BIOTREATMENT OF INDUSTRIAL EFFLUENTS CONTAINING NAPHTHALENE SULPHONATE

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

Signed: 

Date: 25/3/03

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Dedicated to the memory of

Phyllis Tranter

'Pretium victoriae'

- Tacitus

ABSTRACT

Naphthalene-2-sulphonate (NSA) is a frequently reported pollutant of industrial wastewaters. NSA was detected in the biologically treated effluent from a tannery (TE) (61.78 mg L^{-1} ± 0.67) and the untreated effluent from a chemical manufacturing plant (CE) (25.29 mg L^{-1} ± 0.06). Both effluents also contained significant concentrations of naphthalene sulphonate-formaldehyde condensates (SNFC). NSA and SNFC contributed a large fraction of the COD of TE (33.03% of total COD) and CE (48.31% of total COD). BOD_5 determinations indicated that this COD fraction would be refractory to conventional biological treatment and would subsequently be discharged into the environment.

Two NSA-degrading isolates, *Sphingomonas* sp. (RBN) and *Comamonas testosteroni* (BAS), were isolated from acclimated activated sludges, by enrichment with NSA as sole source of carbon and energy. Both isolates degraded NSA via a plasmid-encoded catabolic pathway analogous to the naphthalene degradation pathway. Inoculation of a suspended floc reactor treating a mixed feed containing NSA with RBN, resulted in a 112.5% increase in the NSA removal rate (q_E) and a 29.9% increase in the total organic removal rate (*k*). The survival of RBN after inoculation was determined by chromosomal marking with green fluorescent protein (*gfp*). *Sphingomonas* sp. strain RBN*gfp* persisted in a suspended floc reactor (4.3% of total CFU) for 14 d after inoculation and increased (10.8% of total CFU) in response to shock loading of the reactor with NSA (250 mg L^{-1}). The recovery time from shock loading of the reactor, was reduced compared to a matched reactor inoculated with plasmid–cured RBN*gfp*. The results suggest that the bioaugmentation of biological treatment plants with NSA-degrading isolates would be a promising route for the remediation of NSA-contaminated effluents. It is believed that this is the first report of the use of bioaugmentation of biological treatment processes for the remediation of effluents containing NSA.

Neither RBN nor BAS utilised the SNFC compound #22 as sole source of carbon and energy. Compound #22 was also not degraded cometabolically in the presence of NSA. Photochemical oxidation with Fenton reagent of compound #22 did not increase its biodegradability by RBN or its BOD_5 value. It is proposed that the continued recalcitrance of compound #22, and hence SNFC in effluents, was due to maintenance of the integrity of the polymeric structure, even after extensive photo-Fenton treatment. Depolymerisation of SNFC will therefore be a prerequisite to their biodegradation.

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of variance
BDW	Biomass dry weight
$BOD_{(5)}$	Biological oxygen demand $_{(5\ day)}$
CFU	Colony forming unit
COD	Chemical oxygen demand
CM	Carbon source medium
D	Dilution rate
gfp	Green fluorescent protein
HPLC	High pressure liquid chromatography
HRT	Hydraulic retention time
k	maximum organic removal rate
Km	kanamycin
K_s	Half saturation constant
LAS	Linear alkylbenzene sulphonate
μ	Specific growth rate
μ_{max}	Maximum specific growth rate
m	maintenance
MLSS	Mixed liquor suspended solids
MSM	Mineral salts medium
NSA	Naphthalene-2-sulphonate
OD_{600}	Optical density at 600 nm
PCR	Polymerase chain reaction
PSA	Phenol sulphonic acid
q_E	Specific substrate removal rate
r^2	Least squares regression coefficient
RT-PCR	Reverse transcriptase-PCR
S	Substrate concentration

SNFC	Sulphonated naphthalene-formaldehyde condensate
SPE	Solid phase extraction
SSCP	Single strand conformation polymorphism
ThOD	Theoretical oxygen demand
t_R	Retention time
U	Specific utilisation rate
Y	Yield
Y_E	Theoretical maximum yield
Y_S	Theoretical maximum yield accounting for maintenance

CHAPTER 1

INTRODUCTION

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1.1. Aromatic sulphonates: application and implication

Naturally produced and biologically degradable sulphonated organic compounds, such as taurine and cysteate, are typically aliphatic (Schwitzguébel *et al.*, 2002), and only a few naturally occurring aromatic sulphonates have been documented, e.g. aeruginosin B (Kerr, 2000). However, a wide range of aromatic sulphonates has been synthesised. These are used extensively in the synