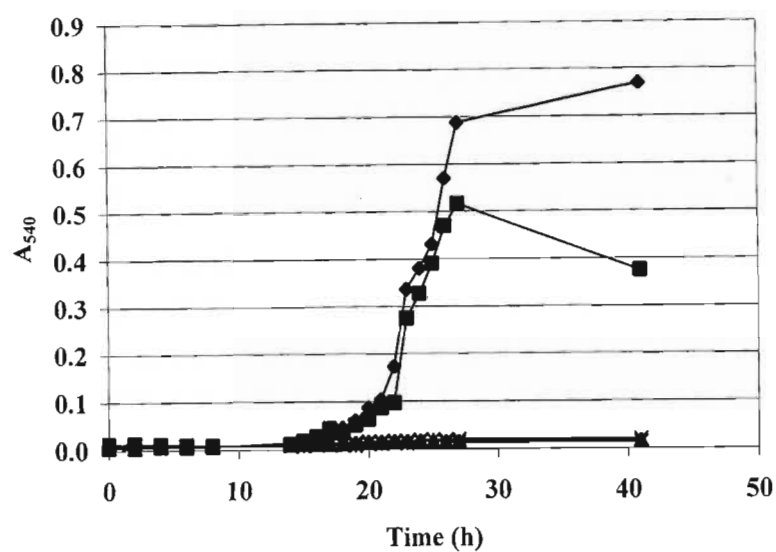


Figure 4.4: Changes in optical densities with time of the combined aerobic *o*-xylene-catabolizing association KRO50 batch cultured (30°C) in the presence of 50mg l^{-1} *o*-xylene and initial pH values of 4(♦), 5 (■), 6(▲), 7(×) and 8(*).

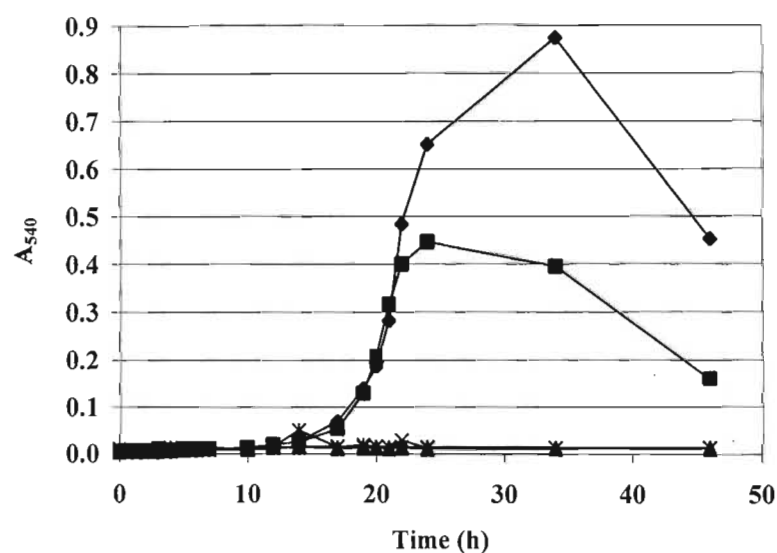


Figure 4.5: Changes in optical densities with time of the combined aerobic *m*-xylene-catabolizing association KRM50 batch cultured (30°C) in the presence of 50mg l^{-1} *m*-xylene and initial pH values of 4(♦), 5 (■), 6(▲), 7(×) and 8(*).

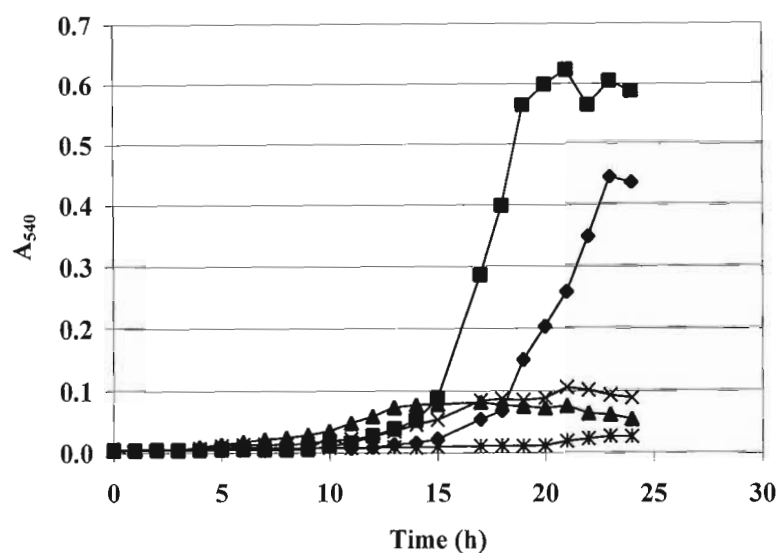


Figure 4.6: Changes in optical densities with time of the combined aerobic *p*-xylene-catabolizing association KRP50 batch cultured (30⁰C) in the presence of 50 mg^l⁻¹ *p*-xylene and initial pH values of 4(♦), 5 (■), 6(▲), 7(×) and 8(*).

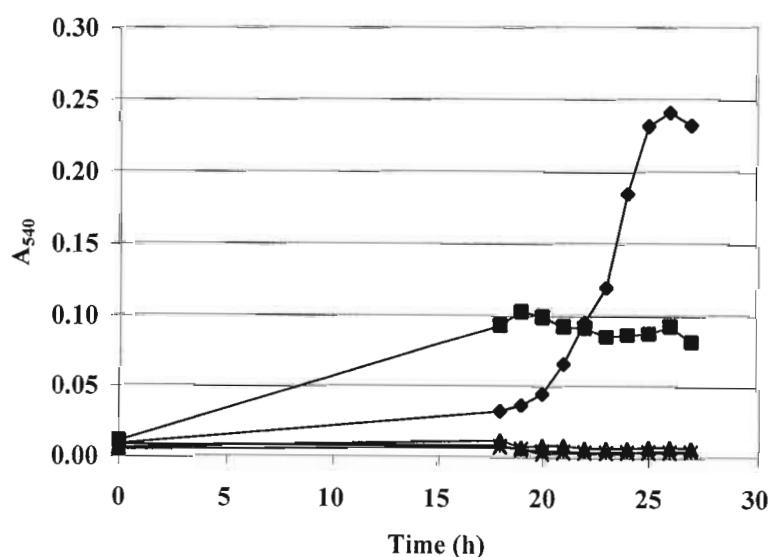


Figure 4.7: Changes in optical densities with time of the combined aerobic benzene-catabolizing association KRB50 batch cultured (30⁰C) in the presence of 50 mg^l⁻¹ benzene and initial pH values of 4(♦), 5 (■), 6(▲), 7(×) and 8(*).

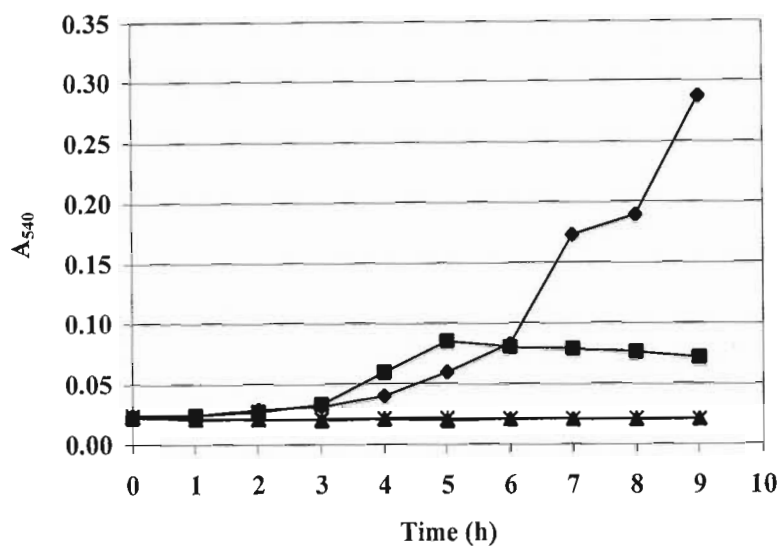


Figure 4.8: Changes in optical densities with time of the combined aerobic toluene-catabolizing association KRT50 batch cultured (30°C) in the presence of 50 mg/l toluene and initial pH values of 4(◆), 5 (■), 6(▲), 7(×) and 8(*).

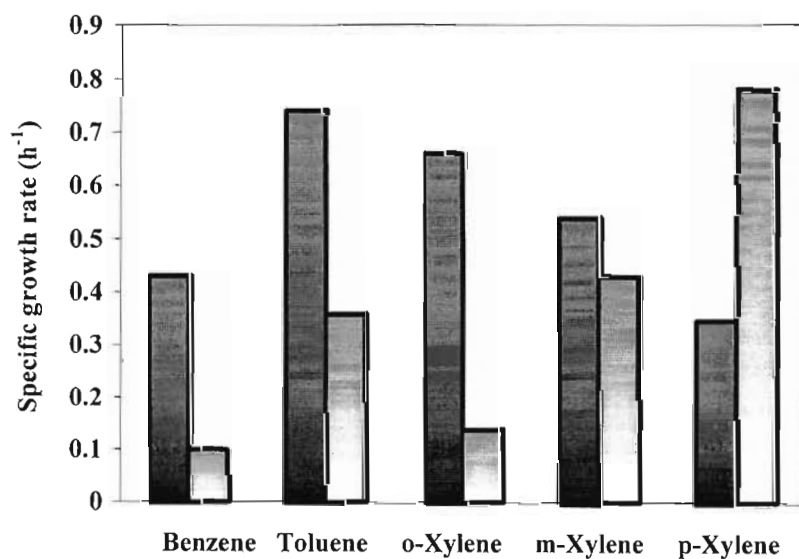


Figure 4.9: Maximum specific growth rates (h⁻¹) of the combined aerobic BTX molecule-catabolizing associations batch cultured (30°C) at initial pH values of 4 (■) and 5(▨).

4.2.2 Environmental Temperature

Temperature, in the field in particular, plays a significant role in controlling the physico-chemical compositions of hydrocarbons, their rates of catabolism and, hence, the extent of catabolism (Margesin and Schinner, 2001).

Atrazine (KRA30)

Perhaps surprisingly, comparable specific growth rates were recorded at 15 and 20°C for the atrazine-catabolizing association although the rate more than doubled for the temperature increment of 20 to 30°C (Figures 4.10 and 4.11). These results partly contrasted those of Skipper and Volk (1972) who reported two- to three-fold catabolism rate increases for the two 10°C temperature increments of 15-25°C and 25-35°C. Erickson and Lee (1989) and Sparling *et al.* (1998) also reported that the rate of atrazine catabolism increased with temperature and doubled with each 10°C increase between 10 and 30°C. Radosevich *et al.* (1996), however, recorded lowered mineralization rates at 10°C possibly due to atrazine sorption changes.

BTX Molecules (KRB50, KRT50, KRO50, KRM50 and KRP50)

Although a few true exponential phases were recorded during the 10-hour incubation period, in general, the combined associations showed increased specific growth rates with increased temperatures (Figures 4.12 - 4.17). Surprisingly, KRB50 recorded its lowest specific growth rate (0.009 h⁻¹) at 25°C. Highest, maximum specific growth rates of 0.032, 0.069, 0.077 and 0.067 h⁻¹ were recorded at 30°C for KRB50, KRO50, KRM50 and KRP50, respectively while for KRT50 the highest maximum specific growth rate of 0.039 h⁻¹ was recorded at 25°C. Of the five cultures, KRM50 recorded the highest maximum specific growth rates for the temperature range of 20-30°C (Figure 4.17).

As observed in the pH optimization study (4.2.1), protracted lag phases characterized many of the cultures. For some (results not shown), these lasted for 15 h unless increased volumes of logarithmic phase cells were used as the inoculum. A typical logarithmic growth phase was of the order of 5 h and was often followed by a very limited stationary phase and a pronounced autolytic phase which suggested that repeat inoculations may be required for *in situ* (bioaugmented) bioremediation.

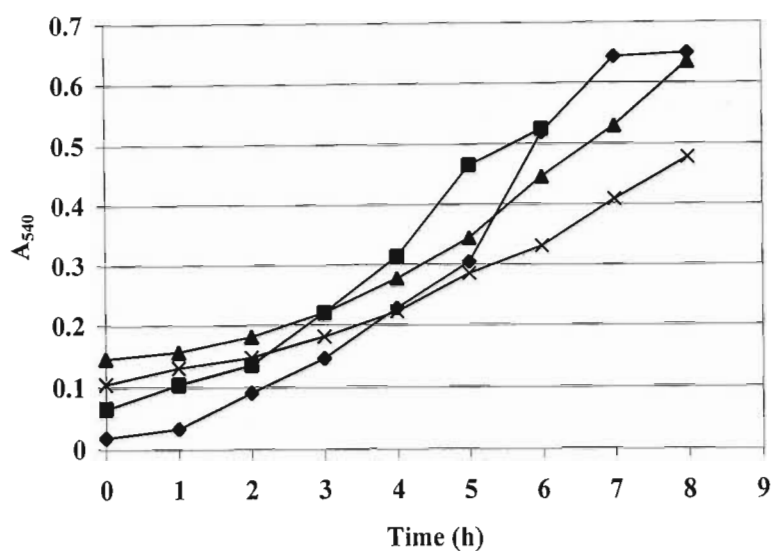


Figure 4.10: Changes in optical densities with time of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (pH 4) in the presence of 30 mg l⁻¹ atrazine at 15 (×), 20 (▲), 25 (■) and 30 (◆) °C.

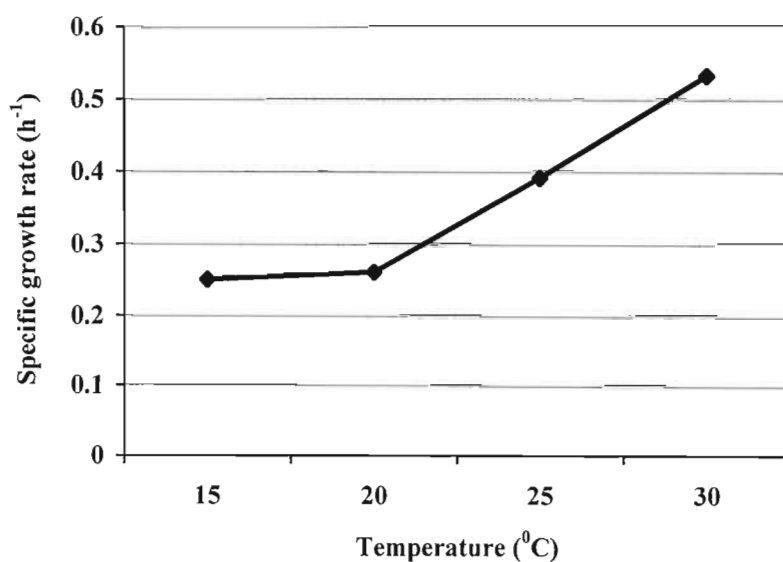


Figure 4.11: Maximum specific growth rates (h⁻¹) of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (pH 4) in the presence of 30 mg l⁻¹ atrazine at different temperatures.

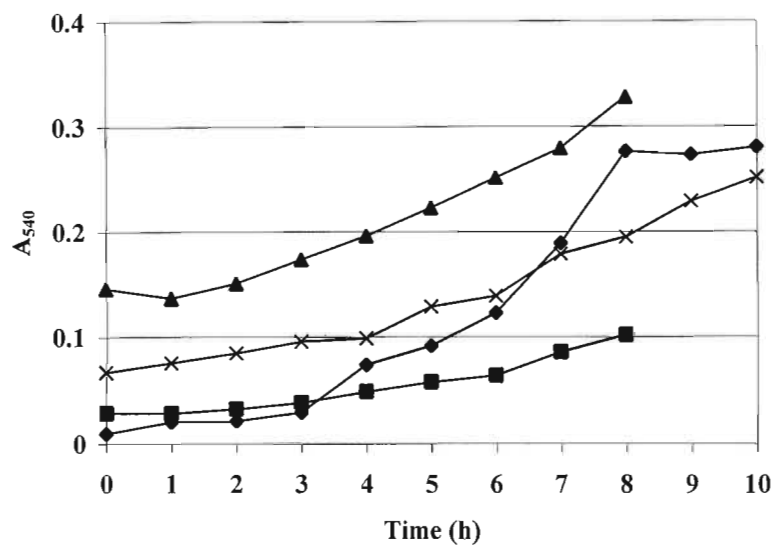


Figure 4.12: Changes in optical densities with time of the combined aerobic benzene-catabolizing association KRB50 batch cultured (pH 4) in the presence of 50 mg l^{-1} benzene at 15 (x), 20 (▲), 25 (■) and 30 (◆) $^{\circ}\text{C}$.

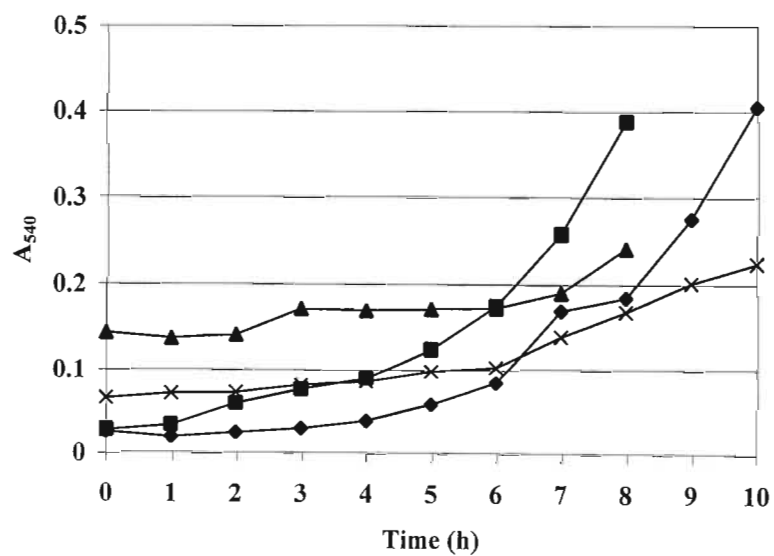


Figure 4.13: Changes in optical densities with time of the combined aerobic toluene-catabolizing association KRT50 batch cultured (pH 4) in the presence of 50 mg l^{-1} toluene at 15 (x), 20 (▲), 25 (■) and 30 (◆) $^{\circ}\text{C}$.

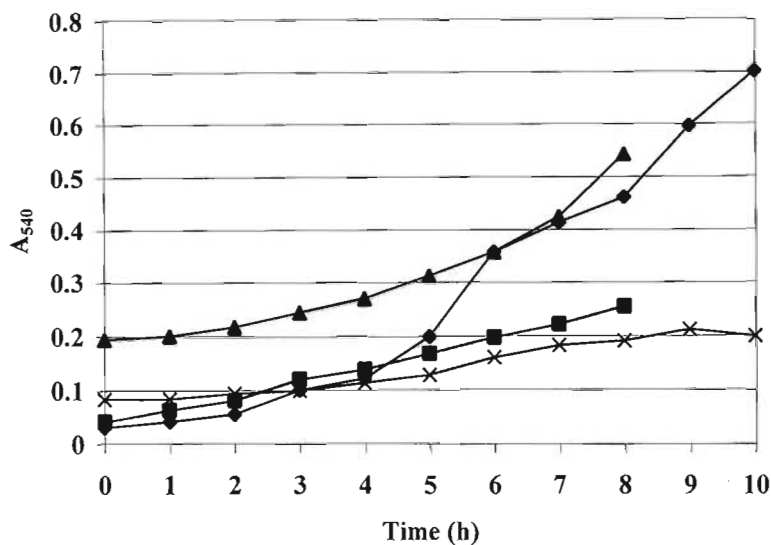


Figure 4.14: Changes in optical densities with time of the combined aerobic *o*-xylene-catabolizing association KRO50 batch cultured (pH 4) in the presence of 50 mg l⁻¹ *o*-xylene at 15 (×), 20 (▲), 25 (■) and 30 (◆) °C.

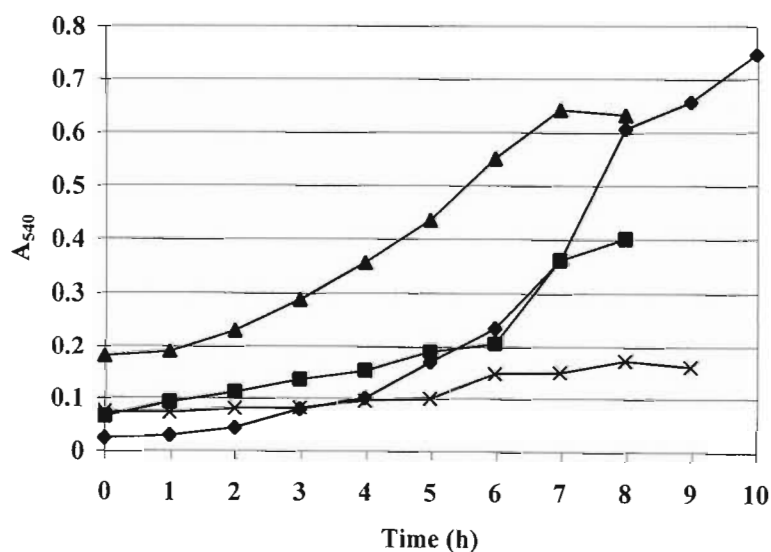


Figure 4.15: Changes in optical densities with time of the combined aerobic *m*-xylene-catabolizing association KRM50 batch cultured (pH 4) in the presence of 50 mg l⁻¹ *m*-xylene at 15 (×), 20 (▲), 25 (■) and 30 (◆) °C.

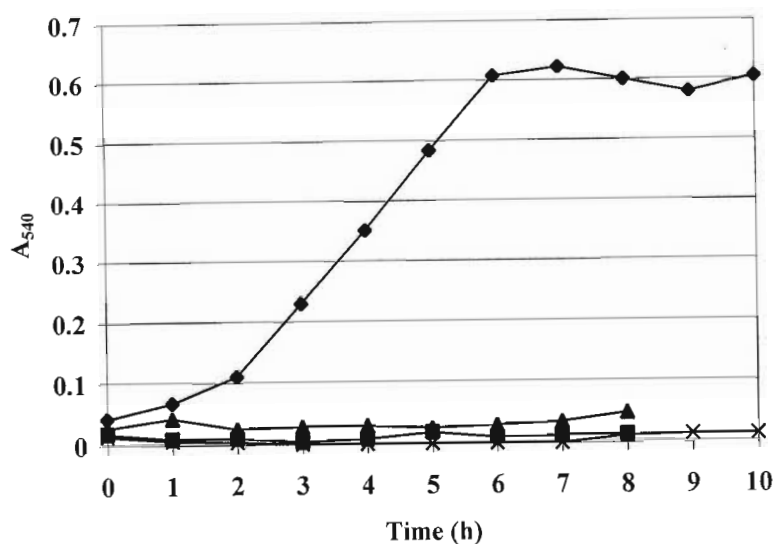


Figure 4.16: Changes in optical densities with time of the combined aerobic *p*-xylene-catabolizing association KRP50 batch cultured (pH 5) in the presence of 50 mg l⁻¹ *p*-xylene at 15 (×), 20 (▲), 25 (■) and 30 (◆) °C.

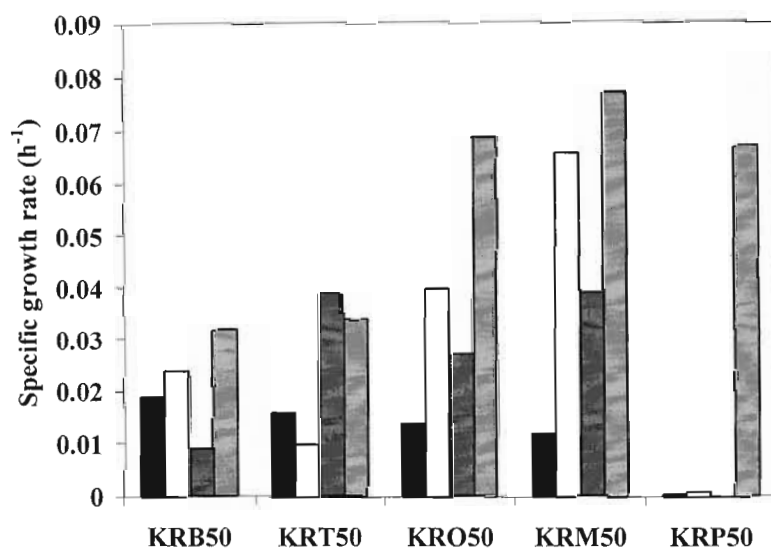


Figure 4.17: Maximum specific growth rates (h⁻¹) of the combined aerobic catabolic associations KRB50, KRT50, KRO50 and KRM50 (pH 4) and KRP50 (pH 5) batch cultured at 15 (■), 20 (□), 25 (■) and 30 (■) °C.

From the results it was apparent that bioremediations *in situ* should proceed at the temperature conditions typical of South African soils although this determinant should be monitored closely. For *ex situ* (bioreactor) bioremediations high volumes of logarithmic-phase inocula may be required to minimize the lag phases.

4.2.3 Scanning Electron Microscopy Visualization

To determine whether the different specific growth rates recorded were effected by different population profiles, logarithmic-phase samples were taken during the pH and temperature optimization studies for examination by scanning electron microscopy and molecular characterization (6.2.5).

Two morphotypes were visible in associations KRA30 and KRO50 cultured at pH 4 and 30⁰C and pH 4 and 15⁰C, respectively (Plates 4.1 and 4.2, Arrows) while association KRT50 recorded 3 morphotypes at pH 4 and both 15 and 30⁰C (Plate 4.3, Arrows 1, 2 and 3). For KRT50, profile changes in response to pH/temperature changes were recorded. The lower temperature supported the growth of, predominantly, rods 2-4 μm long (Arrows 1 and 2) while the increased temperature of 30⁰C facilitated the growth of cocci (Arrow 3).

This suggested that changes in species numerical dominance occurred in the combined microbial associations in response to temperature and, probably, pH changes. These changes were as expected since each species should exhibit optimal growth and substrate catabolism at a specific temperature and pH value (Dragun, 1988).

4.3 Conclusions

Since the results of the batch culture studies showed that the combined catabolic associations exhibited different specific growth rates (and molecule catabolism rates) in response to different pH and temperature values, it may be concluded that the rates of both intrinsic and bioaugmented bioremediation of atrazine- and BTX molecule-contaminated soils should vary depending on the season. For all six molecules, degradation should proceed at increased rates in soils with temperatures near 30⁰C and pH values of 4 to 5. Since increased temperatures can be maintained only in the first 5 cm horizon of surface soil *in situ*, biopiles, co-composting and landfarming

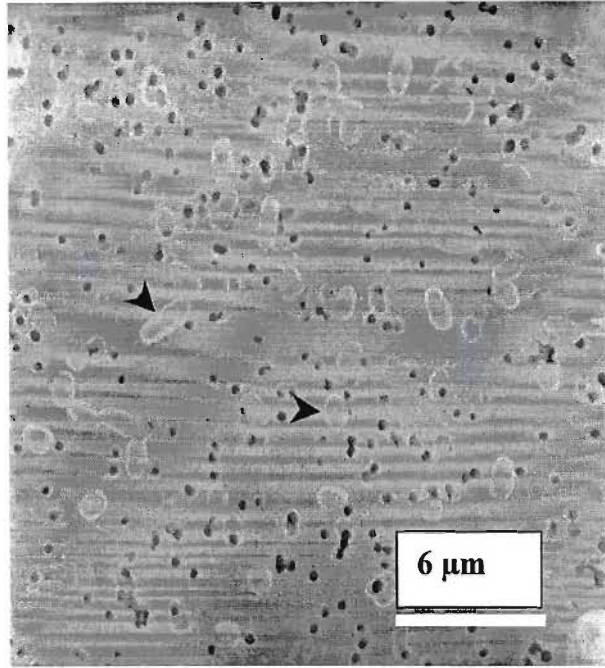


Plate 4.1: Scanning electron micrograph of logarithmic-phase bacterial cells of the combined aerobic atrazine-catabolizing association KRA30 batch cultured (30°C) in the presence of 30 mg l^{-1} atrazine and an initial pH value of 4. The arrows indicate the different morphotypes.

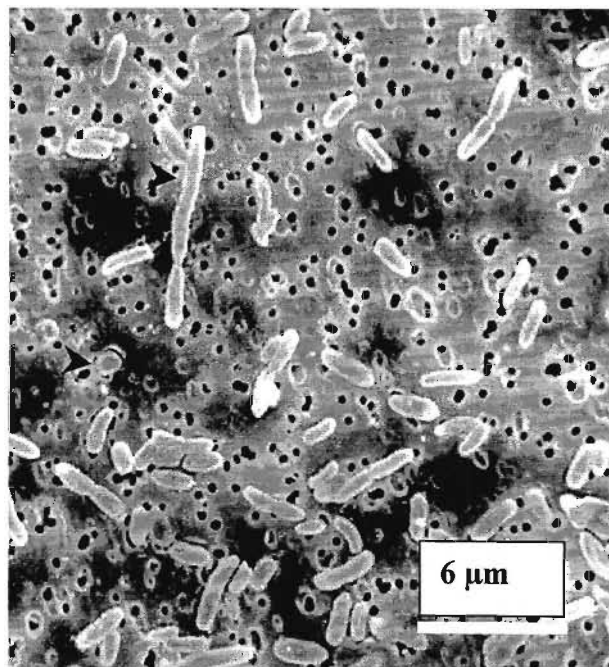


Plate 4.2: Scanning electron micrograph of logarithmic-phase bacterial cells of the combined aerobic *o*-xylene-catabolizing association KRO50 batch cultured (15°C) in the presence of 50 mg l^{-1} *o*-xylene and an initial pH value of 4. The arrows indicate the different morphotypes.

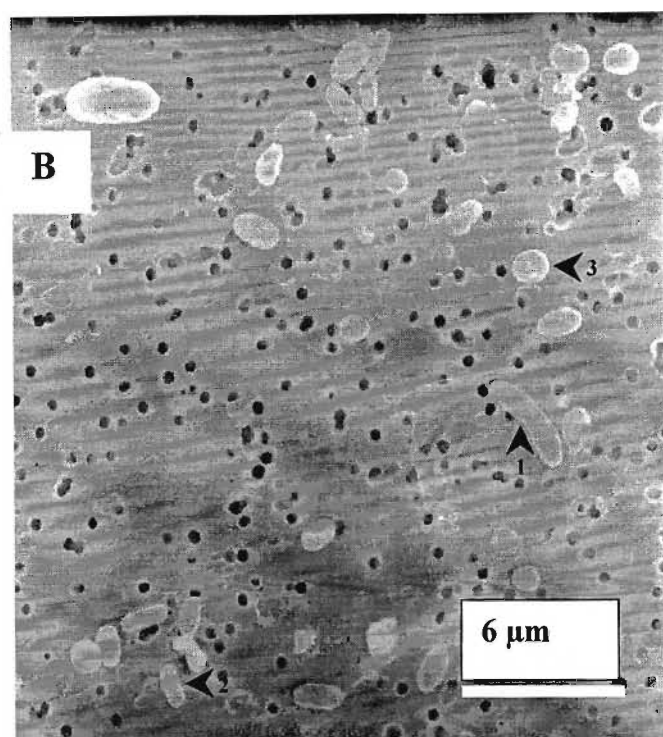
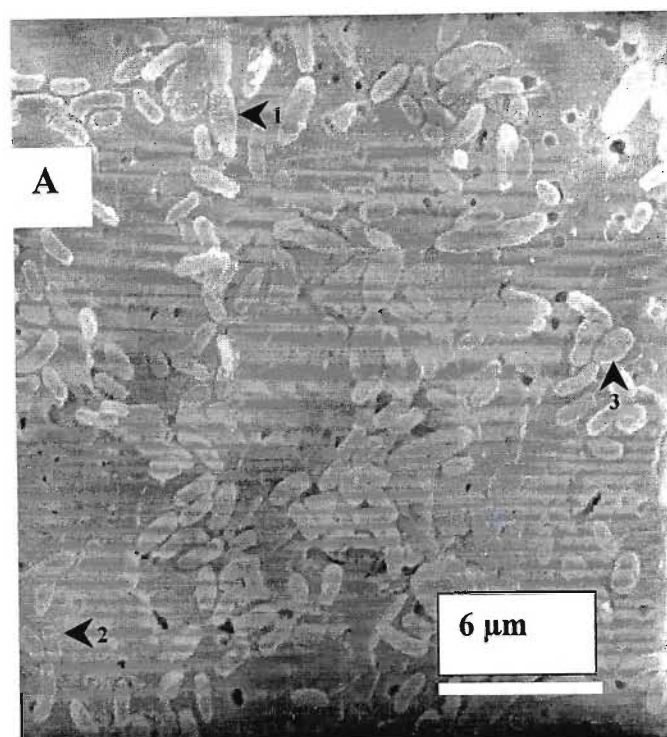


Plate 4. Scanning electron micrographs of logarithmic-phase bacterial cells of the combined aerobic toluene-catabolizing association KRT50 batch cultured in the presence of 50 mg l^{-1} toluene and an initial pH value of 4 at 15°C (A) and 30°C (B). Arrows 1, 2 and 3 indicate long rods, short rods and cocci, respectively.

may be considered together with engineered and covered biopiles particularly during the cold and rainy seasons.

Soil pH would be an easier variable to manage although cost implications would have to be considered carefully.

Smit *et al.* (2001) observed that together with seasonal temperature variations, shifts in species complements of microbial associations can be effected by key factors such as pH, moisture content and nutrient type and concentration. Thus, complete environmental parameter optimization (and association characterization) to identify the optima should be made prior to bioaugmentation.

If cost-effective, *ex situ* bioaugmented bioremediation with soil slurry bioreactors would be the most efficient approach due to the fine control of all key physiological variables. Also, Robertson and Alexander (1994) reported that accelerated biodegradation occurred frequently with pesticides which served as carbon sources, in particular, if elevated population densities of the active catabolic species were maintained and the time intervals were kept brief between the first and subsequent pesticide-contaminated soil additions. To minimize the lag phases of batch-operated bioreactors, inocula should be provided by open (continuous) cultures maintained at specific temperature and pH values relative to the site and seasonal conditions (3.3).

Chapter 5: The Catabolic Potential of Association KRA30 Under Carbon- and Nitrogen-Limited Conditions

5.1 Introduction

The need to estimate the catabolic potentials of soil microbial associations has been recognized (1.4.1, 1.5.3). Open cultures are often the methods of choice since they are able to effect slow specific growth rates which are characteristic of electron donor poor/electron acceptor variable soil ecosystems. Retentostats are considered ideal specifically since the biomass is retained and, therefore, the cultures mirror *in situ* environmental conditions.

Although fully-oxidized ring carbons are relatively poor energy sources (3.1), atrazine dealkylation provides a carbon and energy source for heterotrophic growth (Erickson and Lee, 1989). Nevertheless, bioenergetic limitations, due to low herbicide concentrations, may result but these may be circumvented by the addition of supplemental carbon/energy sources such as sucrose, glucose or citrate with atrazine used as the nitrogen source.

Studies of catabolic potential facilitation/optimization must, however, be complemented by molecular analysis (de Souza *et al.*, 1998a). Following the approach of other research groups (Møller *et al.*, 1996; Ramsing *et al.*, 1996; Teske *et al.*, 1996; Massol-Deyá *et al.*, 1997), retentostat cultures were used in this study to: determine the catabolic potential (2.6) of the atrazine-catabolizing combined association KRA30 following physiological optimization; and assign association form or structure to function by species composition determination after atrazine catabolism under carbon- and nitrogen-limited conditions.

Since its isolation by Mandelbaum *et al.* (1995), *Pseudomonas* sp. strain ADP, which is capable of catabolizing herbicide concentrations $>1\,000\text{mg l}^{-1}$, has become a reference strain and has been used extensively both to study atrazine catabolism under different conditions and elucidate the enzymes involved in one of the aerobic catabolic pathways (Figure 1.1). This strain was, therefore, included as a comparison.

5.2 Results and Discussion

Since the objectives of this section of the programme were to determine the catabolic potentials of KRA30 and *Pseudomonas* sp. strain ADP together with population profile changes of the microbial association in response to different carbon and nitrogen limitations and mineral salts composition, no detailed carbon balances were determined and general observations only were made.

5.2.1 Batch Cultures

Growth in the Presence of Different Citrate/Citric Acid Concentrations

A review by Erickson and Lee (1989) indicated that slow atrazine degradation/dealkylation rates and, therefore, low specific growth rates are common. For example, average specific growth rates of 0.008 and 0.005 h⁻¹ were recorded over 14-day periods for a *Pseudomonas* sp. batch cultured on 60 mg l⁻¹ atrazine at 29°C while a specific growth rate of 0.014 h⁻¹ was reported for a *Nocardia* sp. in the presence of 60 mg l⁻¹ atrazine and an incubation temperature of 30°C. These results identified the possible need for a supplemental carbon/energy source to promote pesticide catabolism.

Since citrate-phosphate buffer was used to poise the culture pH values in the environmental parameter optimization investigation (Chapter 4), the same carbon source was used to determine its influence on atrazine catabolism. Thus, batch cultures (2.6.3) were made to compare growth of the association on 30 mg l⁻¹ (0.14mM) atrazine in the presence of two citrate (as tri-sodium citrate and citric acid) concentrations. Many attempts were made to prepare a medium of citrate-free M-III supplemented with atrazine but these proved unsuccessful due to the presence of an unidentified precipitate. As a result of this, a control which contained atrazine but no citrate was omitted.

Although comparable specific growth rates were recorded for the atrazine plus citrate plus citric acid (0.43 h⁻¹)- and the citrate plus citric acid (0.35 h⁻¹)-supplemented cultures, the former recorded an increased terminal optical density (0.54) compared with the latter (0.30) (Figure 5.1A). In contrast, a slower growth rate (0.067 h⁻¹) and a reduced terminal optical density (0.39) were recorded in the presence of atrazine plus 1 g l⁻¹ tri-sodium citrate. The atrazine degradation rates were, however, comparable (Figure 5.1B) as too were the final residual atrazine concentrations. This suggested the possibility of

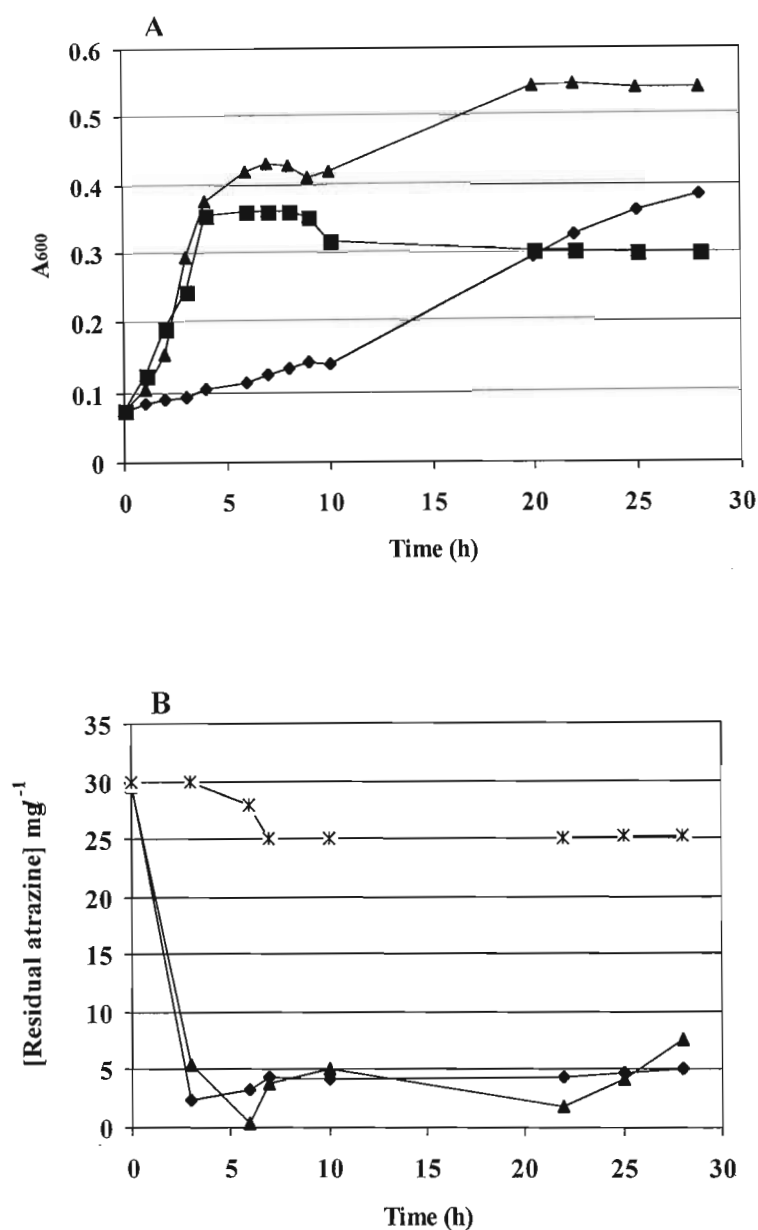


Figure 5.1: Changes in optical densities (A) and residual atrazine concentrations (B) with time of the combined aerobic atrazine-catabolizing association KRA30 in batch cultures (30°C) in mineral salts solution M-III in the presence of: atrazine (30 mg l^{-1}) + citrate (1 g l^{-1}) (◆); atrazine (30 mg l^{-1}) + citrate (1 g l^{-1}) + citric acid (3mM) (▲); and citrate (1 g l^{-1}) + citric acid (3 mM) (■). An uninoculated control of atrazine (30 mg l^{-1})-supplemented M-III (*) was included.

nitrogen limitation. In this eventuality, it would be important to identify the critical citrate/citric acid supplementation concentration and so minimize the costs of biosupplemented/biostimulated remediation.

The appearance of a double (diauxic/biphasic) growth cycle is recorded often with media which contain mixtures of substrates (Schlegel, 1986). Figure 5.1A suggested diauxic growth for KRA30 during cultivation in the presence of atrazine plus citrate plus citric acid and, to a lesser extent, atrazine plus citrate, with the citrate/citric acid possibly utilized first. However, Figure 5.1B shows that atrazine was degraded during the first 5 h of incubation and thus the observed growth pattern was probably not biphasic. The second growth phase recorded from 10 h for the atrazine plus citrate plus citric acid may, therefore, have resulted from the catabolism of the atrazine degradation products.

Although a study of the component species of the microbial association might have identified individual catabolic capabilities, the initial attempt to isolate monocultures was unsuccessful (3.2.4). Other researchers (de Souza *et al.*, 1998a), who adopted both mono- and mixed culture approaches to study an atrazine-catabolizing microbial association, reported an optical density ≈ 0.4 for *Clavibacter michiganese* during cultivation on mineral salts supplemented with glucose ($1\ 000\ \text{mg l}^{-1}$) and atrazine ($100\ \text{mg l}^{-1}$). However, the microbial association attained a maximal cell density >1.2 and an elevated atrazine catabolism rate. Also, the association exhibited an increased capacity to metabolize some degradation products when they were provided as both carbon and nitrogen sources.

While not analyzed in detail in this study, the appearance and disappearance of peaks, during successive HPLC analyses, which corresponded to less polar atrazine degradation intermediates were interpreted as possible circumstantial evidence of the metabolism of these molecules by association KRA30. Also, a relationship between these and 16S rDNA profiling results (5.2.3) implicated the roles of individual species in the degradation pathway.

Comparison of the Growth of Association KRA30 and Pseudomonas sp. strain ADP

Prior to the retentostat studies (5.2.2), batch cultures (2.7) were used to explore and compare the catabolic capabilities of association KRA30 and *Pseudomonas* sp. strain

ADP in the presence of atrazine-supplemented mineral salts solution M-III. A herbicide concentration of 100 mg l^{-1} was chosen since it had been used by other workers (Mandelbaum *et al.*, 1995; de Souza *et al.*, 1998a). A heat-killed (15 lb psi, 121°C , 15 min) inoculum of KRA30 was included and, thus, the possibility of herbicide adsorption to biomass (2.7) was investigated also.

Similar growth trends were observed for the combined association and *Pseudomonas* sp. strain ADP while no growth was recorded with the heat-killed cells (control) (Figure 5.2A). Lag phases of 3 h were recorded for both the cultures and these were then followed by protracted logarithmic phases of 13 h. The residual atrazine concentration plots (Figure 5.2B) mirrored these which indicated that catabolism of the pesticide was probably growth linked. Also, the residual atrazine concentration of the control remained $\geq 95 \text{ mg l}^{-1}$ and indicated that adsorption/photolysis accounted for $\leq 5\%$.

While it is difficult to make comparisons due to differences in mineral salts composition, atrazine concentration, inoculum density and culture conditions, both similar (de Souza *et al.*, 1998a; Bichat *et al.*, 1999) and slower (Behki *et al.*, 1993; Shao and Behki, 1995) atrazine degradation rates have been reported.

5.2.2 Retentostat Cultures

Retentostat cultivation of the combined association KRA30 and the reference *Pseudomonas* sp. strain ADP under C- and N-limited conditions was made in 1 and 5 l vessels with the three types of mineral salts solutions (2.2.1) supplemented with 100 mg l^{-1} atrazine (2.6). Due to increased biofilm formation on the vessel walls with protracted continuous cultivation, some of the optical density and cell number results were interpreted with caution.

1 l Retentostats

The simple mineral salts solution M-I was used to study the effects of providing atrazine as a source of both carbon and nitrogen, and as a source of nitrogen with citric acid added as a supplementary source of carbon for the cultivation of KRA30 and *Pseudomonas* sp. strain ADP. The combined association recorded little growth during cultivation on atrazine alone with an O.D. of 0.2 recorded after 72 h of incubation. Increased growth,

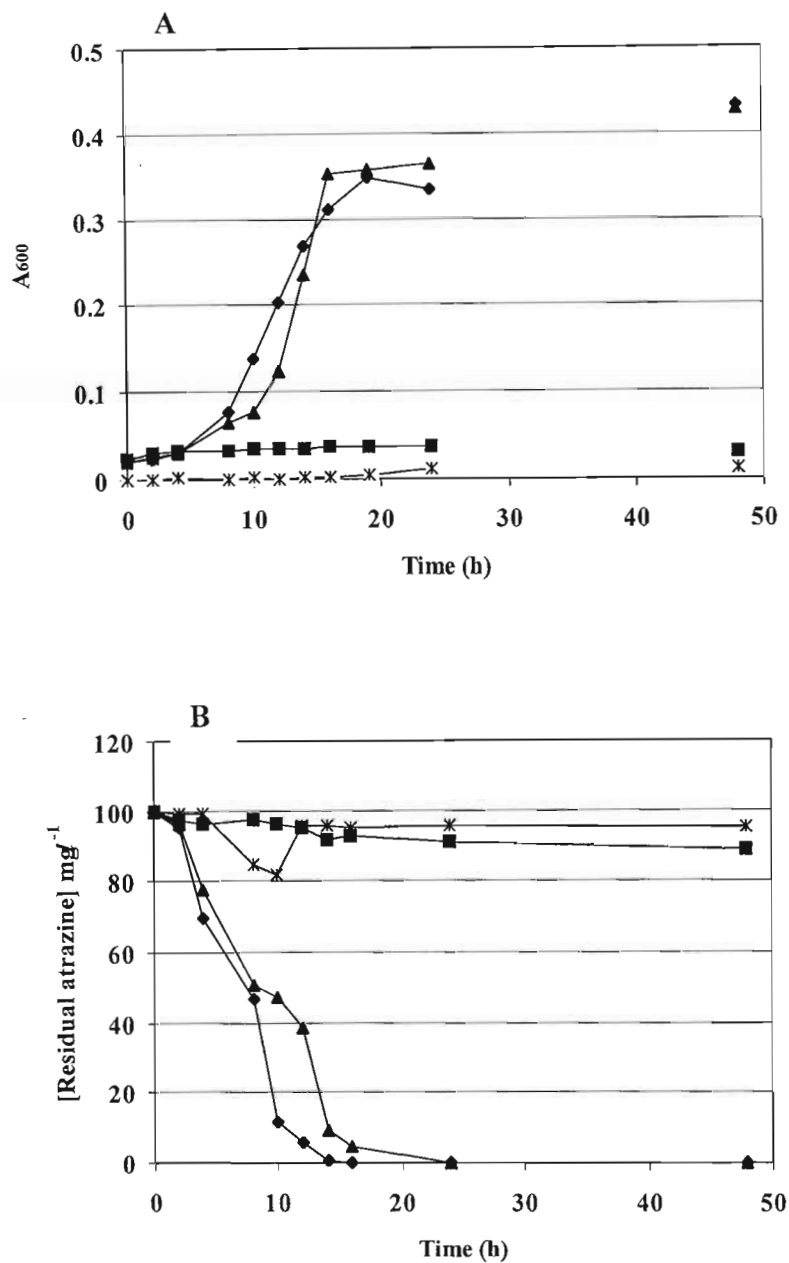


Figure 5.2: Changes in optical densities (A) and residual atrazine concentrations (B) with time of the combined atrazine-catabolizing association KRA30 (▲) and *Pseudomonas* sp. strain ADP (◆) in aerobic batch cultures (30°C) in mineral salts solution M-III supplemented with 100 mg l^{-1} (0.46 mM) atrazine. Control flasks with heat-killed KRA30 inoculum (■) and no inoculum (*) were included.

with a terminal optical density of 1.7, was recorded, however, in the presence of citric acid as a supplementary carbon source (Figure 5.3). The highest terminal optical density of 2.1 was recorded with the combined association cultured in the presence of citric acid as the sole carbon source.

For the reference *Pseudomonas* sp. strain ADP, although an optical density of 0.7 was recorded after 48 h of incubation for the herbicide-supplemented culture, the optical density recorded after 120 h of continuous cultivation was 0.3 which suggested increased autolysis of the retained cells. In contrast, the presence of herbicide plus citric acid resulted in a corresponding optical density of 1.9. Growth of *Pseudomonas* sp. strain ADP in the presence of mineral salts solution M-I was a little unexpected since the species was isolated with the more complex medium M-III (Mandelbaun *et al.*, 1995). Due to the history of the strain, which was enriched/isolated in the presence of tri-sodium citrate, growth in the presence of atrazine as the N source and citric acid as the C source was expected.

Despite biomass retention in the retentostats, particulate total organic carbon concentrations $<1\ 200\ \mu\text{g l}^{-1}$ were recorded for the cultures (KRA30, A-C; *Pseudomonas* sp. strain ADP, D and E) (Figure 5.4) with the lowest concentrations $\leq 35\ \mu\text{g l}^{-1}$ recorded for KRA30 in the presence of atrazine-supplemented M-I (A). If the results of the study had reflected conventional continuous culture rather than retentostat cultivation with biomass retention, the maximum biomass concentrations of each bioreactor would still have been only 0.007-0.08% (Figure 5.4A), 0.27-0.4% (Figure 5.4B), 0.29-0.32% (Figure 5.4C), 0.12-0.49% (Figure 5.4D) and 0.27-0.61% (Figure 5.4E) of the influent carbon. It is important to note, however, that, like the optical density data, the TOC results of retentostats B, C and E reflected the presence of citric acid. With so little of the influent carbon retained in the biomass, possibly due to high autolysis, the balance must have been accounted for by the residual pesticide/citric acid, catabolic intermediates and carbon dioxide. The limited biomass production/retention was possibly indicative of high maintenance energy requirements of both the association and the reference strain.

Since Abdelhafid *et al.* (2000) suggested that the walls and membranes of growing microbial cells were reactive and provided surfaces (sinks) for herbicide retention, this possibility was investigated, partly, with 5 l retentostat cultures.

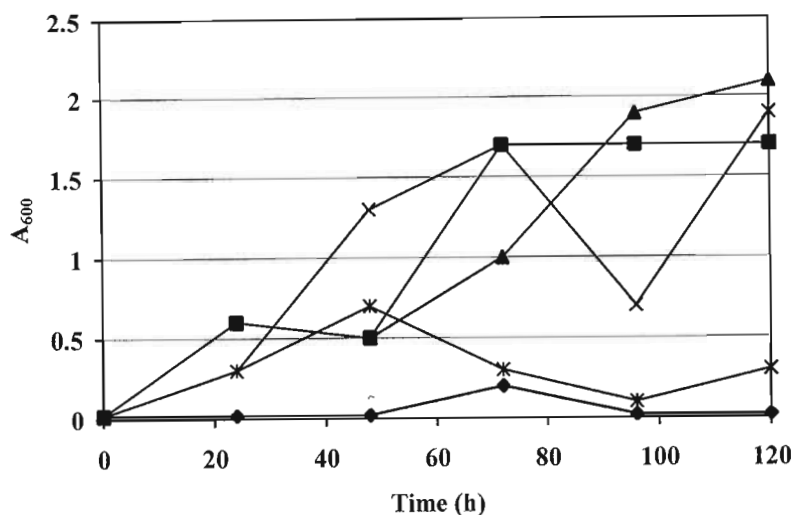


Figure 5.3: Changes in optical densities with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30°C) in 1 l retentostats (working volume 400 ml) with mineral salts solution M-I supplemented with 100 mg l^{-1} atrazine (◆), 100 mg l^{-1} atrazine + 3 mM citric acid (■) and 3 mM citric acid (▲), and *Pseudomonas* sp. strain ADP cultured in the presence of 100 mg l^{-1} atrazine (*)- and 100 mg l^{-1} atrazine + 3 mM citric acid (×)-supplemented M-I.

Figure 5.5 shows the mass spectrometer headspace carbon dioxide concentration results of the first 24 h of cultivation of KRA30 in the presence of M-I supplemented with atrazine plus citric acid. Low CO_2 concentrations were recorded during the start-up period with a peak after 22 h which corresponded to an increase in optical density (Figure 5.3) and, probably, atrazine catabolism. As stated earlier (5.2.1) attempts to prepare a medium of citrate-free M-III supplemented with atrazine were unsuccessful and, thus, analysis of culture headspace carbon dioxide concentration in the absence of supplemental citrate was not made.

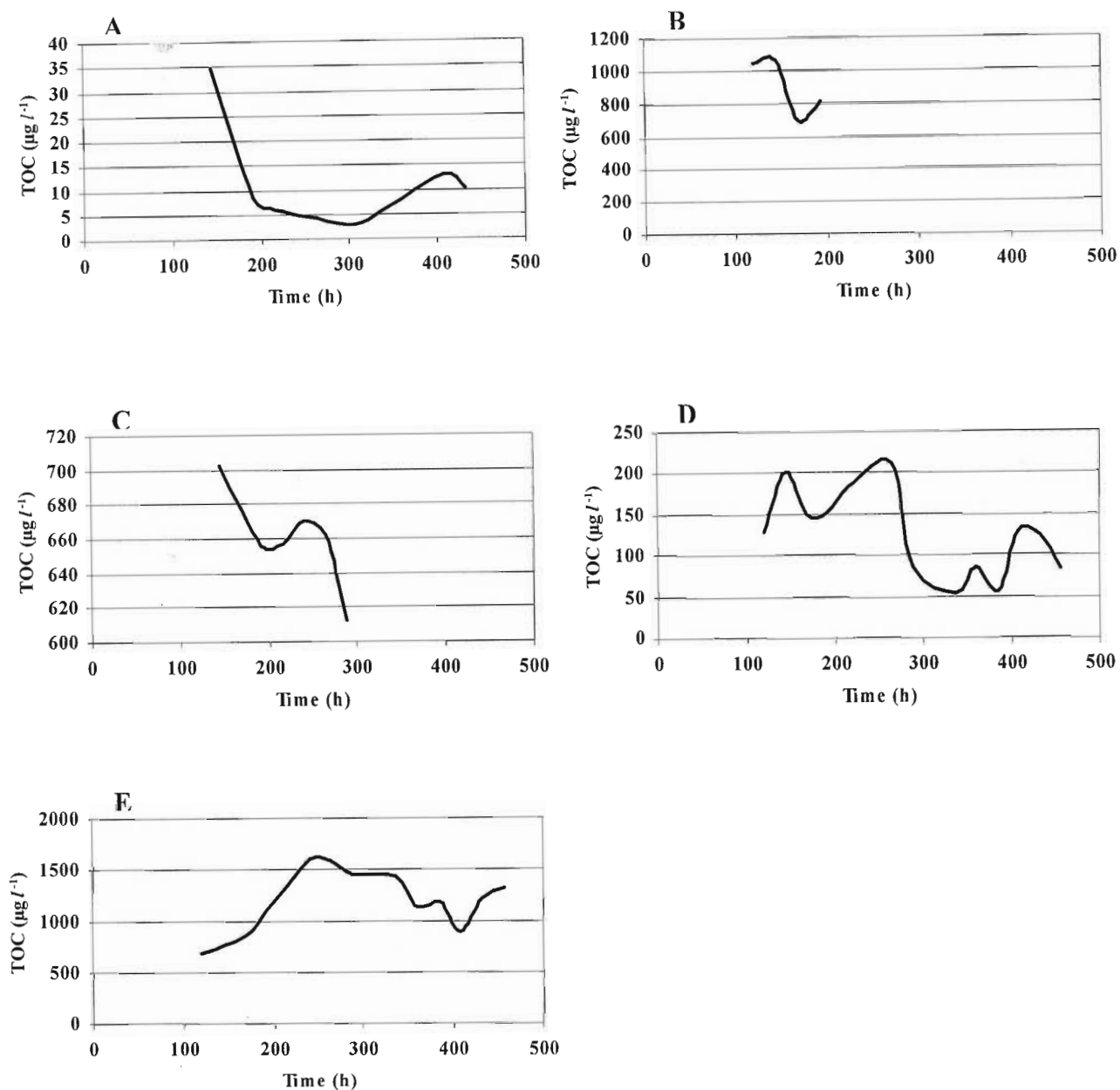


Figure 5.4: Changes in particulate total organic carbon (TOC) with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30⁰C) in 1 l retentostats (400 ml working volume) with mineral salts solution M-I supplemented with 100 mg l⁻¹ atrazine (A), 100 mg l⁻¹ atrazine + 3 mM citric acid (B) and 3 mM citric acid (C), and *Pseudomonas* sp. strain ADP cultured in the presence of 100 mg l⁻¹ atrazine (D)- and 100 mg l⁻¹ atrazine + 3 mM citric acid (E)-supplemented M-I.

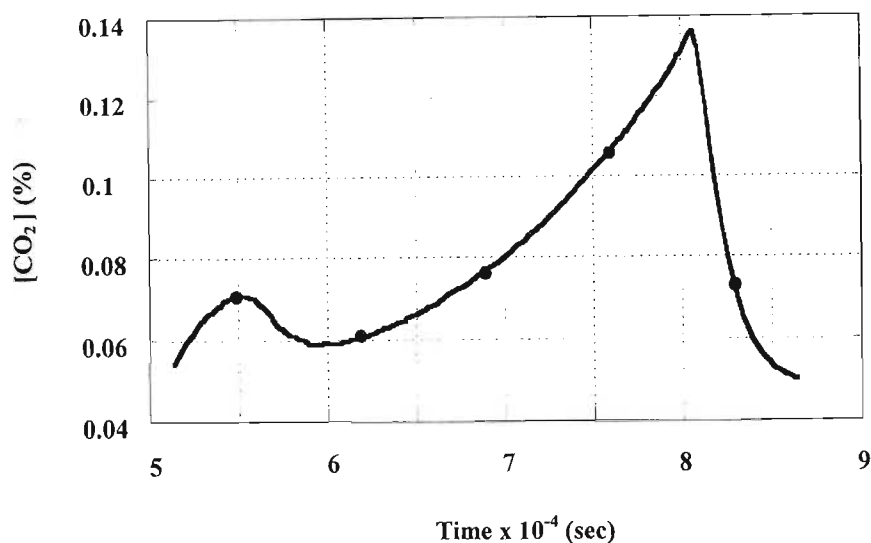


Figure 5.5: Changes in headspace carbon dioxide concentration with time of a 1 l aerobic retentostat culture (working volume 400 ml, 30°C) of the atrazine-catabolizing association KRA30 in the presence of mineral salts solution M-I supplemented with 100 mg l⁻¹ atrazine + 3mM citric acid.

Unspecified increased optical density and >80% ¹⁴CO₂ production from ring-labelled [¹⁴C]-atrazine were recorded for *Pseudomonas* sp. strain ADP by Mandelbaum *et al.* (1995) in batch cultures with M-III. A direct comparison with these results was, however, impossible since complete carbon balances were not determined in this study and the method of cultivation was different. It is possible, however, that cultivation of *Pseudomonas* sp. strain ADP in the less complex mineral salts solution M-I with, possibly, elemental limitation might have resulted in less mineralization of the herbicide. Further studies with [¹⁴C]-atrazine would be required to elucidate the catabolism of atrazine by KRA30.

To explore further the effects of a supplementary carbon source(s) on KRA30 growth in a simple salts solution (M-I) and the more complex M-III, additional retentostat cultures were made.

Cultivation of the combined association in atrazine-supplemented M-III resulted in an optical density of 1.5 after 95 h (Figure 5.6A). When the atrazine was replaced by 3 mM citric acid the equivalent optical density was 0.3 (Figure 5.6B). Growth inhibition or nitrogen limitation due to the supplementary carbon source was assumed. Therefore, as observed with the batch cultures (5.2.1), a critical carbon supplementation concentration was inferred again. The growth pattern recorded with the citric acid-supplemented culture was possibly due to adhesion and detachment of cells from the vessel walls.

Cultivation of the association in M-I supplemented with 3 mM citric acid recorded the highest growth (Figure 5.6C) which suggested that the M-III citric acid-supplemented culture had exceeded the critical substrate concentration. The results also suggested that supplementation with elements other than carbon might be unnecessary for the combined association with mineral salts solution M-I.

If carbon supplementation is deemed necessary to promote bioremediation, the costs must be considered. Since the addition of citric acid would not be cost-effective, the use of alternative carbon sources from waste streams such as waste brewer's yeast could be considered. However, the presence of nitrogen in such materials and its effects on atrazine catabolism would necessitate additional scrutiny.

5 l Retentostats

This study focused specifically on the growth of the combined atrazine-catabolizing association KRA30 under carbon- and nitrogen-limited conditions but in 5 l retentostats charged with 4.5 l of mineral salts solution M-II (2.6). M-II was similar to M-I but contained trace elements and Titriplex I, to enhance atrazine solubility. In contrast to M-III, no tri-sodium citrate was included as a carbon source. Thus M-II could be prepared with atrazine as: the sole carbon and nitrogen source in the absence of inorganic nitrogen; the sole carbon source in the presence of NH_4Cl nitrogen; and the sole nitrogen source in the presence of citric acid or succinic acid as a supplementary carbon source.

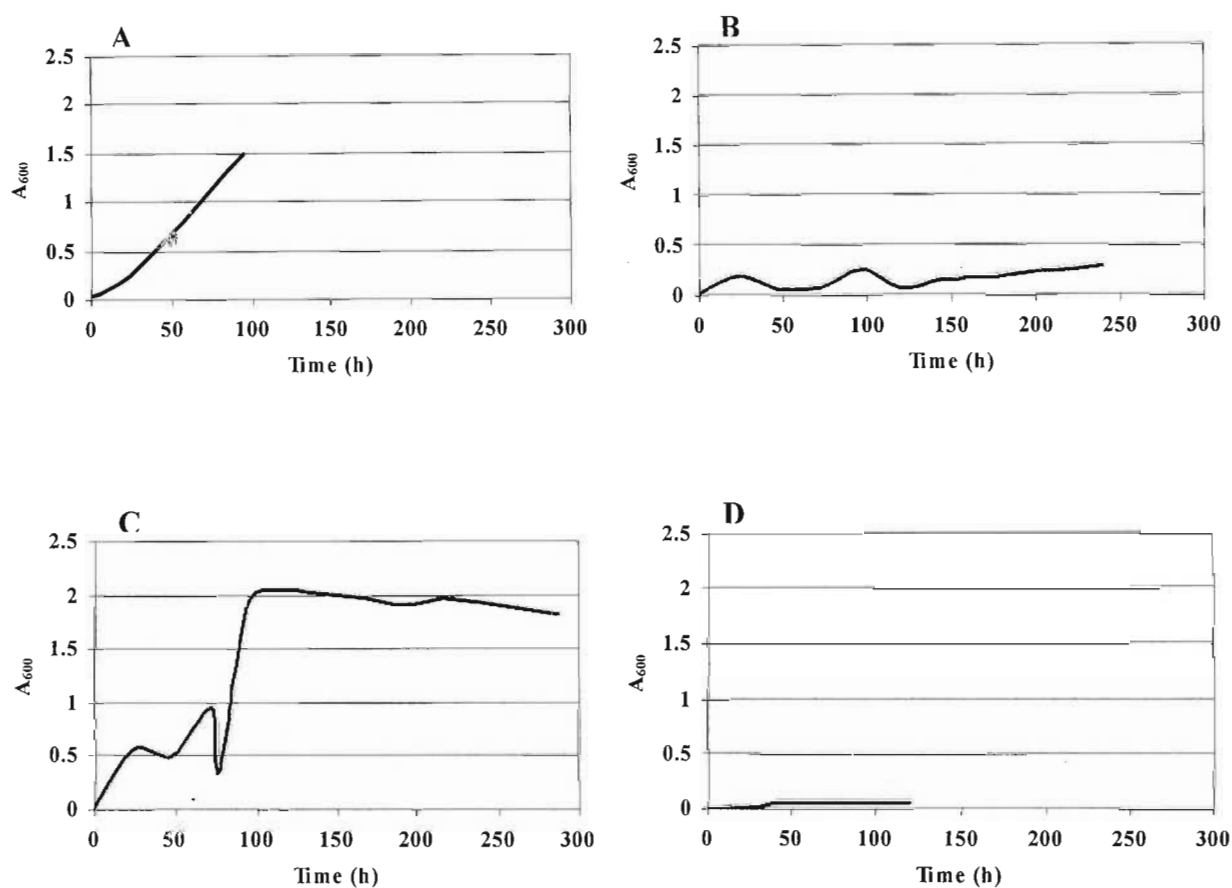


Figure 5.6: Changes in optical densities with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30°C) in 1 l retentostats (working volume 400 ml) in the presence of mineral salts solution M-III supplemented with 100 mg l^{-1} atrazine (A) and 3mM additional citric acid (B), and 3mM citric acid-supplemented mineral salts solution M-I (C). An uninoculated control vessel charged with M-III was included (D).

The optical density results showed a general decrease in biomass when the association was cultured with atrazine as the sole carbon source (Figure 5.7A). A peak (0.41) was recorded after 50 h of cultivation but this was not sustained and reduced optical densities (0.01 to 0.28) were then recorded during the next 450 h of cultivation. Since ring cleavage has been reported when atrazine was provided as a nitrogen source (Mandelbaum *et al.*, 1993; Assaf and Turco, 1994a;b) it is possible that the carbon-limited conditions of the retentostat did not facilitate atrazine catabolism and so there was a progressive reduction of the retained biomass through autolysis.

In contrast, increased optical densities were recorded when atrazine was provided as the source of both carbon and nitrogen (Figure 5.7B) and as a source of nitrogen with citric acid supplementation (Figure 5.7C). The latter culture reached a maximum optical density (0.28) after 50 h of incubation while the former recorded a value of 0.05 at the same sampling time which suggested that the elevated population size was due to the supplemental carbon. In the presence of citric acid-supplementation, cell proliferation resulted in increased biofilm production and blockage of the retentostat filter. The experiment was, therefore, terminated after 120 h.

Since carbon supplementation was made on the basis of molarity rather than carbon equivalents it was assumed that succinic acid would support less growth than citric acid and this proved to be the case (Figure 5.7D). It is possible, however, that the results may have reflected a protracted acclimation phase since the association was cultured in the presence of citric acid but not succinic acid during inoculum preparation.

Residual atrazine analysis indicated incomplete catabolism in the presence (Figure 5.8A) and absence (Figure 5.8B) of inorganic N, where the herbicide provided the sole source of carbon and carbon and nitrogen, respectively. Also, a considerable decrease in residual pesticide concentration in the former reflected a peak in biomass (Figure 5.7A) after 50 hours of cultivation. Similar progressive, but more pronounced, decreases in residual herbicide concentration in the presence of citric acid (Figure 5.8C) and succinic acid (Figure 5.8D) were recorded. Also, for both of these cultures complete catabolism was recorded. Although obtained under different cultivation conditions, the results agreed with those of other researchers (Mandelbaum *et al.*, 1995; Shapir *et al.*, 1998; Katz *et al.*, 2000) who reported increased population sizes and herbicide

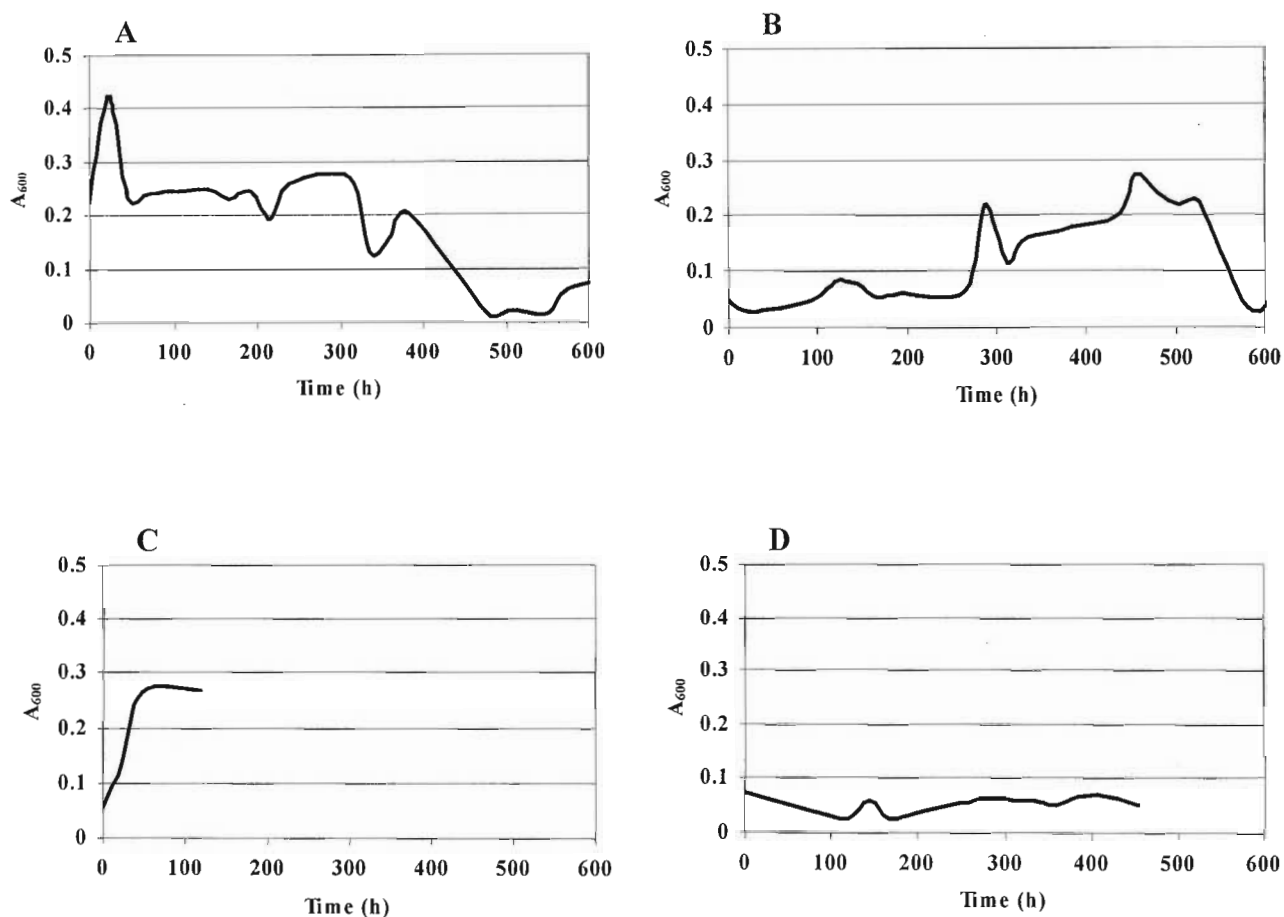


Figure 5.7: Changes in optical densities with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30°C) in 5 l retentostats (working volume 4.5 l) with mineral salts solution M-II supplemented with 100 mg l^{-1} atrazine in the presence (A) and absence (B) of inorganic nitrogen (NH_4Cl) and in the presence of 3mM citric acid (C) and 3 mM succinic acid (D).

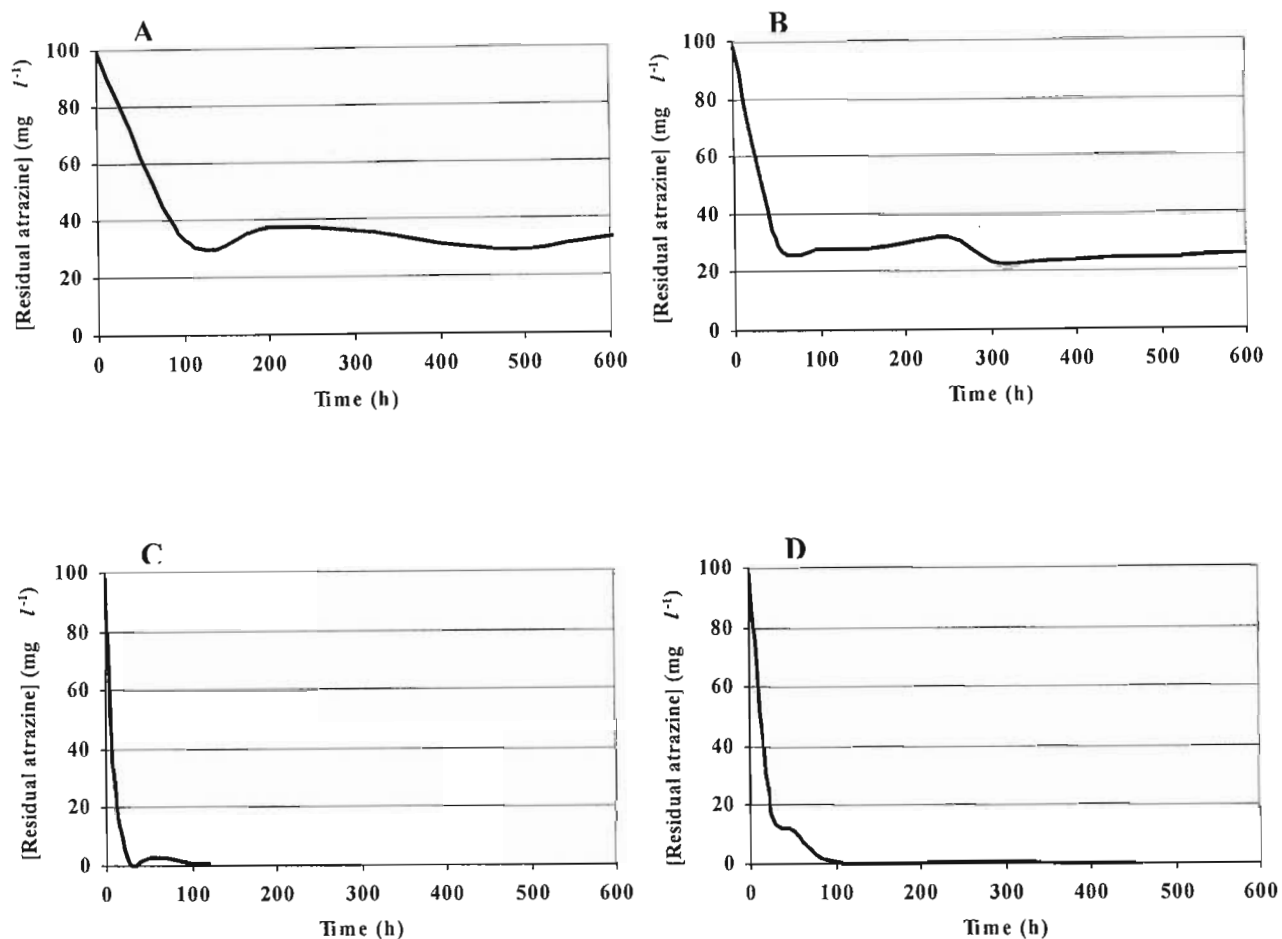


Figure 5.8: Changes in residual atrazine concentrations with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30⁰C) in 5 l retentostats (working volume 4.5 l) with mineral salts solution M-II supplemented with 100 mg l⁻¹ atrazine in the presence (A) and absence (B) of inorganic nitrogen (NH₄Cl) and in the presence of 3mM citric acid (C) and 3 mM succinic acid (D).

degradation with the provision of tri-sodium citrate as a supplementary carbon source. Also, atrazine co-metabolism in the presence of succinic acid was assumed since, according to Robertson and Alexander (1994), increased mineralization in the absence of increased biomass (Figure 5.7D) suggests elevated activity per unit biomass.

While both citrate and succinate supplementation promoted atrazine catabolism, any possible addition *in situ* must be approached with caution since Abdelhafid *et al.* (2000), for example, reported that the addition of glucose to soil facilitated the formation of bound atrazine residues and, thus, reduced its bioavailability.

With the exception of the succinate-supplemented retentostat culture for which the lowest total cell counts (Figure 5.9D) and optical densities (Figure 5.7D) were recorded, very little correlation existed for the other retentostats (Figures 5.9A-C and Figures 5.7A-C). This probably reflected the changing population profile of the association in each of the retentostats. Although attempts were made to capture size distributions and, hence, population changes in the cultures, these proved unsuccessful.

In general, reduced particulate total organic carbon concentrations were recorded during cultivation of KRA30 in the presence of M-II (Figure 5.10) compared with cultivation in the presence of M-I (Figure 5.4) possibly due to the presence of Titriplex I which was added to improved atrazine availability. Since the population size of the atrazine plus citric acid-supplemented M-II culture (Figure 5.7C) was much lower than the atrazine plus citric acid-supplemented M-I culture (Figure 5.6C), the reduced assimilation was probably accounted for by a greater degree of mineralization of the added carbon due to the presence of Titriplex I. Cultivation of KRA30 with atrazine plus citrate-supplemented M-II indicated that on average 1.66 mg l^{-1} (1.66%) of influent atrazine was adsorbed to the cell surfaces (2.7.3). Since retentostats, unlike other continuous cultures, do not facilitate the maintenance of biomass steady-states, a more comprehensive investigation of the rate of herbicide adsorption in relation to biomass age and composition would be essential.

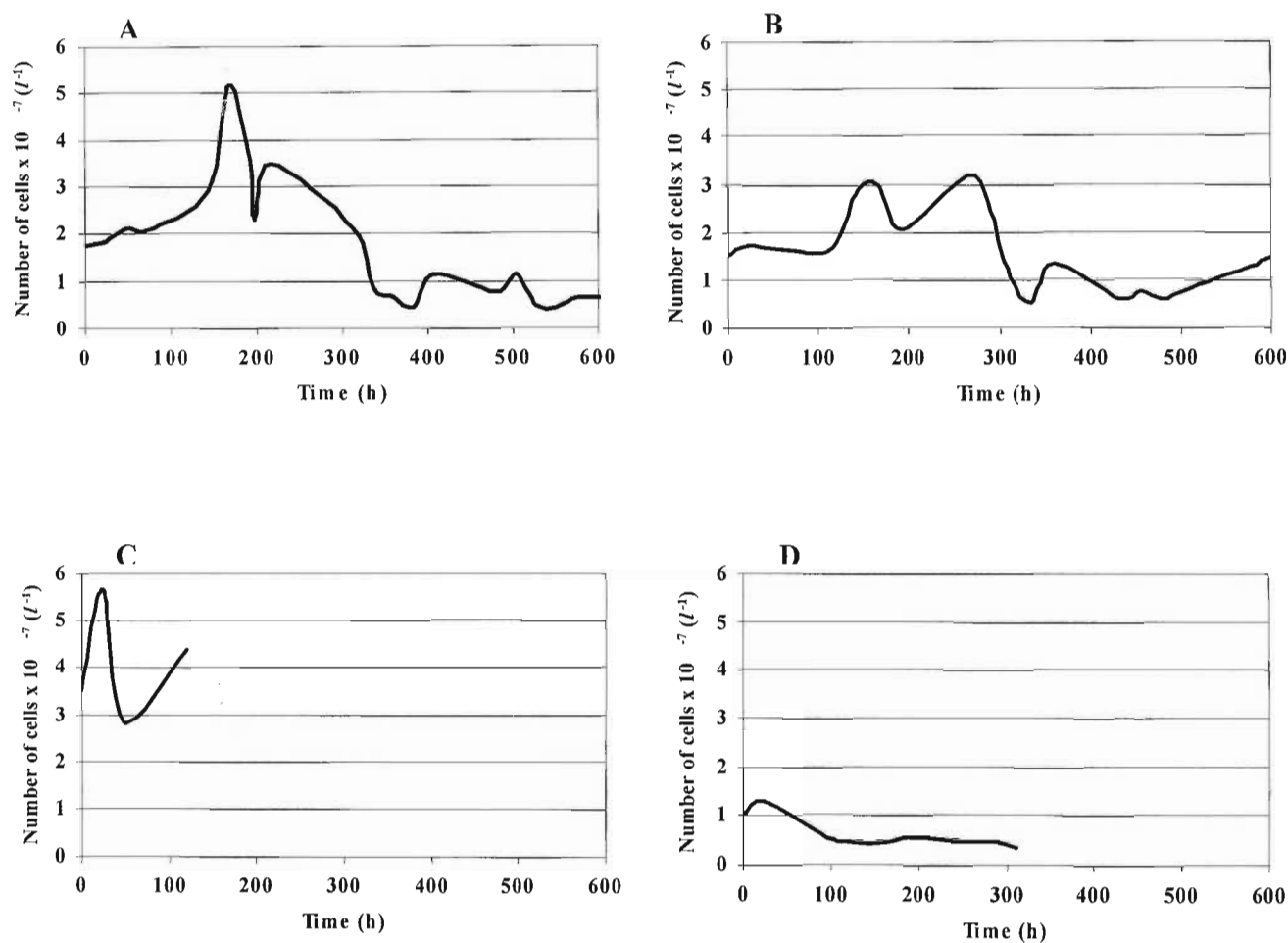


Figure 5.9: Changes in cell numbers with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30⁰C) in 5 l retentostats (working volume 4.5 l) with mineral salts solution M-II supplemented with 100 mg l⁻¹ atrazine in the presence (A) and absence (B) of inorganic nitrogen (NH₄Cl) and in the presence of 3mM citric acid (C) and 3 mM succinic acid (D).

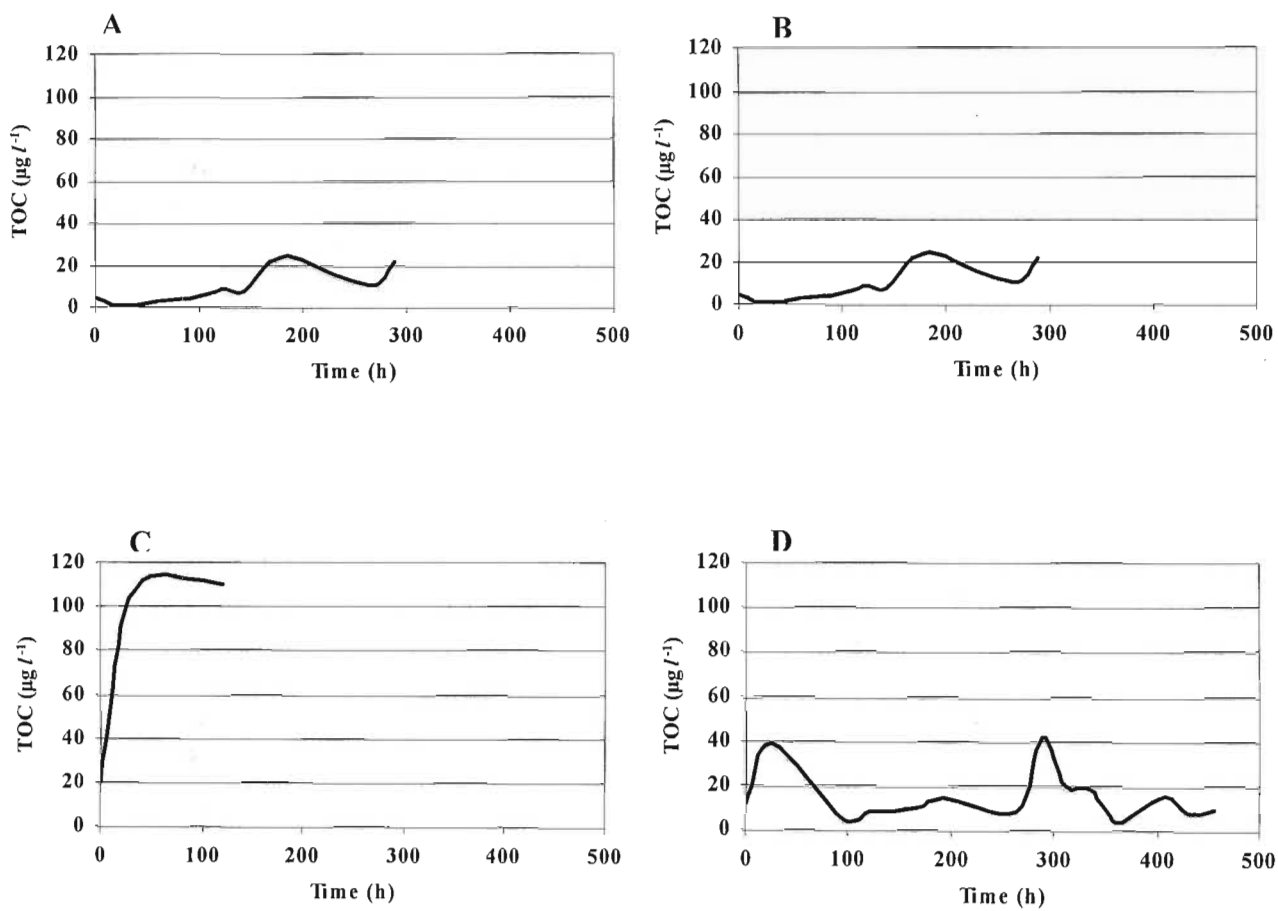


Figure 5.10: Changes in particulate total organic carbon (TOC) with time of the combined aerobic atrazine-catabolizing association KRA30 cultured (30°C) in 5 l retentostats (working volume 4.5 l) with mineral salts solution M-II supplemented with 100 mg l^{-1} atrazine in the presence (A) and absence (B) of inorganic nitrogen (NH_4Cl) and in the presence of 3mM citric acid (C) and 3 mM succinic acid (D).

5.3 Conclusions

Although intrinsic catabolic potential is well established as exemplified by the successful isolation of atrazine-catabolizing microbial associations and monocultures (1.4), the persistence of the herbicide in the environment has not abated. This could be attributed to reduced bioavailability and, hence, limited biodegradation due to sorption and/or nutrient limitation. Also, differences in microbial growth rates are determined by targeting the atrazine molecule as either a carbon or nitrogen source (Smit *et al.*, 1981). As a result, element supplementation (biostimulation/biosupplementation) to stimulate indigenous microorganisms is one of the most widely used bioremediation approaches (Margesin and Schinner, 2001). From the present study it was concluded that with association KRA30 atrazine should be targeted as a nitrogen source in the presence of supplemental carbon.

Further studies to explore the effects of nutrient additions other than carbon *in situ* must be made. Smit *et al.* (1981), for example, reported that some mineral salt solution components provided competitive sorption sites which affected atrazine availability. In particular, a decrease in atrazine adsorption to a sesquioxide soil with an increase in KH_2PO_4 was recorded. The negatively-charged phosphorus ions compete with atrazine for the available sorption sites on the amorphous (Fe,Al,OH) components of the soil at pH values ≤ 5 . Thus, a phosphate-rich mineral salts solution should facilitate atrazine desorption from the soil particles, inhibit its adsorption to cationic components such as Fe and Al and, therefore, increase its bioavailability for microbial degradation.

Alvey and Crowley (1995) and Abdelhafid *et al.* (2000) observed that nitrogen concentrations such as 2.5 gNkg^{-1} soil decreased atrazine mineralization with the addition used as the preferred nitrogen source. Also, Topp *et al.* (2000a) reported approximately 20, 50, 70 and 80% atrazine catabolism- or hydrolase inhibition in a *Nocardioides* sp. by 100 μM Mg, Cu, Co and Zn, respectively. Since no inhibition of *Pseudomonas* sp. strain ADP was reported, it is possible that a different catabolic pathway was operative.

In low nutrient soil it is possible that there is spatial separation of the catabolic species from the target molecule. Therefore, when cost-effective, soil slurry treatment or soil washing/chemical extraction of atrazine could be considered for *ex situ* bioremediation in bioreactors inoculated with the combined association KRA30. Also,

atrazine-compromised aquifers and groundwater could be treated by the pump, treat and re-inject approach (1.4.1).

Further studies to determine the full population fingerprint and, thus, identify any component which is a potential pathogenic species, and the maintenance energy requirement, to elucidate inoculum longevity, should precede *in situ* soil inoculation with association KRA30. A high maintenance energy requirement could justify inclusion of pathogenic species since rapid autolysis should result following atrazine catabolism and, therefore, elimination of these strains from the environment with no threat to groundwater.

Chapter 6: Molecular Characterization and Physiological Profiling of Selected Catabolic Associations

6.1 Introduction

Recognition has been made that the molecular ecology of key catabolic associations has received little attention and understanding the distribution of contaminant-catabolizing microorganisms is of paramount importance (Mason *et al.*, 1998). Molecular techniques have been identified as ideal tools to address this paucity (Heuer and Smalla, 1997a). To appreciate fully sequence diversity, a combination of PCR and DGGE was used to analyse the structures of atrazine- and BTX molecule-catabolizing microbial associations enriched/isolated from soil. Atrazine- and petroleum hydrocarbon-contaminated and uncontaminated soils were also analyzed by PCR-DGGE (2.10.1). A specific objective of this component of the research programme was to determine whether changes in key environmental variables effected species complement changes (qualitative/quantitative) in the microbial associations (Chapter 4).

The environmental Biolog assay has been adopted as a community-level approach to investigate soil microbial communities by assessing their profiles with respect to sole carbon source utilization and, therefore, their physiological capabilities (El Fantroussi *et al.*, 1999; Laverman, 2000). The approach has been employed increasingly to counter the lack of effective methods for describing microbial associations and the limitations of conventional culture-based techniques. Notwithstanding its known limitations (1.5.5), the Biolog assay was used in this study in combination with molecular techniques to characterize the aerobic enriched/isolated atrazine-catabolizing microbial associations (2.10.13).

6.2 Results and Discussion

6.2.1 16S rDNA Profiles of Soils

Characterization of the isolated associations included molecular analysis of the chemically-compromised soils (2.1), from which the primary enrichments were made,

and their pristine equivalents. Thus, DNA was isolated (2.10.3) from soils which had been contaminated with atrazine or petroleum for periods of > 20 and > 2 years, respectively. Two extraction protocols (2.10.3), direct and indirect, were used to determine the most efficient method. Of the two, indirect DNA extraction from soil resulted in a larger amount of product.

Denaturing-gradient gel electrophoresis analysis indicated complex sequence patterns in both the contaminated and uncontaminated soils. The banding showed that contamination effected differences in species dominance in the polluted (Plate 6.1, Lanes 1 and 3) and pristine soils (Plate 6.1, Lanes 2 and 4) as has been recorded by other researchers (Øvreås *et al.*, 1998; MacNaughton *et al.*, 1999). Soil contamination did not necessarily change the complexity of the sequence patterns but effected shifts in quantitative species dominance. For example, exposure to petroleum hydrocarbons increased the expression of species with readily-denatured melting domains as shown by the presence of intense bands in the upper section of the gradient with a reduced concentration of the denaturant (Plate 6.1, Lane 3). In contrast, some researchers (Cheung and Kinkle, 2001) have reported decreases in the diversity of indigenous soil microbial populations in response to, for example, polycyclic aromatic hydrocarbon contamination.

Despite the changes in numerical dominance, as indicated by increased band intensities, some similarities were recorded between the contaminated and pristine soils. Furthermore, some of the numerically-dominant species in the latter were retained in the presence of the pollutant(s) (Plate 6.1, Arrows). This, together with the observed qualitative increases of some species, suggested that the toxicities of the molecules were minimal particularly to the numerically-dominant soil components.

Also, the increases in numerical dominance of some species in the contaminated soils indicated microbial adaptation and *in situ* enrichment. Other researchers (Whyte *et al.*, 1999; Juck *et al.*, 2000) also observed that petroleum hydrocarbons promoted the abundance of some catabolic microorganisms through the addition of carbon and energy sources. Such pre-exposure facilitated the enrichment/isolation of catabolic associations at different pH values and electron donor concentrations.

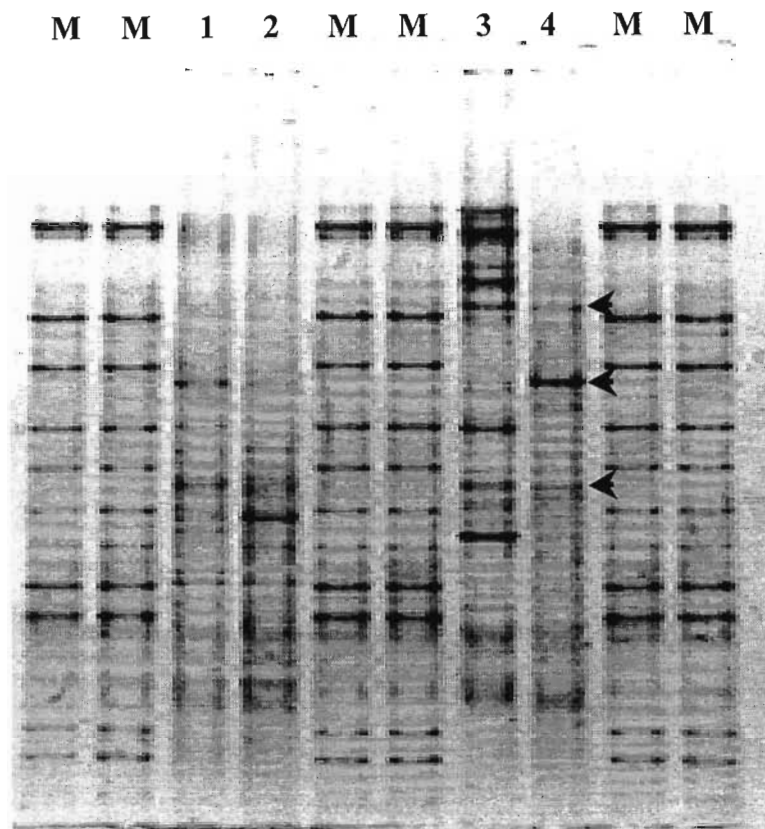


Plate 6.1: 16S rDNA profiles (50 to 65% denaturing gradient) of soils contaminated with atrazine (Lane 1) and petroleum hydrocarbons (Lane 3) and the equivalent uncontaminated soils (Lanes 2 and 4). Quantitatively-dominant species of the pristine soil were retained in the presence of petroleum (Arrows). M denotes the marker.

The results of this study were interpreted with caution since it is recognized that different soil DNA extraction techniques are characterized often by specific biases which are dependent primarily on soil physicochemical properties (Krsek and Wellington, 1999; Tien *et al.*, 1999; Martin-Laurent *et al.*, 2001). Despite the biases, according to Martin-Laurent *et al.* (2001), soil biodiversity can be measured with molecular techniques although the relative abundance of phylotypes in soils may not be estimated accurately. Also, the different methods applied for extracted DNA purification result often in significant losses and decreased recoveries (Tebbe and Vahjen, 1993). Thus, a different extraction protocol and a cleanup technique dissimilar to the Wizard soil DNA method might have given different species complement profiles. The presence of extracellular soil DNA (Tebbe and Vahjen, 1993) possibly from non-viable and/or non-catabolic species, while not pertinent for purposes of this study, was, nonetheless, recognized.

6.2.2 BTX Molecule-Catabolizing Microbial Associations

Plates 6.2, 6.3 and 6.4 show the 16S rDNA banding patterns of aerobic benzene (KRB01-08)-, toluene (KRT01-08)- and *p*-xylene (KRP01-08)-catabolizing microbial associations (Table 2.2) and exemplify the complex DGGE patterns which characterized the BTX molecule-catabolizing associations. Parallel gels were run for *o*- and *m*-xylene and the processed data are shown in Figure 6.1. Similar numerically-dominant (high intensity) bands characterized the associations depending on the enrichment pH. This was particularly evident in the *p*-xylene-catabolizing associations (Plate 6.4) which also exhibited increased similarities between the pH 5.5-poised KRP05 and KRP06 (5 000 and 500 mg l⁻¹) and the KRP07 and KRP08 (50 and 5 mg l⁻¹) associations.

Figure 6.1 shows the sequence patterns after Pearson product-moment (a) and Jaccard coefficient (b) clustering of the aerobic *o*- and *m*-xylene-catabolizing associations cultured at different pH values and molecule concentrations. According to the Jaccard coefficient, each association was constituted by between 10 (5 mg l⁻¹ *o*-xylene, pH 5.5) and 20 (50 mg l⁻¹ *m*-xylene, pH 7.5) members with different sequences. Although the BTX component, concentration and pH of the enrichment medium selected different associations, the incidence of common dominant bands suggested some species similarities. For example, a 30% clustering was recorded

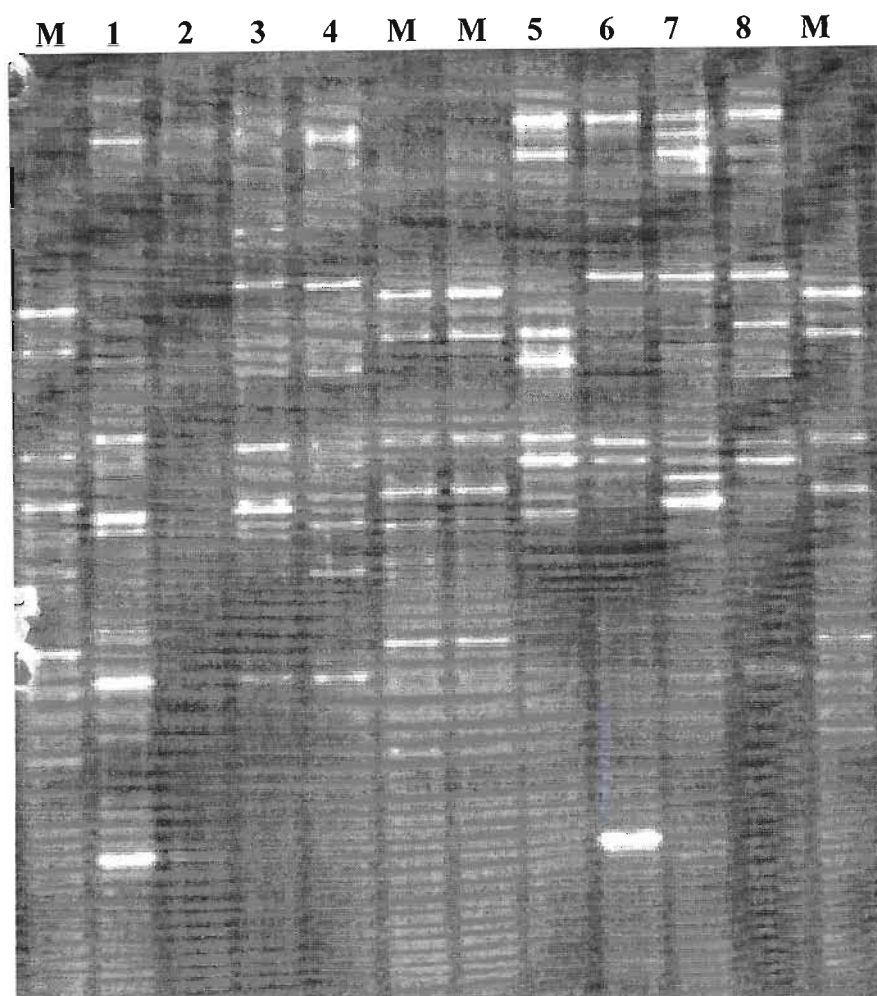


Plate 6.2 16S rDNA profiles (50-65% denaturing gradient) of aerobic benzene-catabolizing microbial associations KRB01-08 enriched/isolated in the presence of 5 000 mg l^{-1} (pH 7.5, 5.5) (Lane 1, 5), 500 mg l^{-1} (pH 7.5, 5.5) (Lane 2, 6), 50 mg l^{-1} (pH 7.5, 5.5) (Lane 3, 7) and 5 mg l^{-1} (pH 7.5, 5.5) (Lane 4, 8) benzene. M denotes the marker.

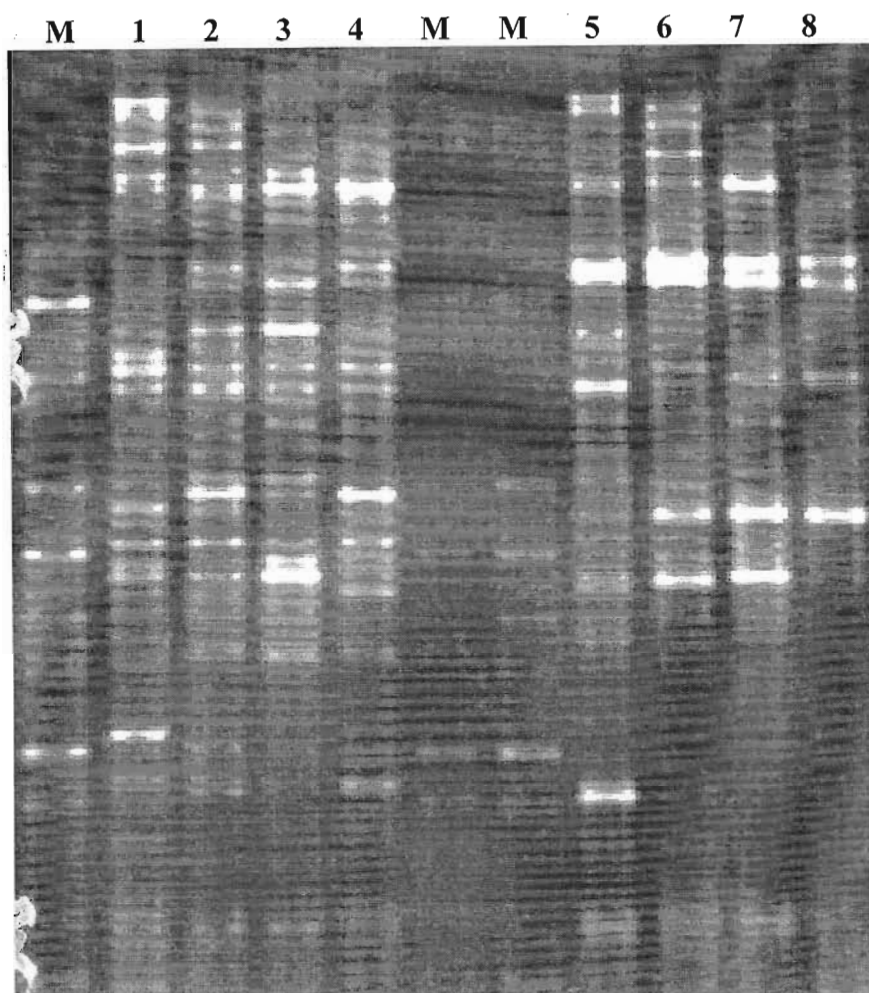


Plate 6.3: 16S rDNA profiles (50-65% denaturing gradient) of aerobic toluene-catabolizing microbial associations KRT01-08 enriched/isolated in the presence of 5 000 mg l^{-1} (pH 7.5, 5.5) (Lane 1, 5), 500 mg l^{-1} (pH 7.5, 5.5) (Lane 2, 6), 50 mg l^{-1} (pH 7.5, 5.5) (Lane 3, 7) and 5 mg l^{-1} (pH 7.5, 5.5) (Lane 4, 8) toluene. M denotes the marker.

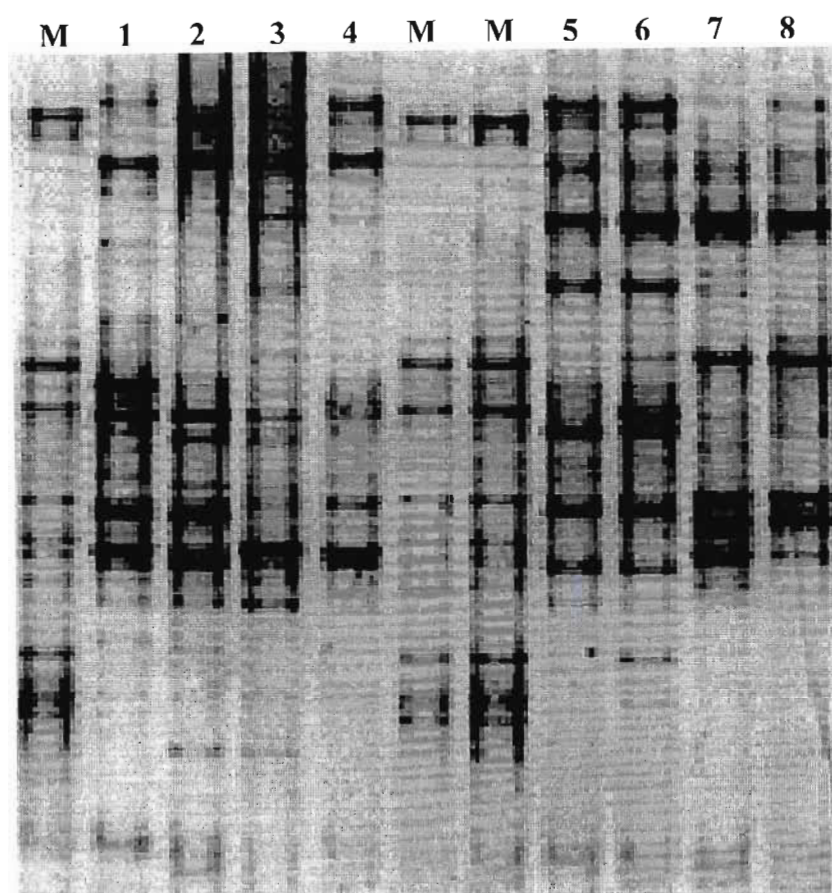


Plate 6.4: 16S rDNA profiles (50-65% denaturing gradient) of aerobic *p*-xylene-catabolizing microbial associations KRP01-08 enriched/isolated in the presence of 5 000 mg l^{-1} (pH 7.5, 5.5) (Lane 1, 5), 500 mg l^{-1} (pH 7.5, 5.5) (Lane 2, 6), 50 mg l^{-1} (pH 7.5, 5.5) (Lane 3, 7) and 5 mg l^{-1} (pH 7.5, 5.5) (Lane 4, 8) *p*-xylene. M denotes the marker.

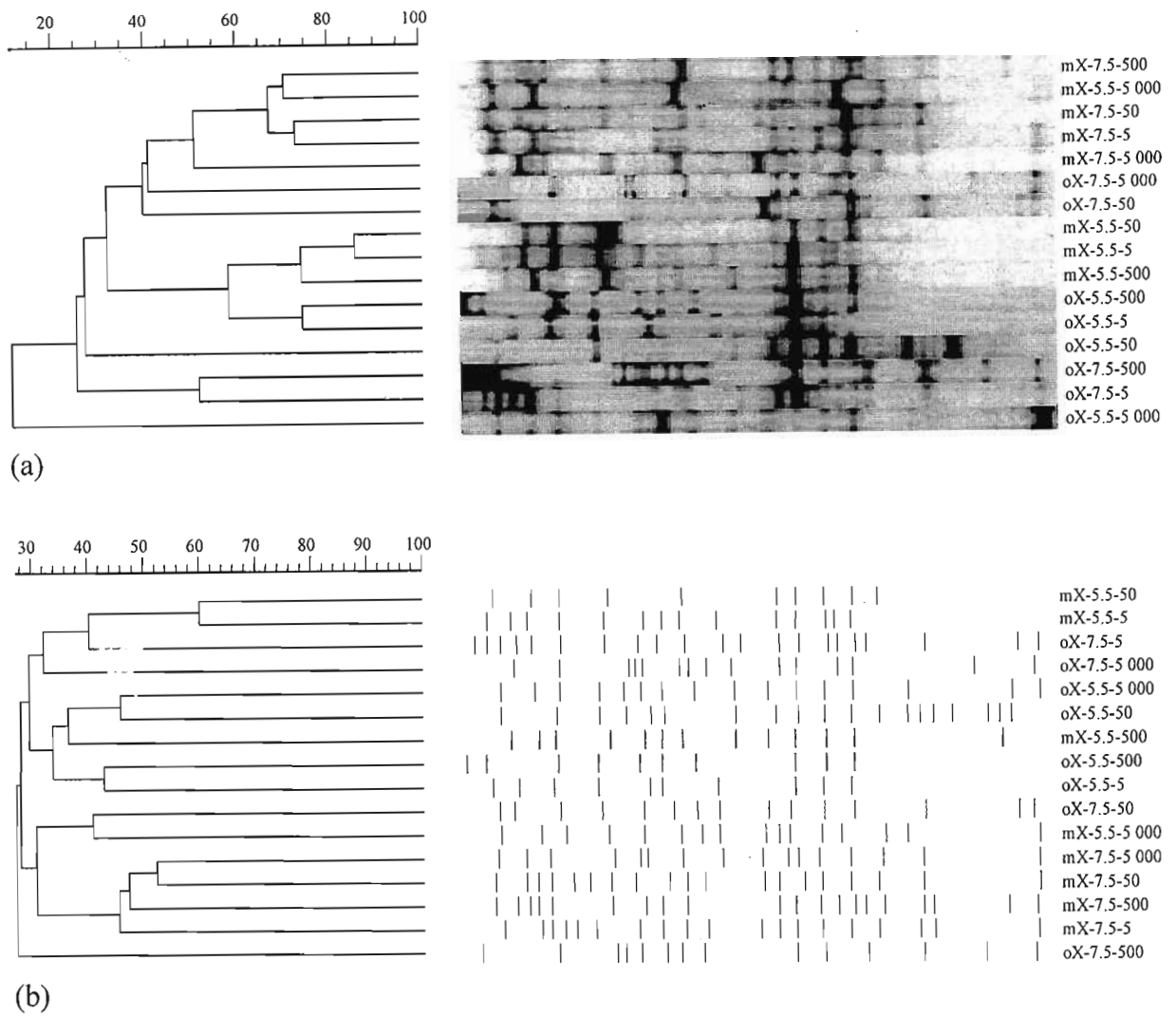


Figure 6.7: Pearson product-moment (a) and Jaccard coefficient (b) clustering of normalized DGGE gels showing the sequence patterns of the *o* (oX)- and *m* (mX)-xylene-catabolizing associations enriched/isolated at different pH values and molecule concentrations.

between the aerobic *o*- and *m*-xylene-catabolizing associations. Compared with *m*-xylene, *o*-xylene seemed to support an additional community member with a non-easily-denatured melting domain as seen from the presence of an extra band in the high-gradient zone of the DGGE gel (Figure 6.1). Some dominant bands indicative of similar species with melting domains characterized by increased GC contents were also visualized in the benzene-catabolizing associations KRB01 and KRB06 (Plate 6.2, Lanes 1 and 6).

6.2.3 Atrazine-Catabolizing Microbial Associations

Complex banding patterns were observed for both the aerobic (KRA0) and anaerobic (PMA0) atrazine-catabolizing associations (Plate 6.5) for all the concentrations and both pH values. The DGGE patterns showed some similarities in sequences between the aerobic and anaerobic associations. Some major differences were, however, evident. Dominant bands in the upper part of the gel characterized the anaerobic atrazine-catabolizing associations while the predominance of bands in the high-gradient section of the gel suggested that the aerobic associations contained species with less-easily denatured melting domains.

Although common bands were seen within and between the aerobic and anaerobic microbial associations, the atrazine concentration and the pH of the primary enrichment appeared to select different associations. It is interesting to note, however, that with 30 and 33 mg l⁻¹ atrazine at pH 7.5 (Lane 2) and 5.5 (Lane 5), respectively, the band patterns in both the aerobic (KRA0) and anaerobic associations were comparable.

Similarities in species dominance of the aerobic and anaerobic atrazine-catabolizing microbial associations seemed to suggest a degree of catabolic versatility. Thus, changes in electron acceptor availability during bioremediation would not necessarily inhibit the biotechnology although the rate might change.

Community-Level Physiological Profiling

Biolog analysis was used to compare the isolated aerobic atrazine-catabolizing associations on the basis of carbon source utilization. The substrate utilization patterns were subjected to Pearson product-moment correlation. The results (Figure 6.2) identified both similarities and differences in the isolated aerobic atrazine-degrading associations KRA01 - KRA08.

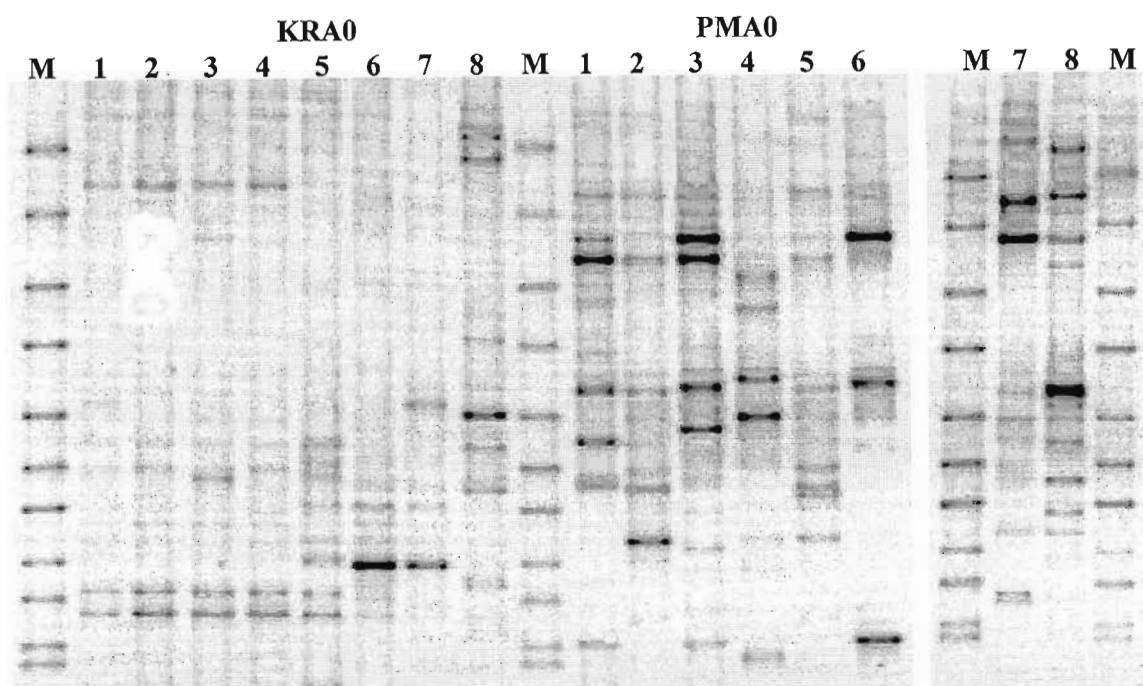


Plate 6.5: 16S rDNA profiles (50-65% denaturing gradient) of microbial associations KRA0 (aerobic) and PMA0 (anaerobic) enriched and isolated in the presence of 33 mg l^{-1} (pH 7.5, 5.5) (Lane 1, 5), 30 mg l^{-1} (pH 7.5, 5.5) (Lane 2, 6), 20 mg l^{-1} (pH 7.5, 5.5) (Lane 3, 7) and 10 mg l^{-1} (pH 7.5, 5.5) (Lane 4, 8) atrazine. M denotes the marker.

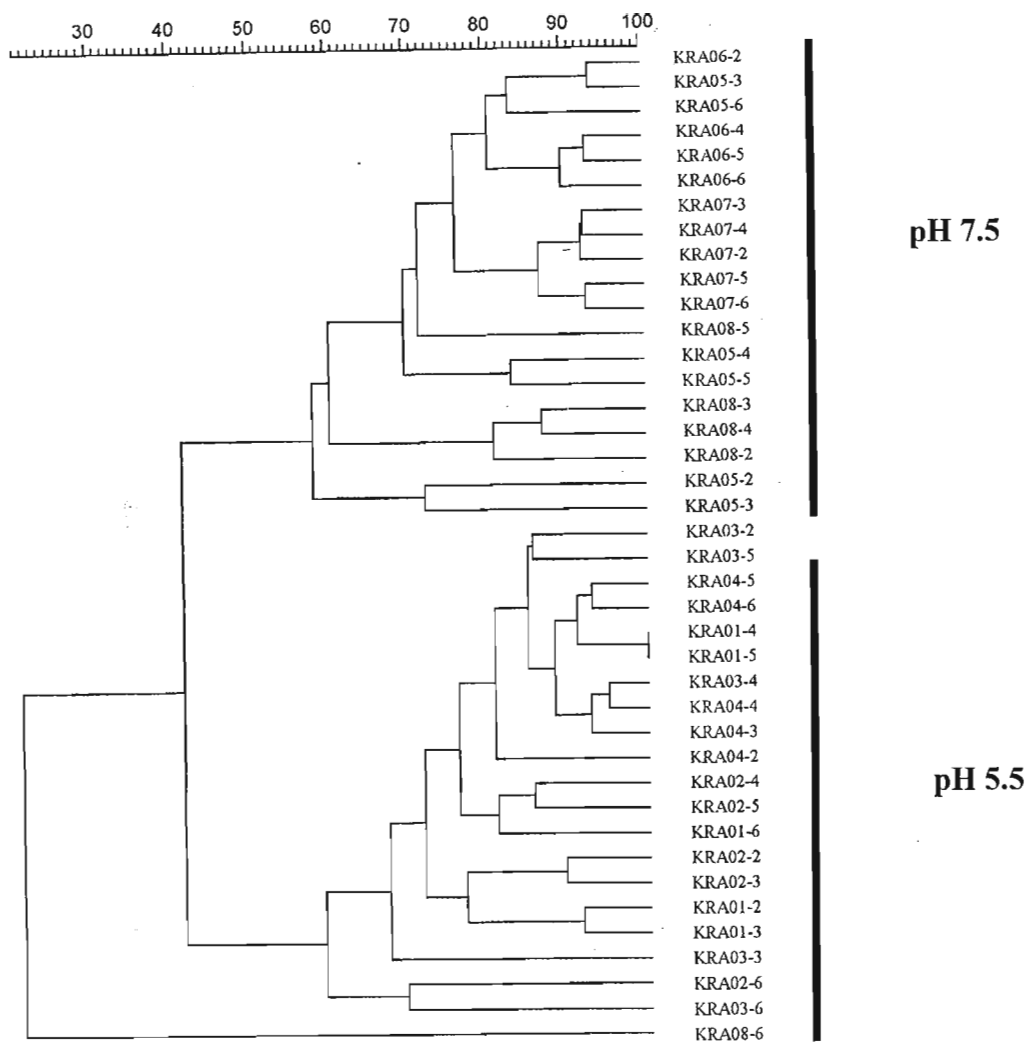


Figure 6.2: Pearson product-moment correlation (Röling *et al.*, 2000a) showing the physiological variability observed in the aerobic atrazine-catabolizing associations KRA01-KRA08 (Table 2.1) after 4 days of incubation (30⁰C) in Biolog EcoPlates. (-2, -3, -4, -5, -6 designate the logarithmic (base 10) dilution factor).

Different pH values and atrazine concentrations of the primary enrichments selected different associations. The microbial associations enriched/isolated at pH 5.5 clustered at approximately 40% with those enriched/isolated at pH 7.5, irrespective of the pesticide concentration, and, thus, emphasized the differences effected by pH (Figure 6.2).

Although clustering for each discrete pH value increased, and ranged from 55 to 90%, the concentration of atrazine appeared to effect considerable differences in the associations. Dilution of the microbial inoculum also seemed to effect changes in clustering within and between the different pH and concentration groups. For example, by diluting association KRA03 from 10^{-2} to 10^{-6} a clustering reduction >10% resulted.

As discussed above, for all molecule concentrations and pH values examined, the enriched/isolated atrazine- and BTX molecule-catabolizing associations were characterized by complex banding patterns typical of microbial associations with high numbers of equally-abundant species (Heuer and Smalla, 1997a). Some numerically-dominant bands common to the source soils and the catabolic microbial associations were also recorded. Although differences were recorded in response to each molecule type, the common selection pressures of molecule concentration and pH, during the primary enrichments, possibly effected the recorded 30% similarity of the isolated *o*- and *m*-xylene-catabolizing associations.

Gelsomino *et al.* (1999) examined a Flavo silt loam soil over a 1-year period and showed by DGGE that population profile changes in response to seasonal fluctuations were small and suggested that the soil bacterial community was characterized by a limited number of dominant and stable species. However, if population profiles changes are effected by season (Smit *et al.*, 2001), the time of sampling could be critical. Also, enriched/isolated catabolic associations are imperfect since exact duplication of an ecological niche is impossible. Therefore, an isolated population may not mirror exactly the *in situ* population (Senior, 1977; Dunbar *et al.*, 1997; Hengstmann *et al.*, 1999). Thus, bioaugmented site amelioration efficacy may be dependent on using an inoculum which is specific to the season.

6.2.4 *Analysis of Single Colonies*

Qualitative profiling of complex microbial associations is facilitated by DGGE. Although analysis of DNA extracted from single colonies is limited to the components of an association which are culturable on solid medium, it can help to identify the numerically-dominant species. Single colonies from mineral salts agar supplemented with the target molecules, and tryptic soy agar plates (2.10.8) were, therefore, analyzed by DGGE and compared to the total DNA profile of the specific microbial association. The results showed that some of the dominant members of the associations were culturable on defined growth medium and, most importantly, were not eliminated during the enrichment/isolation programme since they correlated to dominant bands in the original soils.

As discussed in 3.2.4, the occasional proximity of two or three colony types on atrazine- and BTX molecule-supplemented mineral salts agar and 1% (m/v) tryptic soy agar plates posed some difficulty in selecting single discrete colonies. This often resulted in multiple bands on the DGGE gels (Plate 6.6, Lanes 1-3, 8-9). Also, DGGE analysis of some discrete colonies still revealed more than one band (Lanes 4, 5, 7 and 11-13). Although some single colonies exhibited only one band, which suggested successful monoculture isolation, no corresponding molecule catabolism was recorded (3.2.4).

From this study it appeared essential to confirm by DGGE the isolation of monocultures prior to elucidating the catabolic roles of each.

6.2.5 *DGGE Profiles of Log Phase Cultures*

To explore key rate-limiting factors exemplary of South African soils, combined associations (Table 2.3) were used (4.2). The calculated specific growth rates and scanning electron microscopy visualizations suggested changes in the combined aerobic catabolic association profiles in response to pH and temperature changes. To investigate this further, aliquots (1.5 ml) were taken during the log phase of each batch culture and subjected to PCR/DGGE which, according to Head *et al.* (1998), should rapidly assess species fluctuations in a microbial population due to environmental perturbations.

Differences in numerical and, possibly, activity dominance were observed in the 16S rDNA profiles of the atrazine-catabolizing association KRA30 (Plate 6.7) in

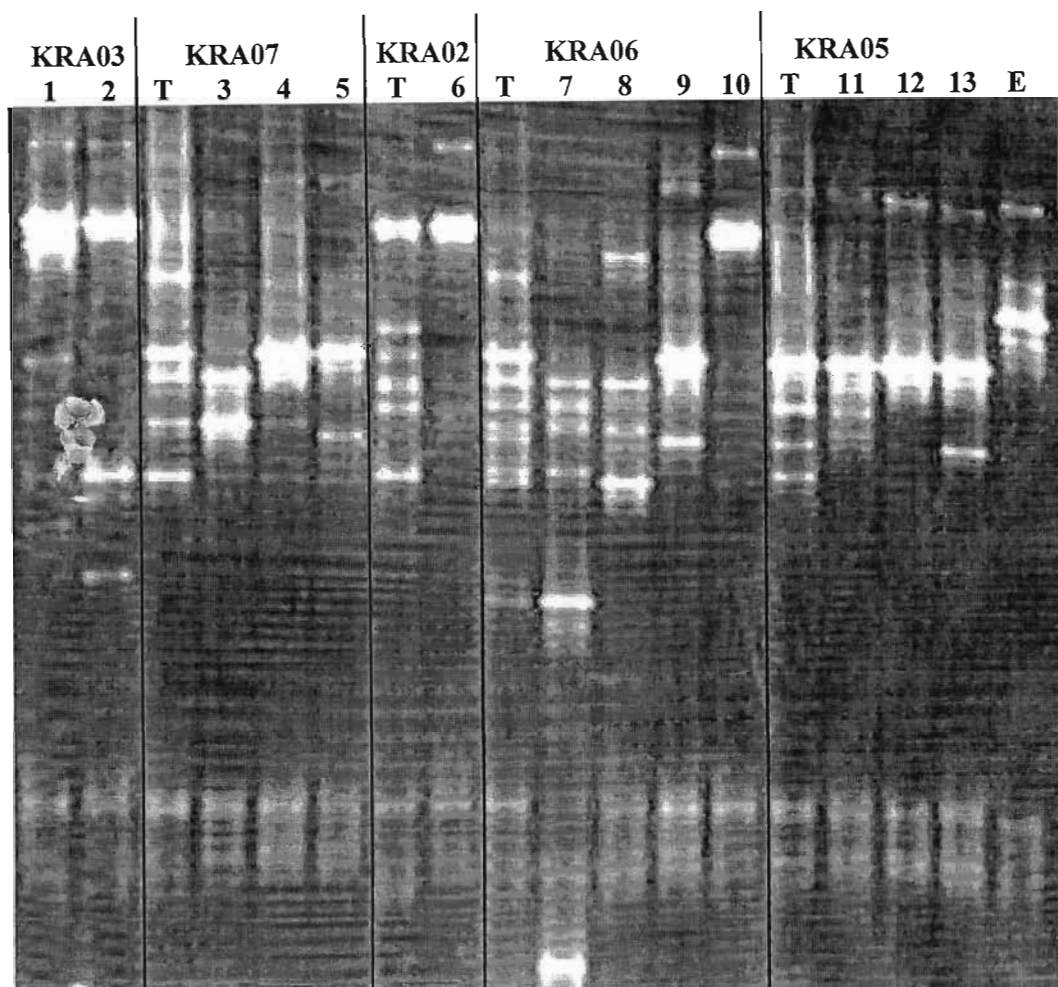


Plate 6.6: 16S rDNA profiles (50-65% denaturing gradient) of single colonies/association members of aerobic atrazine-catabolizing microbial associations cultured on atrazine-supplemented mineral salts agar after enrichment/isolation in the presence of 20 mg l^{-1} (KRA03, pH 7.5, Lanes 1 and 2), 20 mg l^{-1} (KRA07, pH 5.5, Lanes 3-5), 30 mg l^{-1} (KRA02, pH 7.5, Lane 6), 30 mg l^{-1} (KRA06, pH 5.5, Lanes 7-10) and 33 mg l^{-1} (KRA05, pH 5.5, Lanes 11-13) atrazine. 'T' and 'E' designate total DNA from cultured colonies and *Escherichia coli* DNA, respectively.

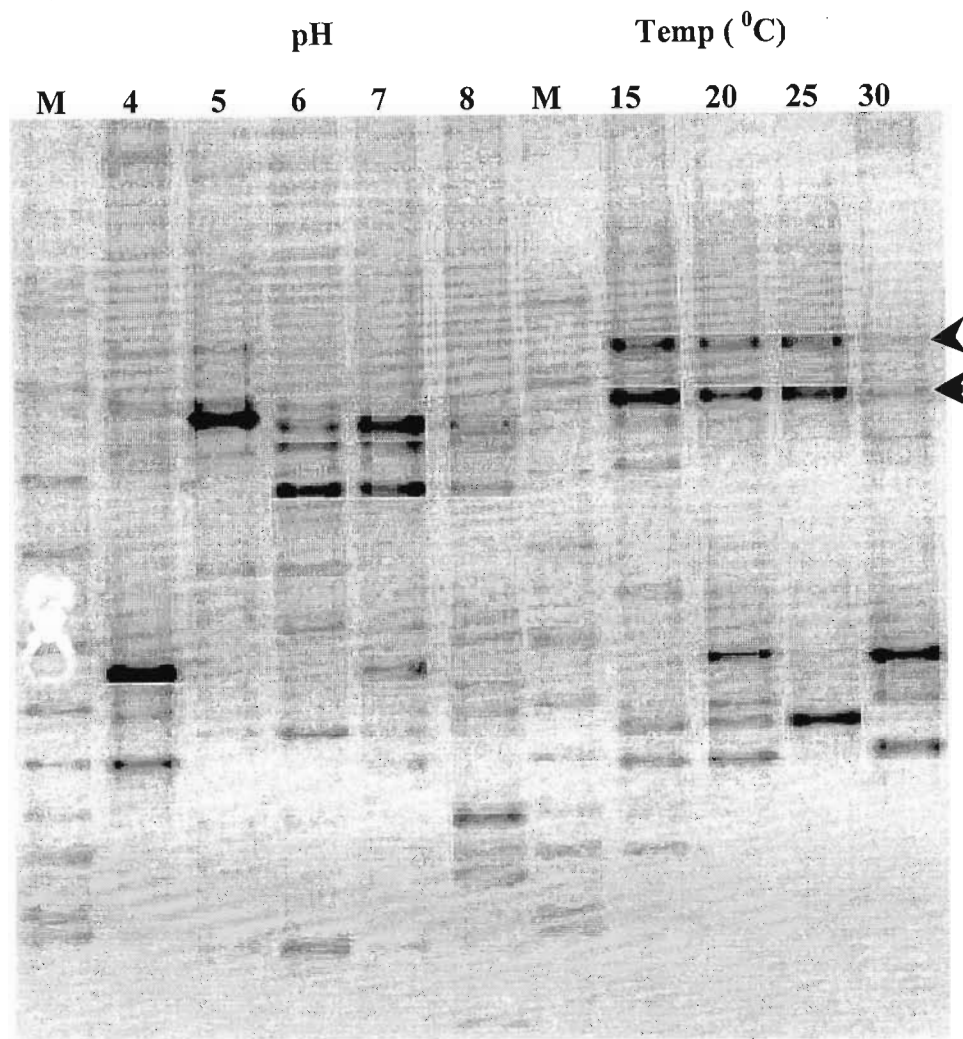


Plate 6.7: 16S rDNA profiles (50-65% denaturing gradient) of exponential phase batch cultures (medium M-I, 30 mg l^{-1} atrazine) of the combined aerobic atrazine-catabolizing association KRA30 incubated at 30 $^{\circ}$ C and buffered at pH values of 4, 5, 6, 7 and 8, and buffered at pH 4 and incubated at temperatures of 15, 20, 25 and 30 $^{\circ}$ C. M denotes the marker and the arrows indicate dominant members retained in the association irrespective of the temperature.

response to changes in pH and temperature. The complexity of the microbial association, as indicated by the number of bands, seemed to be maintained at pH 6, 7 and 8 (30°C), and at 15°C (pH 4). For the two environmental variables examined, visualization of dominant members was consolidated generally in the upper part of the denaturing gradient. Furthermore, the common dominant members were maintained in the combined catabolic association irrespective of the pH or temperature. This was particularly apparent for temperature where the expression of two dominant members (Plate 6.7, Arrows) remained at 15, 20 and 25°C.

Similar dominant bands (Arrows 1) characterized the individual hydrocarbon molecule-catabolizing associations (KRB50, KRT50, KRO50, KRM50 and KRP50, Table 2.3) (Plates 6.8A and B) despite changes in temperature. A band common to all the different BTX molecule-catabolizing associations was also evident (Arrows 2). Despite these, Pearson product-moment correlations indicated that temperature changes effected a 65% difference in each of the BTX-molecule catabolizing associations.

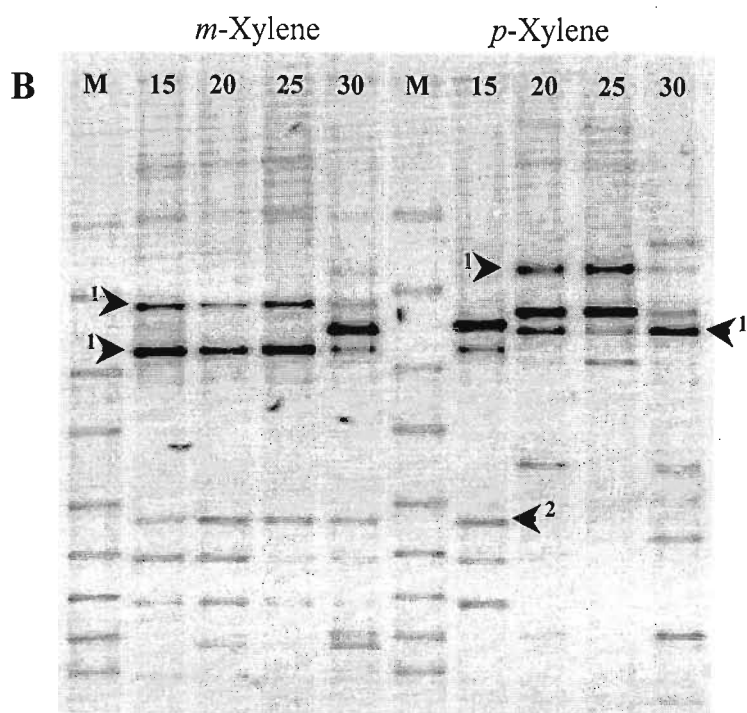
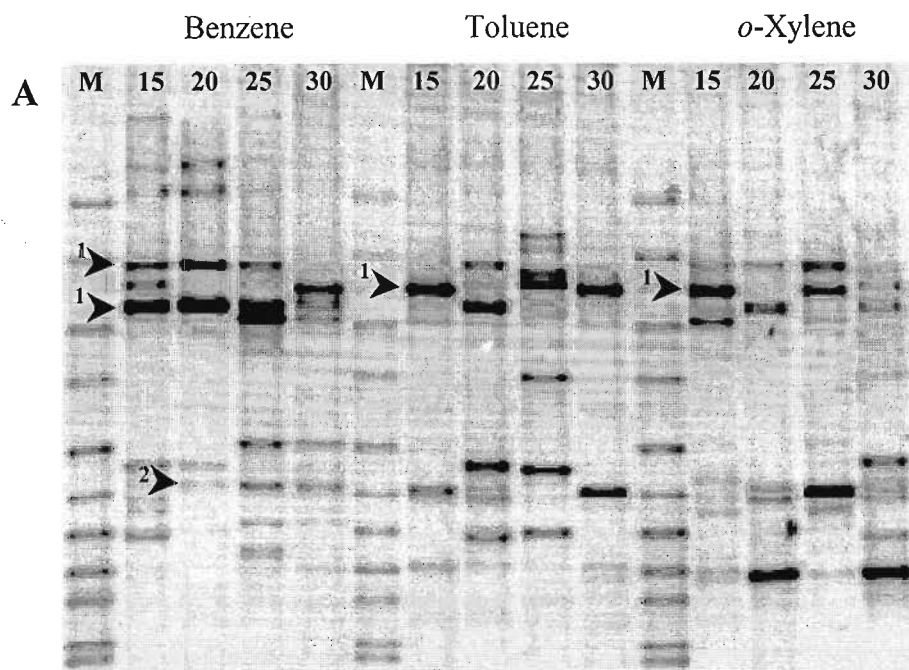
The associations recorded different specific growth rates concomitant with changes in the environmental parameters (4.2.1 and 4.2.2) which also suggested possible variations in the population profiles. This was confirmed by 16S rDNA band pattern differences which were indicative of changes in numerical and, probably, activity dominance.

The same analysis also revealed that some dominant species were common to both the aerobic atrazine- and BTX molecule-catabolizing associations under different pH and temperature conditions which suggested that both sites had common selection determinants.

The results of this study and the conflicting observations of other research groups suggested that environmental parameter optimization and characterization of catabolic association structure must precede bioaugmentation so that control of key variables may be considered to facilitate the catabolic activity of the dominant site-specific species.

6.2.6 *Retentostat Cultivation of Association KRA30*

Following retentostat cultivation of association KRA30 under carbon- and nitrogen-limited conditions (Chapter 5), 16S rDNA-based PCR-DGGE analysis was made on



selected cultures to explore possible shifts in numerical dominance of the component species.

In general, association profile changes resulted when atrazine was provided as the sole source of both carbon and nitrogen and when the molecule was the N source in the presence of citric acid as a supplementary C source. Under the former conditions, the complexity of KRA30 was maintained with most of the components, which included 6-8 numerically-dominant species, still visualized after 408 h of cultivation (Plate 6.9). Although, some species were not affected (Arrow 1), the numerical dominance of others either increased or decreased (Arrows 2) with protracted cultivation. In contrast, the addition of citric acid and, thus, nitrogen limitation in the presence of mineral salts solution M-I, reduced the complexity of the atrazine-catabolizing association profile (Plate 6.10) with four numerically-dominant members visualized. Of these, three species were also numerically dominant during cultivation in the presence of citric acid only (Plate 6.11).

To examine further the population profile changes effected by supplementary carbon (citric acid) addition, KRA30 was cultured in the presence of atrazine plus citric acid-supplemented M-I (168 h) and also in the presence of citric acid-supplemented M-I (168 h) followed by atrazine plus citric acid-supplemented M-I (120 h). For the latter, changes in numerical dominance and increased profile complexity were recorded compared with the culture which was exposed continuously to atrazine plus citric acid (Plate 6.12). This suggested that enhanced catabolic potential could be attained by an initial cultivation step with citric acid-supplemented M-I. It is probable also that an increased population size would result.

Plate 6.13 shows the association profile changes during cultivation in the presence of M-I and M-III supplemented with 100 mg l^{-1} atrazine which were accompanied by reduced and increased optical densities (Figure 5.3 and 5.6A), respectively. For both media, numerical band increases were recorded with cultivation time and these were accompanied by changes in species dominance. For M-I, 2-4 species dominated numerically while 5-7 species dominated the M-III culture. Shifts in numerical dominance were highlighted further by comparison of the association profiles at selected cultivation time intervals. Of the dominant species, only one (Arrow) was common throughout both cultivations with increased intensity recorded in the presence of M-I after 168 h of cultivation (Plate 6.14).

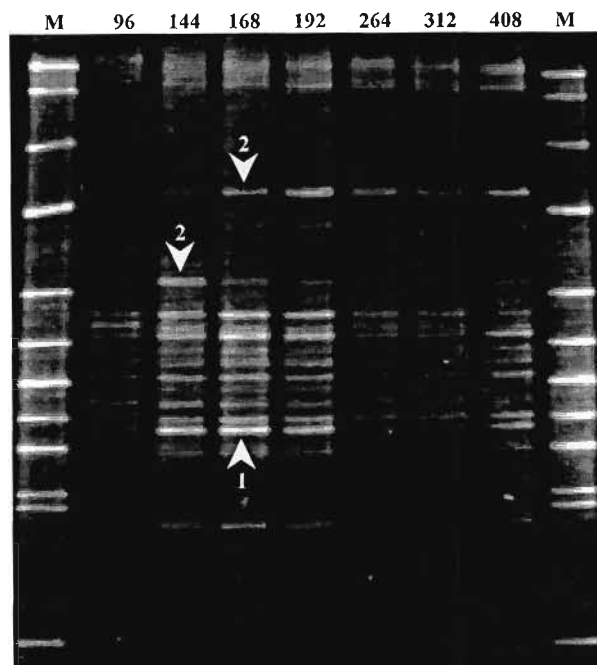


Plate 6.9: Changes with time (96-408 h) in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 *l* retentostat (400 ml working volume) cultivation (30⁰C) in the presence of mineral salts solution M-I supplemented with atrazine (100 mg⁻¹). M denotes the marker while Arrows 1 and 2 indicate species which showed sustained and increased/decreased numerical dominance, respectively, with time.

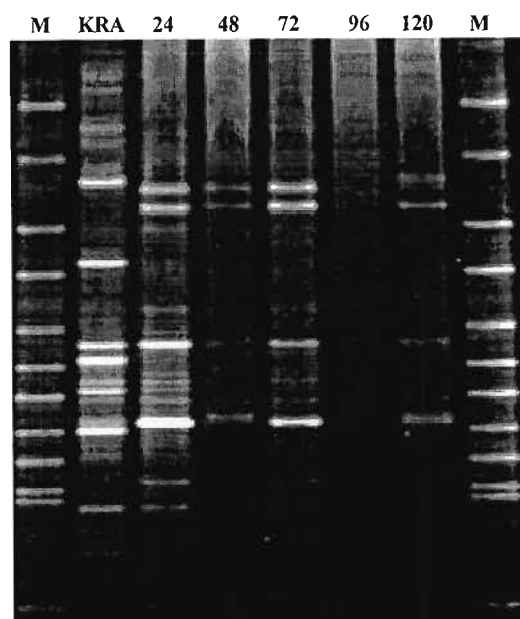


Plate 6.10: Changes with time (24-120 h) in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 l retentostat (400 ml working volume) cultivation (30⁰C) in the presence of mineral salts solution M-I supplemented with atrazine (100 mg l⁻¹) plus citric acid (3 mM). M denotes the marker while KRA indicates the complete profile of KRA30.

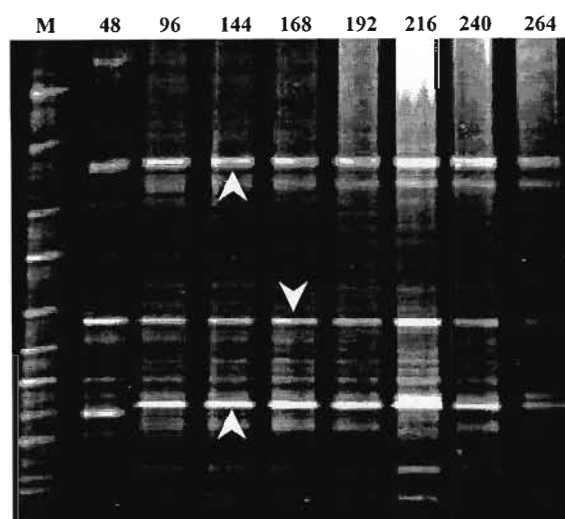


Plate 6.11: Changes with time (48-264 h) in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 l retentostat (400 ml working volume) cultivation (30⁰C) in the presence of mineral salts solution M-I supplemented with citric acid (3 mM). M denotes the marker while the arrows indicate association components which showed constant numerical dominance with time.

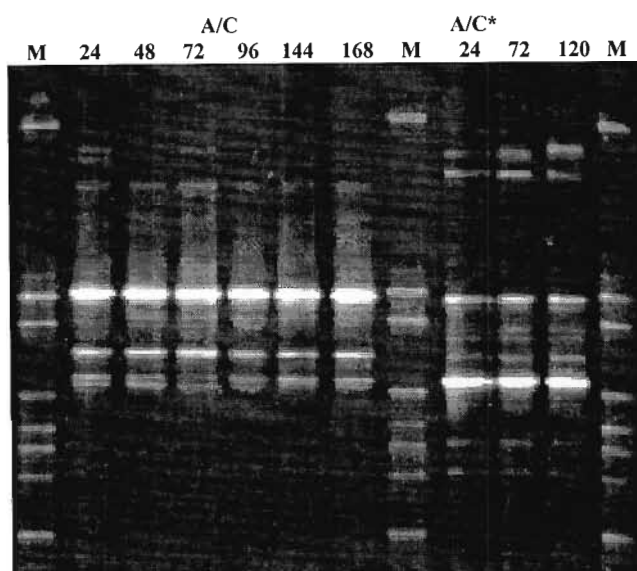


Plate 6.12: Changes in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 l retentostat (400 ml working volume) cultivation (30⁰C) for: 24-168 h in the presence of mineral salts solution M-I supplemented with atrazine (100 mg/l) plus citric acid (3 mM) (A/C); and 24-120 h in the presence of the same medium following initial cultivation (168 h) in the presence of citric acid (3 mM)-supplemented M-I (A/C*). M denotes the marker.

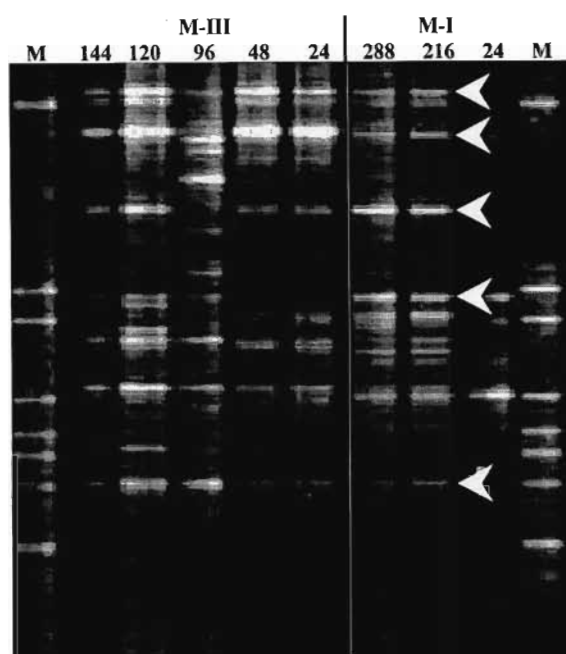


Plate 6.4.5: Changes in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 l retentostat (400 ml working volume) cultivation (30⁰C) for: 24-288 h in the presence of atrazine (100 mg l⁻¹)-supplemented mineral salts solution M-I; and 24-144 h in the presence of atrazine (100 mg l⁻¹)-supplemented M-III. M denotes the marker while the arrows depict species which were common after both cultivations.

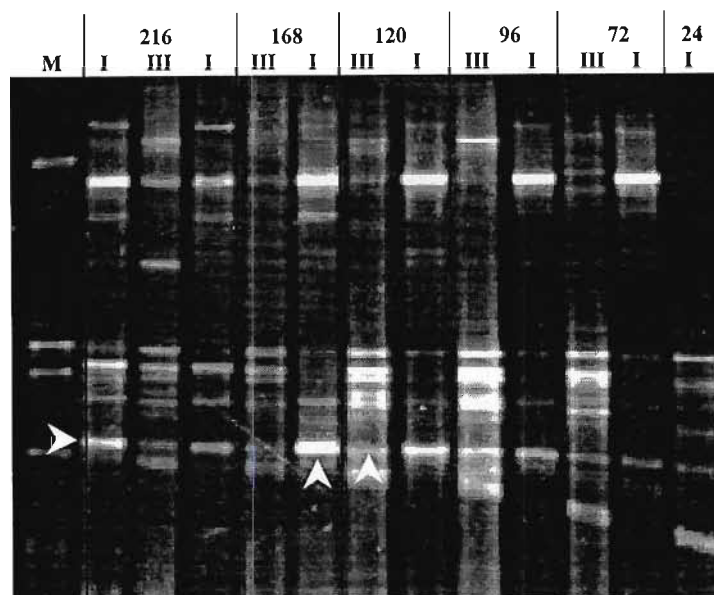


Plate 6.14: Changes with time (24-216 h) of the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 l retentostat (400 ml working volume) cultivation (30°C) in the presence of atrazine (100 mg l⁻¹)-supplemented M-I (I) and M-III (III) mineral salts solutions. M denotes the marker while the arrows depict species which were common throughout both cultivations.

Since enhanced atrazine catabolism was recorded in the presence of a supplemental carbon source (5.2.2) and increased association complexity was maintained with the more complex medium M-III, culture maintenance of association KRA30 under such conditions prior to inoculum preparation for *in situ* and *ex situ* bioaugmented remediation may be considered. The cost implications of culture maintenance and inoculum preparation must, however, be considered.

To study further the effect of medium composition on the association profile, a 1 l retentostat was charged with atrazine (100 mg l^{-1})-supplemented mineral salts solution M-I and association KRA30 was cultured for 120 h before the influent medium was changed to atrazine (100 mg l^{-1})-supplemented M-III.

Population profile changes and changes in numerical dominance resulted in response to both time and medium composition (Plate 6.15). As observed earlier (Plates 6.9 and 6.13), the 16S rDNA banding pattern was less complex when atrazine was targeted as both a carbon and nitrogen source in the presence of M-I than when nitrogen-limitation (M-III) was imposed. Also, changes in the number of bands and numerical dominance of individual species were recorded 24 h after M-I was replaced by M-III.

In general, KRA30 cultivation in atrazine-supplemented mineral salts solution M-I resulted in reduced or no visualization of bands in the upper part of the denaturing gradient possibly due to the absence of supplemental citrate and/or a reduced planktonic population due to increased wall growth (5.2.2). By replacing the influent medium with M-III an increased planktonic population resulted which was accompanied by proliferation of species with readily-denatured 16S rDNA melting domains (Plate 6.15, Arrows 1). In contrast, the numerical dominance of some species with less easily-denatured 16S rDNA melting domains decreased (Plate 6.15, Arrows 2).

Although nutrient availability has been reported to be one of the parameters which effect shifts in component species of microbial associations (4.3), wall growth/biofilm formation within fermenters has been implicated also. For example, Senior (1977) reported that, in general, increased wall growth led to decreased molecule catabolism. Wall growth did, however, facilitate the establishment of atypical ecological niches so that both competing and interacting association components were able to coexist. Thus, stability was maintained even though steady states were not reached.

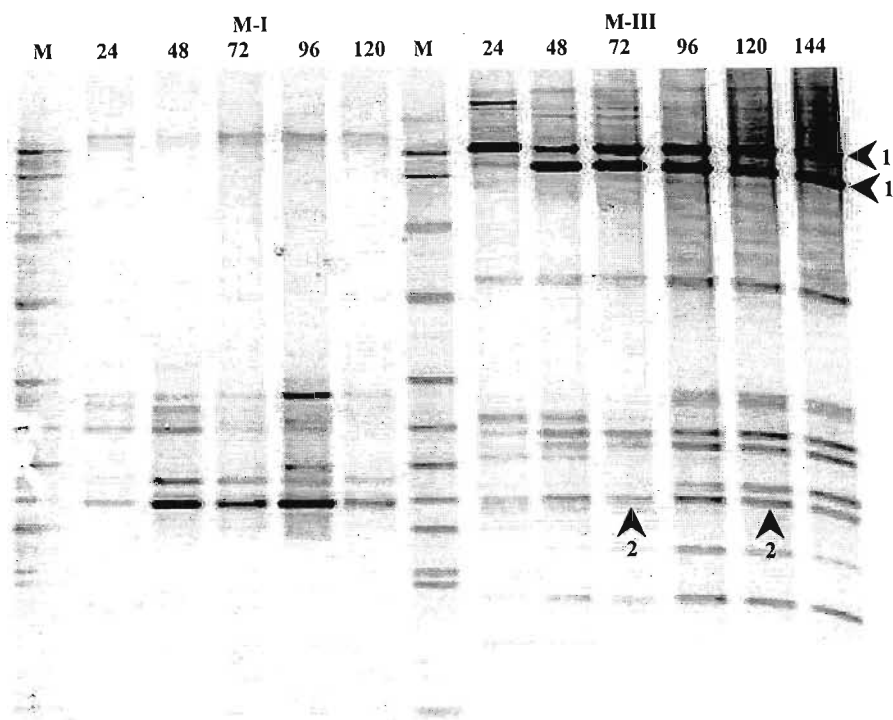


Plate 6.15: Changes with time of the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 during 1 *l* retentostat (400 ml working volume) cultivation (24-120 h) (30⁰C) in the presence of atrazine (100 mg/l)-supplemented M-I followed by atrazine (100 mg/l)-supplemented M-III (24-144h). M denotes the marker while Arrows 1 and 2 depict species which showed increased and decreased numerical dominance, respectively, in the presence of M-III.

For the current study, the effect of biofilm formation on the microbial association profile was investigated with 1 l retentostat cultures of KRA30 cultivated in the presence of: M-I supplemented with 100 mg l⁻¹ atrazine and 3 mM citric acid; the same medium before a change to M-III supplemented with 100 mg l⁻¹ atrazine; and M-III supplemented with 100 mg l⁻¹ atrazine. Following cultivation, samples of planktonic and biofilm populations were taken and characterized as before.

With M-I, more bands were detected with the planktonic sample than the biofilm (Plate 6.16, Lanes 1, 2 and 7) although, numerically, the three dominant species were comparable (Arrows 1). Growth in the presence of atrazine (100 mg l⁻¹)-supplemented M-III following initial cultivation in atrazine (100 mg l⁻¹)/citric acid (3 mM)-supplemented M-I gave comparable profiles (Lane 3, biofilm; Lane 4, planktonic population). Shifts in numerical dominance were, however, recorded between the suspension (Lane 5) and biofilm (Lane 6) populations when KRA30 was cultured throughout in the presence of atrazine-supplemented M-III. For example, two components (Arrows 2) were numerically-dominant in the biofilm but not in the planktonic population.

In general, although 16S rRNA analysis was not made, the complexity of the association and the numerical dominance of key component species which were maintained suggested maintenance of atrazine catabolic potential despite considerable biofilm formation in the retentostats. This was confirmed partly by the M-II retentostat cultures (5.2.2) for which, although DGGE fingerprinting was not made, increased atrazine catabolism was recorded despite extensive wall growth.

Although some components remained unchanged, shifts in numerical dominance of the KRA30 profile were observed, generally, in response to changes in nutrient limitation and mineral salts composition. Also, as indicated by reduced residual atrazine and total organic carbon (TOC) concentrations (5.2.2), molecule catabolism capability was maintained.

6.2.7 Creation of Clone Banks and Sequencing

Cloning: Microbial Associations PMA03, KRT04 and KRT07

Identification of the dominant members of the isolated associations was attempted by characterizing the dominant bands (2.10.9) from DGGE gels (Plates 6.3 and 6.5) and cloning (2.10.10) and sequencing (2.10.12) individual clones. Analysis of the bands

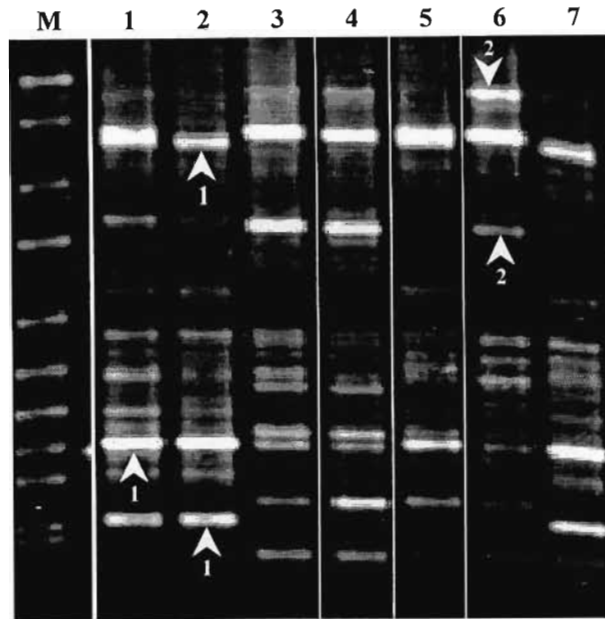


Plate 6.16: Changes in the 16S rDNA profile, visualized after DGGE separation (50-65% denaturing gradient), of the combined aerobic atrazine-catabolizing association KRA30 following 1 l retentostat (400 ml working volume) cultivation (30°C) in the presence of: mineral salts solution M-I supplemented with atrazine (100 mg l⁻¹) plus citric acid (3 mM) (Lane 1, planktonic; Lanes 2 and 7, biofilm population); atrazine (100 mg l⁻¹)-supplemented M-III following initial cultivation in atrazine (100 mg l⁻¹)/citric acid (3 mM)-supplemented M-I (Lane 4, planktonic; Lane 3, biofilm); and atrazine (100 mg l⁻¹)-supplemented M-III (Lane 5, planktonic; Lane 6, biofilm). M denotes the marker. Arrows 1 and 2 indicate species which showed maintained and reduced numerical dominance, respectively, in the planktonic and the corresponding biofilm populations.

cut from gels showed that possible co-migration of whole bands had occurred. Thus, the creation of a clone bank was more specific for the component species.

The subsequent comparison of a few of the randomly-selected clones with the total 16S rDNA profile of the respective association on DGGE gels emphasized the numerical dominance of some of the component species. For example, Plate 6.17 shows that for association KRT07 seven of eight selected recombinants (Y17-1 to -7) migrated the same distance along the denaturant gradient which suggested a common dominant species. For association PMA03, some of the clones (Y5-4 and -6) indicated that the non-dominant members of the association were hardly detected (if at all) within the total population profile.

Sequencing

To initiate identification of the different component species of the catabolic associations, some of the clones were selected for sequencing (2.10.12). Difference in sequence, as determined by clone location or banding along the denaturing gradient, was used as the selection criterion to start the protocol. The sequencing results indicated that the various clones were characterized generally by different sequences (Table 6.1). This was congruent with their different loci recorded in the denaturing-gradient gels.

Although clones Y17-1 and -3 to -7 were not included for sequencing, they exhibited bands in the same positions along the denaturing gradient as the sequenced Y17-2 (Plate 6.17) and were probably characterized by the same sequence which was 98% similar to *Hyphomicrobium denitrificans*. Supporting evidence for this was found with Clones Y14-2 and Y17-8 which, although loaded originally on different gels, exhibited bands in the same region along the same denaturing gradient with a 99% similarity to the same species from the Gamma Proteobacteria family, *Rhodanobacter lindanoclasticus*.

Some researchers (Kowalchuk *et al.*, 1997; Laverman, 2000) have shown, however, that fragments with different sequences can migrate to the same location on a gradient.

The disparities which exist often in the numbers of submitted and GenBank base pairs may suggest discrepancies in comparisons of the corresponding sequence data. Nonetheless, it is accepted generally that a sequence similarity $\geq 97\%$

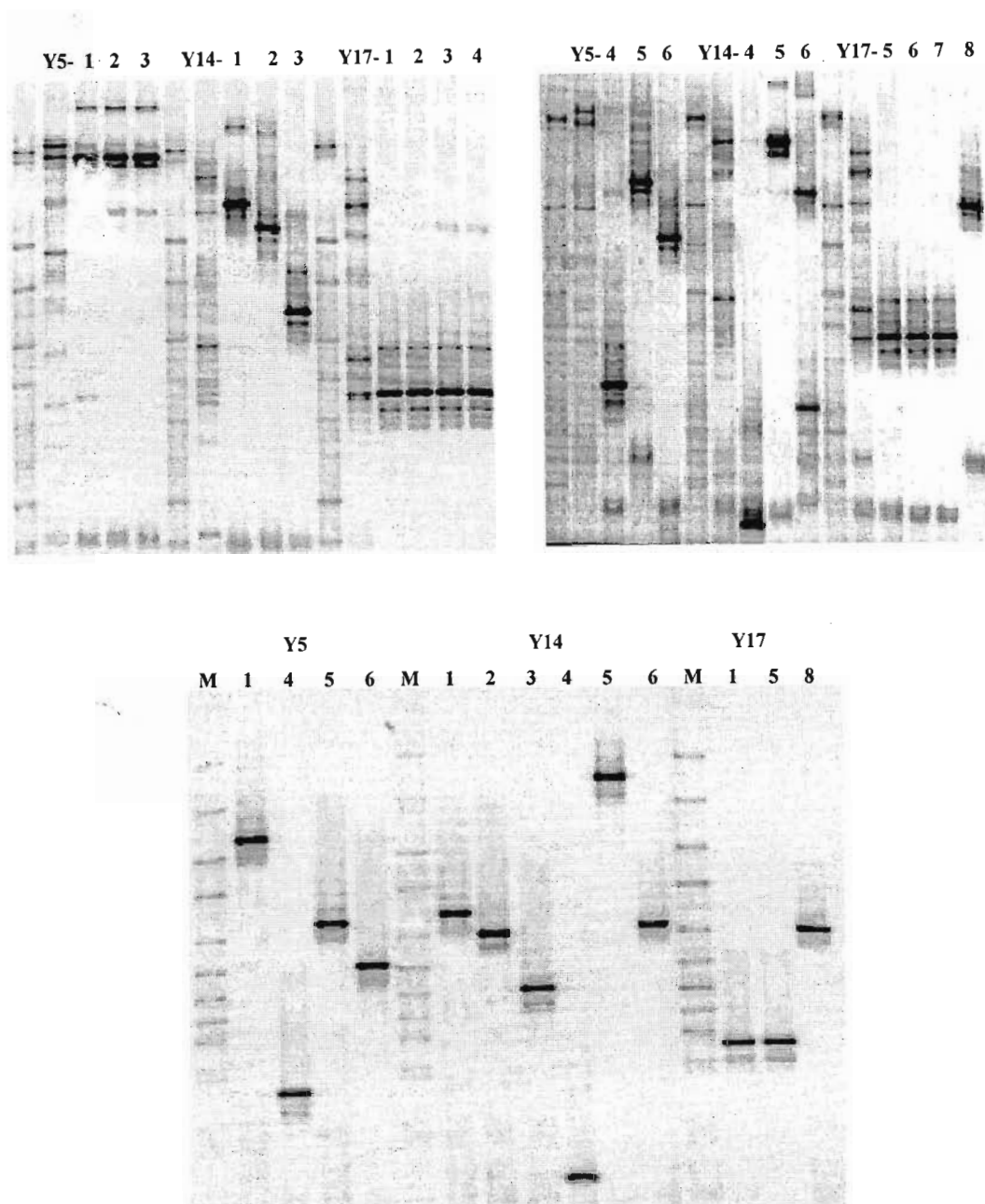


Plate 6.17: 16S rDNA profiles (50-65% denaturing gradient) of clones selected randomly from associations PMA03 (Y5), KRT04 (Y14) and KRT07 (Y17). M denotes the marker.

Table 6.1: Percentage similarities and closest relatives and their accession numbers, as determined by BLAST Network services, of randomly-selected clones from anaerobic atrazine (PMA03)- and aerobic toluene (KRT04/KRT07)-catabolizing associations. Sequencing was made with the Muyzer primers (F357-GC/R518).

Association Designation	Clone #	Class/Family	% Similarity	Closest Relative	Accession #
PMA03	Y5-2	Clostridia/ Peptococcaceae	94	<i>Desulfitobacterium</i> sp	X95972
PMA03	Y5-4	Environmental sample	89	Uncultured bacterium BC13	AF129868
PMA03	Y5-5	Clostridia/ Acidaminococcaceae	100	<i>Sporomusa silvacetica</i>	Y09976
PMA03	Y5-6	Clostridia/ Clostridiaceae	98	<i>Clostridium hydroxybenzoicum</i>	L11305
KRT04	Y14-1	Bacilli/Bacillaceae	86	<i>Bacillus mucilaginosus</i>	AF006077.1
KRT04	Y14-2	Proteobacteria/ Xanthomonadales	99	<i>Rhodanobacter lindanoclasticus</i>	AF039167
KRT04	Y14-3	Bacilli/Bacillaceae	87	<i>Bacillus</i> sp. '112442 JS2'	AF071857
KRT04	Y14-4	Alphaproteobacteria	97	Uncultured eubacterium WCHB1-55	AF050531
KRT04	Y14-5	Bacteria/unclassified	99	Bacterial species 16S rRNA gene	Z95709
KRT04	Y14-6	Clostridia/ Peptococcaceae	85	<i>Dehalobacterium formicoaceticum</i>	X86690
KRT07	Y17-2	Proteobacteria/ Rhizobiales	98	<i>Hyphomicrobium denitrificans</i>	Y14308
KRT07	Y17-8	Proteobacteria/ Xanthomonadales	99	<i>Rhodanobacter lindanoclasticus</i>	AF039167

indicates that the submitted sequence is the same as that of the closest relative while a similarity of $\leq 97\%$ reflects a novel species. Thus, clones Y5-2 and 4, Y14-1, 3 and 6 were new sequences hitherto not isolated or cultured. Also, clones Y5-2 and Y5-4 were new atrazine-catabolizing microorganisms.

As shown in Plate 6.3, both the molecule concentration and enrichment pH effected some similarities in the profiles of the isolated toluene-catabolizing microbial associations. The closely comparable sequences of Clones Y14-2 and Y17-8 (Table 6.1), which were obtained from associations KRT04 (5 mg l^{-1} , pH 7.5) and KRT07 (50 mg l^{-1} , pH 5.5), confirmed this.

In general, the sequencing results supported the observations made with light and scanning electron microscopy of mixtures of Gram-negative and Gram-positive rods and cocci, with the former the predominant morphotype.

The 16S rDNA sequences of the clones from associations KRT04 and KRT07 were submitted subsequently to GenBank and were designated specific accession numbers as shown in Table 6.2.

Table 6.2: Enriched/isolated catabolic microbial associations and the new GenBank accession numbers for some sequenced clones.

Association Designation	Clone Number	GenBank Accession Number
KRT04	Y14-1	AF312215
	Y14-2	AF312216
	Y14-3	AF312217
	Y14-4	AF312218
	Y14-5	AF312219
	Y14-6	AF312220
KRT07	Y17-2	AF312221
	Y17-8	AF312222

Effect of Methanol on the Combined Atrazine-Catabolizing Association KRA30

The solubility of atrazine in water at 30°C is approximately 30 mg l⁻¹ (Erickson and Lee, 1989). To exceed this concentration for enrichment/isolation programmes and catabolic association culture studies methanol is often used to further solubilize the herbicide. The combined atrazine-catabolizing microbial association KRA30 was, therefore, selected to examine the effects of this solvent on species dominance in the 16S rDNA profile.

The association was cultured in 100 mg l⁻¹ atrazine-supplemented mineral salts solution M-I (50 ml) with and without methanol (≤5 ml, 100 % v/v). Two clone banks were then made from each of the resultant cultures and a total of 70 clones were selected randomly from each bank (2.10.11). Only clones with different motilities on the denaturing gradient gel were selected, sequenced and used for the comparative study.

Of the 70 clones selected from each clone bank, only 10 seemed to be characterized by different sequences as visualized on the DGGE plates (Plates 6.18A

and B) and their designations after sequencing are shown in Tables 6.3 and 6.4. The results suggested sequence redundancy within the association irrespective of the presence or absence of methanol. For example, the presence of methanol effected a predominance of Gammaproteobacteria/Xanthomonadaceae species. Also, some of the clones were visualized in the respective association profiles while others were not (Plates 6.18 and 6.19). This highlighted the numerical dominance of the former group of association species.

Table 6.3: Percentage similarities and closest relatives, as determined by BLAST Network Services, of clones of species constituting KRA30 cultured in 100 mg/l¹ atrazine-supplemented mineral salts solution M-I in the presence of methanol.

Clone #	Proteobacteria Sub-division/Family	% Similarity	Closest Relative	Accession #
11	Beta/Alcaligenaceae	99	<i>Alcaligenes</i> sp.	AJ002802
16	Beta/Alcaligenaceae	98	<i>Alcaligenes</i> sp.	AJ002802
05	Beta/Alcaligenaceae	97	<i>Alcaligenes</i> sp.	AJ002802
18	Beta/Alcaligenaceae	98	<i>Alcaligenes</i> sp.	AJ002804
14	Alpha/Sphingomonadaceae	99	<i>Sphingomonas</i> sp. SA-3	AF327069
6A	Betaproteobacteria	96	Arsenite-oxidizing bacterium	AY027506
12	Beta/Comamonadaceae	97	<i>Comamonas acidovorans</i>	AF181575
17	Gamma/Pseudomonadaceae	97	<i>Pseudomonas putida</i>	AF094744
01	Beta/Alcaligenaceae	95	<i>Bordetella broniseptica</i>	X57026
04	Gamma/Pseudomonadaceae	99	<i>Pseudomonas</i> sp. ML2	AF378011
03	CFB group/ Flavobacteriaceae	97	<i>Flavobacterium</i> sp. 3A5	AF368756
15	Proteobacteria	98	Nitrogen-fixing bacterium M1753	AF214644
6T	Proteobacteria	99	Nitrogen-fixing bacterium M1753	AF214644
19	CFB group/ Flavobacteriaceae	99	<i>Flavobacterium</i> sp. 3A5	AF368756

In general, a shift in numerical dominance, effected by the presence or absence of methanol, was recorded in association KRA30 (Plate 6.19). However, one clone was present in both profiles which highlighted a species that could grow on both atrazine and methanol or grow on atrazine in the presence and absence of methanol (Arrows 1). In the presence of methanol band intensity increase was probably indicative of catabolism of this molecule and/or catabolism of the elevated concentration of atrazine available to the association.

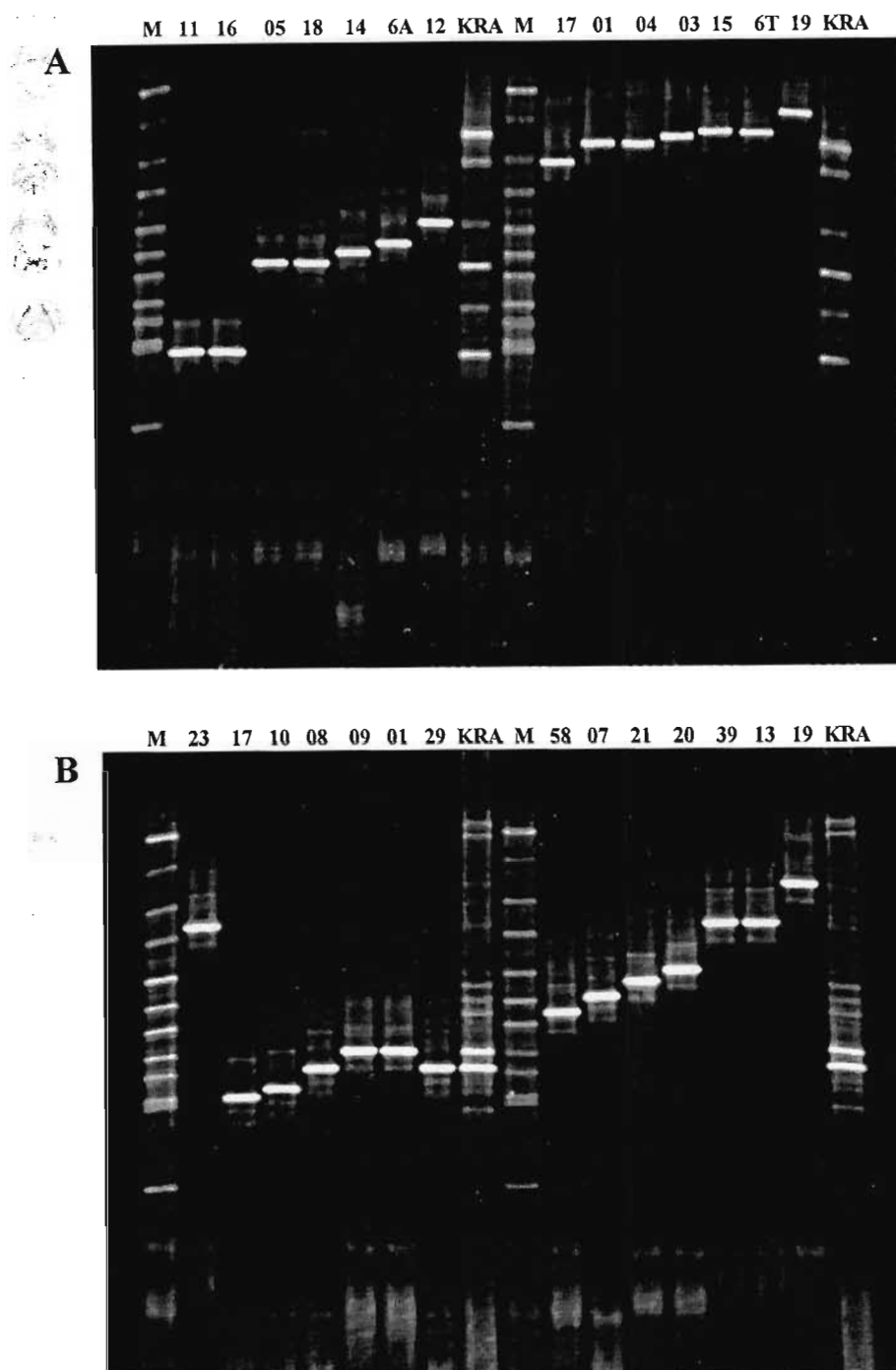


Plate 6.18: 16S rDNA profiles (50-65% denaturing gradient) of combined atrazine-catabolizing microbial association KRA30 (KRA) cultured in 100 mg/l⁻¹ atrazine-supplemented mineral salts solution M-I (50 ml) in the presence (A) and absence (B) of methanol (≤ 5 ml, 100 % v/v). M denotes the marker, while numbers designate clones.

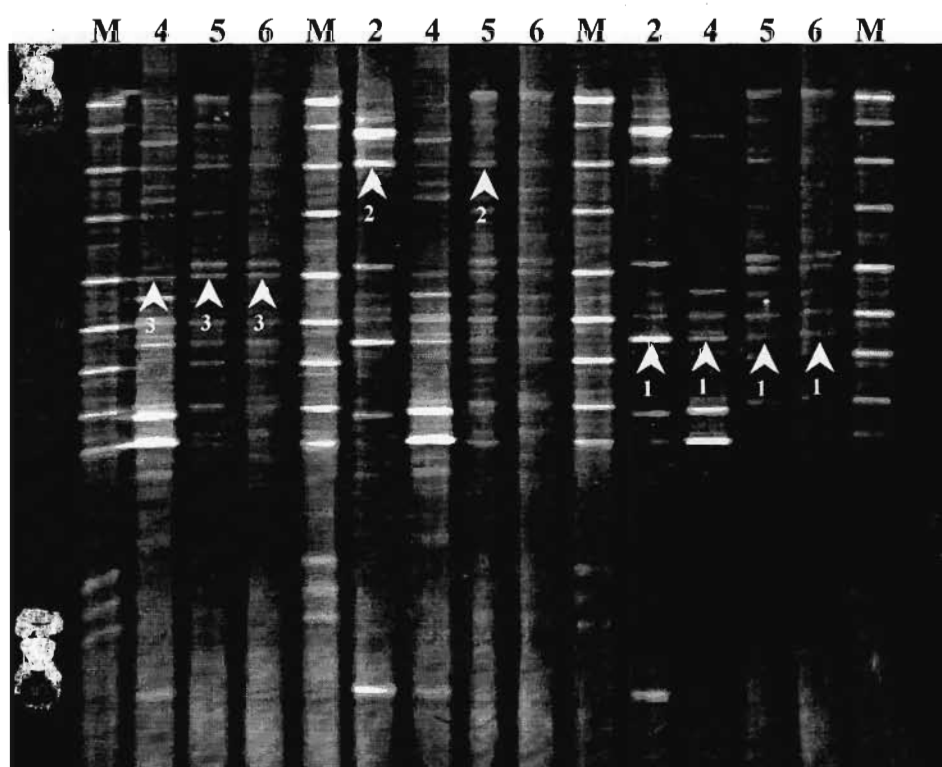
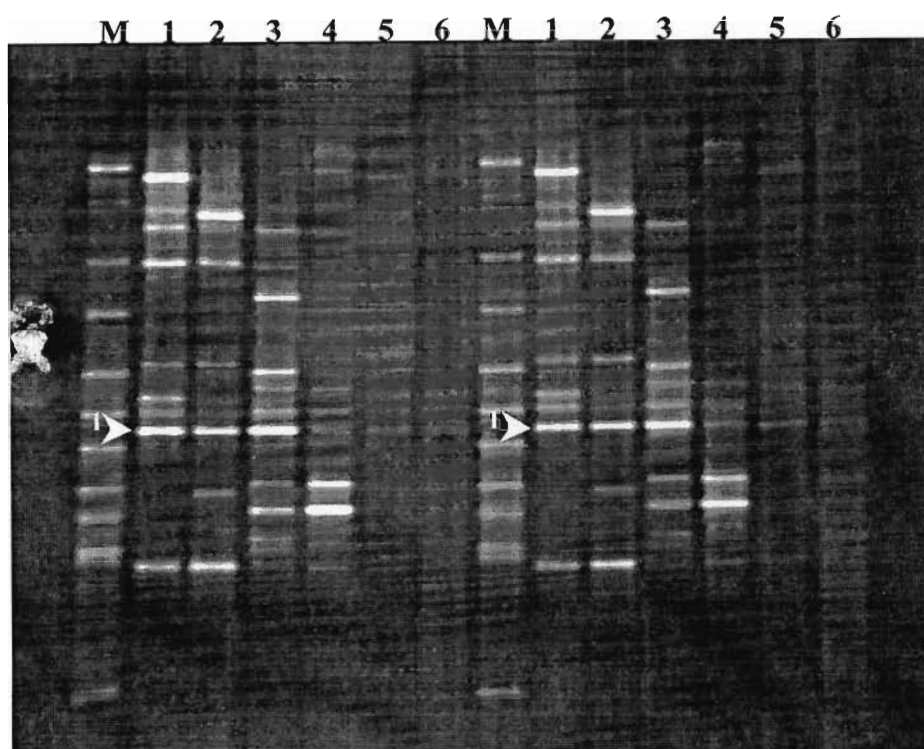
Table 6.4: Percentage similarities and closest relatives, as determined by BLAST Network Services, of clones of species constituting KRA30 cultured in 100 mg l⁻¹ atrazine-supplemented mineral salts solution M-I in the absence of methanol.

Clone #	Proteobacteria Sub-division/Family	% Similarity	Closest Relative	Accession #
23	Gamma/Xanthomonadaceae	99	<i>Stenotrophomonas</i> sp. P91630	AF214139
17	Alpha/Rhizobiaceae	98	<i>Hyphomicrobium facilis</i>	Y14311
10	Alpha/Rhizobiaceae	98	<i>Hyphomicrobium facilis</i>	Y14311
08	Alpha/Rhizobiaceae	96	<i>Hyphomicrobium facilis</i>	Y14311
09	Beta/Burkholderiaceae	97	<i>Burkholderia</i> sp. A6.33GD	AF247492
01	Beta/Burkholderiaceae	98	<i>Burkholderia</i> sp. A6.33GD	AF247492
29	Alpha/Rhizobiaceae	98	<i>Hyphomicrobium facilis</i>	Y14311
58	Beta/Alcaligenaceae	99	<i>Bordetella hinzii</i>	AF177667
07	Alpha/Sphingomonadaceae	97	<i>Caulobacter subvibrioides</i> CB81	M83797
21	Gamma/Xanthomonadaceae	99	<i>Stenotrophomonas</i> sp.	AJ002814
20	Gamma/Xanthomonadaceae	98	<i>Rhodanobacter lindanoclasticus</i>	AF250415
39	Gamma/Xanthomonadaceae	99	<i>Stenotrophomonas</i> sp. P91630	AF214139
13	Gamma/Xanthomonadaceae	99	<i>Stenotrophomonas</i> sp. P91630	AF214139
19	Gamma/Pseudomonadaceae	98	<i>Pseudomonas cedrella</i>	AF064461

To compare the 16S rDNA profile of the atrazine-catabolizing microbial association, in the presence or absence of methanol, to the source soil, and to confirm the earlier observations of numerical dominance in the enriched/isolated associations and the soils (6.2.1 - 6.2.3), the selected clones were mixed (2.10.11) prior to PCR-DGGE (Plate 6.19).

The arrows labelled 1 in Plate 6.19 identify an atrazine-catabolizing association species which was visualized after cultivation in both the presence (Lane2) and absence (Lane 4) of methanol and was also detected in the soil profiles (Lanes 5 and 6). The arrows labelled 2 show a numerically-dominant species in the presence of methanol (Lane 2) which was also visualized in the profile of the atrazine-contaminated and pristine soils (Lanes 5 and 6) while the arrows labelled 3 indicate an association component which was visualized in the absence of the solvent (Lane 4) and was also detected in the soils (Lanes 5 and 6).

Although shifts in numerical dominance were seen in the association profile in response to the presence of methanol and/or elevated available atrazine concentration,



direct correlations with the atrazine-contaminated source soil were recorded following cultivation in both the presence and absence of the solvent.

6.3 Conclusions

The results of analyzing the isolated aerobic atrazine-degrading associations by community-level physiological profiling (CLPP) highlighted both similarities and differences. In particular, different pH conditions and atrazine concentrations of the primary enrichments selected different associations.

16S rDNA-based PCR-DGGE was employed to: elucidate the sequence diversities and structures of the atrazine- and BTX molecule-catabolizing associations and the source soils and their pristine equivalents; study association dynamics in response to selected conditions; and investigate the impact of methanol as an atrazine solvent.

Considerable band similarities were observed between the pristine and the chemically-compromised soils. Since atrazine and petroleum had been introduced to the soils at different times and in different sections of the agricultural and industrial areas, temporal and spatial changes may have accounted for the differences in the profiles.

In recognition of the inherent biases of the different DNA isolation techniques and to minimize variability and facilitate comparison, the same DNA isolation protocol was adopted for the soils and the enriched/isolated catabolic microbial associations.

Despite the known biases and limitations of enrichment culture techniques, the isolated associations were characterized by complex banding patterns indicative of high numbers of equally abundant species (Heuer and Smalla, 1997a). Similar and common numerically-dominant bands also characterized the contaminated soils. In general, van Verseveld *et al.* (1999) found by DGGE profiling that isolated communities, particularly the dominant members, from the same site were very stable. Although differences were recorded for the isolated associations in response to each molecule, the common selection pressures, of molecule concentration and pH, of the primary enrichments, effected the recorded similarities which suggested some metabolic versatility. Numerical dominance does not necessarily reflect catabolic activity dominance. If, however, the numerically-dominant members (intense bands)

were the predominant active catabolic species, the associations could be exploited in different situations. Thus, for example, the toluene-catabolizing associations could facilitate sub-optimal enhanced (bioaugmented) remediation of petroleum hydrocarbon-contaminated soils in the absence of other petroleum component-catabolizing associations. Also, a robust/versatile microbial association could be constructed by selecting the components with the highest activities.

Possible microbial diversity changes in response to seasonal changes and the influence of environmental factors on the subsequently enriched/isolated catabolic associations was considered. Analysis of association profiles following environmental parameter changes identified possible variations in numerical and activity dominance which could be expected in South African soils. Specific growth rate changes, in response to environmental variable changes, were accompanied by DGGE band profile changes although some common patterns persisted. The results, therefore, exemplified the importance of site-specific determinants. Thus, determination of optimum values for key environmental variables to maximize growth/catabolic rates should precede bioaugmentation.

Following retentostat cultivation, shifts in numerical dominance of the KRA30 profile were observed, generally, in response to changes in nutrient limitation and mineral salts composition. In contrast, some components remained unchanged which suggested maintenance of catabolic potential independent of elemental limitations. Also, shifts in the numerical dominance of some association components resulted in response to biomass adhesion to the vessel walls although the species complement was retained which emphasised the difficulty in displacing a component species.

Since monoculture isolations proved unsuccessful, 16S rDNA-based PCR-DGGE may be used to make a provisional identification of the different roles of specific association components in atrazine degradation under carbon- and nitrogen-limited conditions. Cultivation of association KRA30 on selected atrazine metabolites, which are provided as carbon and/or nitrogen sources, should, therefore, be coupled with subsequent 16S rDNA and 16S rRNA DGGE analyses to identify the numerical dominance and activities of the association component species. Also, future studies which include more detailed analyses of catabolic intermediates and enzyme complements should reveal the operative atrazine catabolic pathway.

Characterizing the dominant bands from the DGGE gels and cloning and sequencing individual clones should have facilitated identification of the dominant

species of the isolated associations (Ferris *et al.*, 1996; Cheung and Kinkle, 2001). Analysis of the dominant bands excised from the gels showed that possible co-migration of bands had occurred. Therefore, precise band excision must be practised and should be complemented by cloning, particularly for sequencing purposes, since this proved more reliable to characterize the component species of the isolated associations. Species dominance was often emphasized while the presence of other non-dominant association members, which were hardly detected (if at all) within the total association profile, was observed. This highlighted a limitation of PCR-DGGE visualization when numerically-dominant species “overshadow” the non-dominant ones and, thus, give an incomplete picture of association diversity. Thus, a combination of PCR-DGGE, PCR-based subtractive hybridization (Akopyants *et al.*, 1998) and semi-nested PCR (Heuer *et al.*, 1997), for example, could be used to make a complete characterization of the species complement. Also, PCR-DGGE of 16S rRNA in conjunction with 16S rDNA, following co-extraction from culture and environmental samples (Griffiths *et al.*, 2000), should provide a more comprehensive evaluation of the numerical and activity characteristics of the constitutive species and, consequently, the complete association.

Some of the numerically-dominant and -non-dominant association members were identified through cloning and sequencing. Banding pattern variations, congruent with sequence variations, exemplified the potential use of sequencing in conjunction with DGGE prior to implementation of enhanced bioremediation. Denaturing-gradient gel electrophoresis could then be used, without the expense of sequencing, to monitor the key catabolic species.

Some research groups (Smit *et al.*, 2001) have recorded that bacterial diversity study by molecular and culture-based methods revealed different populations while others (El Fantroussi *et al.*, 1999) observed a correlation, after a specific incubation period, between Biolog and DGGE analyses in soils contaminated with different herbicides. The comparable results of our study with CLPP and DGGE analyses suggested that the two methods could be used in combination and, thus, minimize the limitations of a single method approach. Community-level physiological profiling focuses on the soil culturable fraction which can also utilize the individual substrates. The assay is, however, biased towards fast-growing species and the results reflect these. Conversely, DGGE visualizes both the culturable and non-culturable members irregardless of their growth rates although the numerically-dominant, and not

necessarily the activity-dominant, species can overshadow the less numerically-dominant components.

Denaturing-gradient gel electrophoresis, as exemplified by use of the combined association KRA30, facilitated establishment of a correlation between the 16S rDNA of the enriched/isolated microbial associations and the source soils. In general, the dominant bands of the atrazine-contaminated soil were retained by the enriched/isolated associations which supported the use of contaminated soils as inocula to enrich/isolate local catabolic microbial associations for efficient bioaugmented site amelioration.

If numerical dominance is proven to be correlated directly with activity, the use of PCR-DGGE to identify catabolic potential for, and monitor, intrinsic and bioaugmented remediation would be justified. Soil samples could be collected from a contaminated site and the resultant PCR-DGGE profile used to identify the potential for intrinsic bioremediation or the need to inoculate with a catabolic association(s). Awareness of the limitation of DGGE for this application, where the numerically-dominant members of the association “overshadow” the non-dominant ones, would be paramount. The presence of extracellular soil DNA which is free or reversibly bound to clay particles would give an unrepresentative profile of the site catabolic potential and would, therefore, also be crucial to result interpretation.

While accepted widely that *in situ* treatments rarely yield undesirable by-products, precautions and preliminary baseline tests are always recommended (Korda *et al.*, 1997). The results of this study suggested that PCR-DGGE combined with analyses which link structure and function, such as PCR-amplification of DNA and mRNA functional genes (Muyzer, 1998) and rRNA (Head *et al.*, 1998), bromodeoxyuridine (BrdU) and ^{13}C tagging of metabolically-active bacteria (Borneman, 1999; Urbach *et al.*, 1999; Whitby *et al.*, 2001), measurement of lipid biomarkers, specifically, phospholipid fatty acids (Parkes, 1987; Hanson *et al.*, 1999; MacNaughton *et al.*, 1999), and detection of specific enzymes (Shapir *et al.*, 2000), could be useful.

It has been recognized that the genotypic and phenotypic technologies employed increasingly by environmental microbiologists/molecular microbial ecologists often generate complex patterns which necessitate multi-variate statistical interpretation for classification and identification purposes (Noble *et al.*, 2000; Moschetti *et al.*, 2001). In addition, it has been suggested that DGGE patterns lend

themselves to computerized programmes such as artificial neural networks (Almeida *et al.*, 1999; Cho and Kim, 2000). Thus, this analytical tool could be used, following the establishment and identification of stable specific catabolic association profiles, with online monitoring of *ex situ* (bioreactors) bioaugmented bioremediation, as was implemented with PCR-single-stranded-conformation polymorphisms (Cho and Kim, 2000), randomly-amplified polymorphic DNA patterns (Moschetti *et al.*, 2001), two-dimensional scanning fluorometry (Wolf *et al.*, 2001) and phospholipid fatty acid profiles (Noble *et al.*, 2000), to meet the growing needs of practitioners of bioremediation technologies.

Chapter 7: General Conclusions and Future Prospects

7.1 General Conclusions

Since the pioneering studies of the 1970's of microorganism-microorganism interspecies interactions effecting xenobiotic molecule catabolism, successive tiers of complexity have been added to facilitate ever more comprehensive definitive fundamental studies so that molecular approaches have, increasingly, become the norm. Each new development has motivated a scrutiny of the basic tenets of the research protocol so that a number of problems have been identified and addressed. The net result is that bioremediation can be practised with increasing confidence or intrinsic bioremediation can proceed in a more predictable manner.

In soils and aquifers, the fates of organic compounds such as pesticides and BTX molecules are governed by hydraulic transport coupled with adsorption, desorption and degradation processes. The marked differences between soils do, however, prevent generalizations about the rates and extent of sequestration of organic compounds. Since their subsequent bioavailabilities are the major determinant of the enrichment of catabolic microbial associations, it is essential to elucidate the adsorption/desorption characteristics of contaminant molecules in the specific soil to be remediated. For example, atrazine adsorption to the soil investigated in the current study was dependent on molecule concentration. Irreversible sorption was minimal, however, with 100% recovery effected by three extractions for the initial herbicide concentrations $\leq 100 \text{ mg l}^{-1}$ while the higher concentrations $\geq 500 \text{ mg l}^{-1}$ required further extractions. Also, the soil was characterized by elevated phosphorus and low organic carbon concentrations which effected a low atrazine adsorption coefficient which, in turn, facilitated increased herbicide bioavailability and, thus, successful enrichment/isolation of catabolic microbial associations. Therefore, depending on local soil conditions, site manipulation of parameters such as moisture content, temperature, pH and cation exchange capacity (Huang *et al.*, 1984; Sparling *et al.*, 1998) should be considered, to circumvent bound-residue formation/irreversible sorption of atrazine and BTX molecules which may inhibit microbial catabolism and hamper residual concentration analysis and, thus, bioremediation efficacy monitoring.

Although extensive toxicological investigations have effected different conclusions, the potential toxicity of the *s*-triazine herbicide atrazine motivates

continuous bioremediation-directed research. Several indigenous soil atrazine-catabolizing microbial associations and monocultures have been enriched/isolated from compromised sites. Since petroleum hydrocarbons exist naturally in the environment in reduced concentrations, microorganisms which catabolize them are widespread. Bioremediation was, therefore, developed by the petrochemical industry with the result that BTX molecule catabolisms have been well researched and numerous microorganisms studied.

The limitations and biases of enrichment cultures, such as the non-cultivation of some catabolic species due to the use of selective media or the inherent non-culturability of some species, are well documented. Nonetheless, enriched/isolated microbial associations are often used to predict *in situ* catabolic potential, the optimal environmental conditions for catabolism, and the effects of changing environmental variables on association species profiles and efficacies. As a consequence, the enrichment/isolation of indigenous catabolic microbial populations has been practised extensively and advocated for use in bioaugmentation remediation programmes.

In this study, 48 aerobic microbial associations which catabolized atrazine (8) and selected petroleum hydrocarbons (benzene, toluene and *o*-, *m*- and *p*-xylene) (40) were enriched and isolated from two South African soil types both with a history of contamination. For the two molecule types, catabolism was operative for the enrichment/isolation selection pressures of pH values of 5.5 and 7.5 and electron donor concentration ranges of 10-33 mg l⁻¹ (atrazine) and 5-5 000 mg l⁻¹ (BTX molecules). The pH values studied were within the range (4-8) found normally in South African soils. The atrazine concentrations reflected the solubility range in normal dosages used in agricultural soils (Pick *et al.*, 1992; Yanze-Kontchou and Gschwind, 1994) while the BTX range was comparable with recorded/simulated soil contamination concentrations (Atlas, 1981; Widrig and Manning, 1995; Gardin *et al.*, 1999). Characterization by light and scanning electron microscopy of the culturable components showed a predominance of rods independent of the molecule concentration and enrichment pH. Isolation of catabolic association component species on solid medium proved unsuccessful.

To explore key environmental variables, specifically pH and temperature, exemplary of South African soils, combined associations were used. Growth rate changes were recorded in response to environmental variable changes and, therefore,

if catabolism of each molecule was growth-linked, the catabolic rates *in situ* would be influenced by pH and temperature changes.

Retentostat studies were used to investigate atrazine catabolism with the molecule targeted as a carbon or nitrogen source. Initial studies of the catabolic potentials of the component species were made also. Increased atrazine catabolism by the combined association KRA30 occurred when the herbicide was targeted as a nitrogen source in the presence of supplemental citrate as the carbon source. Co-metabolism in the presence of succinate was implicated also. Thus, as observed by other researchers (Katz *et al.*, 2000), carbon supplementation should be considered for efficient bioaugmented remediation of atrazine-compromised sites with enriched/isolated associations. The same approach should be considered also for the *ex situ* (bioreactor) bioremediation of atrazine-contaminated soil or rinsate with microbial associations or monocultures such as *Agrobacterium radiobacter* strain J14A (Protzman *et al.*, 1999).

For both intrinsic and directed bioremediation monitoring, traditional microbial association studies must be complemented by more advanced physiological and molecular protocols. Although not researched in this programme, an aerobic catabolic pathway for atrazine has been elucidated with *Pseudomonas* strain ADP. Also, the essential catabolic enzymes *atzA*, *atzB* and *atzC* (and now *atzD*) have been identified and sequenced (de Souza *et al.*, 1996; Boundy-Mills *et al.*, 1997; Sadowsky *et al.*, 1998) and probes for the genes which encode these enzymes developed (de Souza *et al.*, 1998c). The location of the genes on a self-transmissible plasmid has been identified also (de Souza *et al.*, 1998b). Genes controlling N-dealkylation have been described (Shao *et al.*, 1995) and one alternative pathway with a new hydrolase for the degradation of a range of *s*-triazines by a *Nocardioides* sp. has been reported (Topp *et al.*, 2000a). In contrast, the pathways, catabolic enzymes and plasmids involved in BTX molecule degradations have been researched/elucidated more extensively with a number of catabolic microorganisms reported including *Azoarcus* sp. strain EB1 (Johnson *et al.*, 2001), *Rhodococcus* sp. (Vanderberg *et al.*, 2000) and *Pseudomonas* spp (Subramanian *et al.*, 1985; Guo *et al.*, 1997; Bertoni *et al.*, 1998; Arengi *et al.*, 2001). Polymerase chain reaction primer sets have been developed for genes which encode catabolic enzymes, such as alkane hydroxylase (*alkB*), catechol-2,3-dioxygenase (*xylE*) and naphthalene dioxygenase (*nahAc*, *doxB*, *ndoB* and

pahAc), to detect (species-specific) hydrocarbon-catabolizing bacteria (Chandler and Brockman, 1996; Wilson *et al.*, 1999).

Despite the above studies, hitherto unknown or novel microorganisms, with unique sequences and different enzyme-mediated operative pathways, particularly for atrazine catabolism, warrant continued investigation to underpin effective site bioremediation.

Molecular biological techniques have become increasingly important to overcome the considerable limitations of culture-dependent methods to elucidate bacterial diversity. These tools have great potential to identify, as yet, uncultured bacteria with known and/or newly-identified rRNA sequences (Heuer *et al.*, 1997; Head *et al.*, 1998). Hölfe and Abraham (1999) postulated that DNA technologies will accelerate analysis and may lead to quantum leaps in understanding the structures and functions of microbial associations. In this study, molecular techniques were used to complement, but not replace, more conventional microbiological approaches.

To resolve fully the sequence diversities and structures of the isolated atrazine- and BTX molecule-catabolizing associations a combination of PCR and DGGE was used. The results showed that despite the different, but chemically similar, petroleum hydrocarbon molecules, the common selection pressures of the primary enrichments effected some similarities in the microbial associations. In general, however, the pollutant type and concentration and the enrichment pH all effected some differences in the species compositions of the different microbial associations.

The results of 16S rDNA-based DGGE gave comparatively comprehensive qualitative profiles of the isolated microbial associations. Where similarities in species dominance, as indicated by intense bands on the DGGE gels, of the aerobic and anaerobic atrazine-catabolizing-, and the different BTX molecule-catabolizing microbial associations existed, this suggested potential for interchangeable use of the isolates. For example, the aerobic atrazine-catabolizing associations could be used under anaerobic conditions. Also, the isolated aerobic benzene-catabolizing associations could be used for enhanced (bioaugmented) remediation of petroleum hydrocarbon-contaminated soils in the absence of other petroleum component-catabolizing associations.

Some dominant bands were common to the atrazine- and BTX molecule-catabolizing associations despite the different molecule structures. It can be speculated, therefore, that either each association could effect some catabolism of the

other molecule or the bands were species which targeted a common intermediate(s) of the different catabolic pathways.

The marked differences in banding patterns of the different BTX molecule-catabolizing associations suggested that a combination of the isolated associations would, possibly, be more effective for the bioremediation of petroleum hydrocarbon-contaminated soil. Thus, robust/versatile BTX-catabolizing microbial associations could be constructed by selecting all the numerically- (activity-) dominant members from the isolated catabolic associations. In general, the complexities of the isolated microbial associations implied a broad biodegradative capacity which would be ideal for bioaugmented remediation.

Total DNA extraction from soil samples facilitates direct monitoring of genotypic diversity changes without the need for cultivation and isolation (Tebbe and Vahjen, 1993). Denaturing-gradient gel electrophoresis was, therefore, used to compare the populations of the atrazine- and petroleum hydrocarbon-contaminated soils and their pristine equivalents. Considerable band similarities were observed and it is possible that the limited differences recorded were due to spatial or temporal differences specific to the two sampling sites.

Common numerically-dominant bands also characterized the contaminated soils and the equivalent enriched/isolated associations. In general, Gelsomino *et al.* (1999) and van Verseveld *et al.* (1999) found, by DGGE profiling, that isolated associations, particularly the dominant members, from the same site were very stable.

The results of this study highlighted the applicability of PCR-DGGE in assessing biodegradative potential and monitoring *in situ* and augmented bioremediation. This would be validated further if band intensity was correlated directly with activity. Polymerase chain reaction-DGGE profiles of contaminated soil samples could then identify the potential for intrinsic bioremediation or the need to inoculate with a catabolic association(s). Awareness of the limitation of DGGE analysis for this application, where the numerically-dominant members of the association may occlude the non-dominant ones, would, however, be paramount.

As discussed above, the combined associations recorded specific growth rate changes in response to different temperature and pH values typical of South African soils. These changes coincided with numerical and, possibly, activity dominance changes. Changes in the association profiles in response to different pH values and temperatures seemed to commend optimization studies for physiological determinants

in the laboratory. More particularly, the pH and temperature values of the contaminated soil should dictate their values in the enrichment/isolation programme. Once isolation has been completed then the optima of the key variables should be identified for possible site manipulation.

Intense bands along the denaturant gradient indicated dominant components and this was interpreted as strong evidence of numerical dominance although this is not necessarily equivalent to activity dominance. Nomura *et al.* (1984) postulated that metabolically-active cells usually contain increased numbers of ribosomes compared with quiescent cells, while Nogales *et al.* (2001) added that total microbial association rRNA analysis may be considered to reflect predominantly the diversity of the metabolically-active species. The latter workers reported also that although some differences in composition existed, similar major constituents characterized the rDNA and rRNA clone libraries from the same biphenyl-polluted soil despite the inherent differences in the generation of the corresponding PCR and RT-PCR amplification products. However, Felske *et al.* (1998) and Griffiths *et al.* (2000) reported profile differences which corresponded to the nucleic acid template (RNA or DNA) and attributed these to differences in activity and total diversity. Therefore, despite possible loss during the additional purification steps for RNA isolation, analyses based on this would be crucial in future studies. 16S rDNA and rRNA from enriched/isolated associations must be subjected to DGGE to visualize the total profile since the less numerically-abundant members might, possibly, be the most metabolically active.

Although inconclusive, attempts were made to establish a link between numerical dominance and activity. Urbach *et al.* (1999) used bromodeoxyuridine (BrdU), a thymidine analogue, to demonstrate the isolation of DNA from metabolically-active bacteria. This approach of analogue incorporation by the component species of catabolic associations warrants further exploration.

Since the isolation on solid medium of the component species was unsuccessful, with single colonies consisting of more than one morphological form, cloning was used to elucidate the compositions of selected associations. Several of the numerically-dominant and non-dominant association members were identified through DGGE analysis of some of the recombinants from the created clone banks. A comparison of these with the original total profiles of the specific isolated associations showed that the diversities or complexities of the associations could be

underestimated. This was highlighted by the bands of specific clones which could not be detected, or were poorly detected, in the band patterns of the complete communities. Alternative gel stains, such as vistra green and silver stains, may be explored to improve band detection.

More sophisticated approaches, such as PCR-based subtractive hybridization, may be used, possibly, to alter the ratio of the 16S rDNA fragments in the isolated microbial associations to enhance the visualization and, consequently, the characterization of the non-dominant members. Another strategy for analysis of the less dominant components is the application of group-specific primers as used by Heuer *et al.* (1997). The protocol entails amplification of the community DNA with primers specific for the enrichment of certain taxonomic groups (*e.g.* high G+C Gram-positive bacteria) followed by amplification of the diluted PCR-product in a semi-nested PCR with DGGE primers.

The preliminary results of sequencing selected clones indicated that although some of the association members were related to other, as yet, unidentified soil microorganisms, some were classified as “unknown” or not related to any identified sequences. The similarities in sequences of the isolates compared with isolates of other research groups highlighted the conservation of genetic profiles across the globe. In contrast, the uniqueness of some isolates (while very exciting) emphasized, and further justified, the need to enrich and isolate site-specific microorganisms. This approach also obviates the possible restrictions of importing “foreign” isolates from other geographic regions. If bioaugmented bioremediation is considered, the sequencing of new isolates is imperative for identification in relation to known human, animal and plant pathogens.

Complete sequence analysis is important to explore the possibility of band co-migration during DGGE separation. Some PCR fragments with different sequences have been shown to have similar mobilities along the denaturing gradient. Thus, any overlap could mean that discrete bands do not reflect accurately the number of different sequences in a given mixture (Kowalchuk *et al.*, 1997). Sequence data can be used also to investigate the possible presence of homologous genes in the atrazine- and hydrocarbon-catabolizing isolates (de Souza *et al.*, 1998c; Mason *et al.*, 1998). New primers specific for targeted association members which define effective catabolic profiles can be designed and so provide a tool for bioremediation progress monitoring or identification of the amenability of a site for augmented amelioration.

Extensive cloning and sequencing of the combined atrazine-catabolizing association KRA30 and subsequent comparison with the DGGE profiles of the atrazine-contaminated soil and its pristine equivalent were used to investigate the impacts of methanol as an atrazine solvent. Notable shifts in numerical dominance were recorded in the 16S rDNA profile in response to the presence of methanol. Despite these, profile similarities were recorded for the combined association, in the presence/absence of the solvent, and the source soil. Thus, as recorded by other researchers (Mandelbaum *et al.*, 1993; Topp *et al.*, 2000a), the key soil microbial components and the intrinsic atrazine catabolic capacity may be harnessed successfully independent of the presence/absence of methanol during enrichment/isolation programmes. Physico-chemical technologies which include the use of methanol may, therefore, be combined with bioaugmented remediation for efficient clean up of atrazine-contaminated soils and sediments.

By correlating DGGE profiling with sequence identification, the latter could be, subsequently, omitted from catabolic species/microbial association monitoring protocols. Central to this application would be an awareness of the limitation of DGGE analysis where species with different sequences but similar melting properties (T_m) may migrate to the same location along the denaturant gradient. The combined approach could be used to develop hybridization probes to facilitate efficient monitoring of augmented bioremediation (Brockman, 1995; Heuer *et al.*, 1997; Mason *et al.*, 1998; Shapir *et al.*, 1998). Ronald *et al.* (1992) reported that environmental monitoring of microorganisms is necessary for public health considerations and for following the activities of microorganisms with genes which encode specific metabolic activities.

Since molecular techniques based on 16S rDNA do not give information on physiological capabilities and quantitative distribution (Röling *et al.*, 2000a), community-level physiological profiling (CLPP) may be used to elucidate carbon source utilization patterns (Garland, 1997; Gamo and Shoji, 1999).

While some researchers have stated that CLPP, via the Biolog assay, does not give fundamental insights of the structure and operation of microbial associations, the results of this present study, in agreement with the deliberations of other workers (Juck *et al.*, 2000), suggested that a combination of DGGE visualization of 16S rDNA/rRNA, CLPP and detection of specific enzymes (Shapir *et al.*, 2000) would be the best approach to plan, implement and monitor augmented bioremediation.

In this study, denaturing-gradient gel electrophoresis facilitated rapid, qualitative and comprehensive profiling of complex enriched/isolated atrazine- and BTX molecule-catabolizing microbial associations. Although the recognized limitations of the culture-independent (DGGE, cloning, sequencing) and culture-dependent (Gram characterization, morphological differentiation, CLPP) microbiological techniques were apparent, their applicabilities in bioremediation were emphasized also.

While widely-accepted that *in situ* treatments rarely yield undesirable by-products, precautions and preliminary baseline tests are always recommended (Korda *et al.*, 1997). For example, detailed site characterization in relation to the type and extent of contamination, hydrogeochemistry, pollutant bioavailability and catabolic capacity is essential. Also, empirical evidence is required to provide insight of how this technology can be effective under one set of circumstances but yet quite inappropriate under another (Stegmann *et al.*, 1994). The results of this study exemplified the potential application and relevance of PCR-DGGE to address these central concerns.

As stated by other researchers (Kozdrój and van Elsas, 2001), the results of the present study led to the conclusion that a combination of molecular and culture-based techniques should be ideal for planning, implementing and monitoring bioremediation programmes for atrazine- and petroleum hydrocarbon-contaminated soils in South Africa. In particular, these methods could be used to understand the long-term effects of the additions of microorganisms and nutrients during bioremediation programmes (Mishra *et al.*, 2001). By use of these tools, the bioremediation protocol should: ascertain the indigenous (intrinsic) biodegradative potential of the site; monitor the biodegradation process; and track introduced microbial associations during bioaugmentation.

7.2 Future Prospects

Despite recommendations for controlled and managed herbicide applications (Gerstl *et al.*, 1998), atrazine usage is likely to continue and result in further compromised soil, surface- and ground-water. Major progress in bioremediation of atrazine and BTX molecules could come from a variety of sources. For example, new microbial associations may be isolated with high specific growth rates and/or high critical

substrate concentrations and/or low maintenance energy requirements. Together with methods to promote indigenous microbial activity, the introduction of genetically-engineered strains (bioaugmentation) may be considered also. Shao *et al.* (1995) and Strong *et al.* (2000), for example, conferred catabolic capacity by constructing recombinant *Rhodococcus* strains and *Escherichia coli* which were capable of dealkylating and dechlorinating atrazine. Alternatively, novel enzymes involved in alternative catabolic pathways may be isolated and exploited via: immobilization on an insoluble cellulose matrix; or over-expression in killed whole-cell suspensions of recombinant microorganisms, as was done with atrazine chlorohydrolase (AtzA) for the treatment of herbicide-compromised groundwater (Kauffmann *et al.*, 2000; Strong *et al.*, 2000).

While it is essential to study the catabolism of individual BTX molecules, the degradation of hydrocarbon mixtures by the different enriched/isolated microbial associations should be explored. The catabolic capabilities of the atrazine-catabolizing associations in the presence of different electron donor and electron acceptor concentrations should be investigated also. For both molecule types, the critical substrate concentrations (S_{crit}) should be determined. Denaturing-gradient gel electrophoresis profiles of microbial associations cultured in the presence of critical substrate concentrations should be used to identify the susceptible component species.

Bench-scale modelling of augmented *in situ* (microcosms) and *ex situ* (slurry bioreactors) bioremediations with catabolic microbial associations enriched/isolated from soils with a history of contamination should be used to test the efficacies of the inoculants. A combination of 16S rDNA and 16S rRNA analyses should then be used to monitor population profile changes with time. To further refine the PCR-DGGE visualization procedure, the relative population sizes of the component species could be determined by incorporating ribosomal intergenic spacer analysis (Yu and Mohn, 2001) to measure band intensity.

Other molecular techniques, in combination with PCR-DGGE, may be considered also. For example, the employment of increasingly sensitive techniques, which use peptide nucleic acid clamps and oligomers under specific hybridization conditions, would be ideal for affinity purification and, thus, detection and quantification of low concentrations (picograms) of DNA and RNA of catabolic species in environmental samples (Chandler *et al.*, 2000).

Hybridization probes and PCR primers which target newly-identified catabolic genetic sequences may be developed as described by Stapleton *et al.* (1998) and Wilson *et al.* (1999). According to Weber *et al.* (2001), direct hybridization avoids the possible biases introduced by DNA extraction and clean up methods, which can distort the visualized community structure and component species dominance (Martin-Laurent *et al.*, 2001), and preferential amplification of more abundant species by PCR (Gordon and Giovannoni, 1996; Suzuki and Giovannoni, 1996; Wilson, 1997). Instead, it facilitates quantitative estimation by whole cell *in situ* hybridization techniques, such as fluorescent *in situ* hybridization, which can give absolute enumeration and identify spatial localization and distribution of the metabolic activities of specific microorganisms in natural environments.

Heuer *et al.* (1999) suggested the use of excised TGGE/DGGE bands to generate highly specific digoxigen (DIG)-labeled probes which target the V6 region of the 16S rRNA gene. The probes can be used to detect bacterial species which correspond to specific bands in the association fingerprints, in environmental samples, without prior DNA sequence knowledge. Together with measuring the occurrence of genes which encode, for example, atrazine and petroleum hydrocarbon molecule catabolic enzymes, gene probe technology may be used to identify: the concentration of a contaminant(s) which effects changes in a microbial association; and soils that have been affected by exposure to the molecule(s) (Guo *et al.*, 1997).

Despite the documented limitations of Gram-negative bacteria community structure characterization, techniques such as ester-linked phospholipid fatty acids analysis may be used to estimate the viable cells (assuming rapid degradation of intact phospholipids upon cell death) and fingerprint the microbial community *in situ* (Green and Scow, 2000; Ringelberg *et al.*, 2001).

The detection of active cells may be made with different nucleic acid-specific fluorescent dyes (SYBR I, SYBR II and SYTO 13), leucine incorporation, flow-cytometry and other fluorescent physiological probes (Lebaron *et al.*, 2001). Dye incorporation by the target catabolic population would have to be elucidated first, however, with enriched/isolated microbial associations prior to use *in situ*.

The sustained effectiveness of natural (intrinsic) and enhanced (bioaugmented/biosupplemented) attenuations must be demonstrated continually so regular site evaluations and results analyses, despite the limitations of chemical extraction methodologies, are crucial. Also, the contaminant concentration limits

permitted by current regulations are becoming increasingly stringent as exemplified by the $0.1 \mu\text{g l}^{-1}$ allowed in Europe for a single pesticide in drinking water (Martín-Esteban *et al.*, 1997). In contrast, the South African Water Quality Guidelines (1996) stipulated an atrazine concentration of $\leq 2 \mu\text{g l}^{-1}$ in domestic water (Anon, 1996). Therefore, while mathematical models may be used to assess quantitatively pesticide mobility and fate *in situ* (Zhang *et al.*, 2000), for example, the routine use of sensitive, rapid and inexpensive detection and screening methods, such as immunoassays/immunosensors (Turiel *et al.*, 1999; Kramer *et al.*, 2001), improved/more sophisticated and up-scaled pollution (water) analytical techniques, such as immunoaffinity chromatography (Frischenschlager *et al.*, 1997; Martín-Esteban *et al.*, 1997; Müller *et al.*, 1997), and extraction methods to predict bioavailability (Kelsey *et al.*, 1997; Chung and Alexander, 1998), are central to environmental monitoring/protection.

In general, a combination of conventional culture-dependent microbial techniques, various relevant molecular microbial ecology approaches and improved pollutant extraction and analysis methods should underpin all bioremediation protocols and so facilitate a comprehensive Code of Practice for the bioremediation of soil and groundwater.

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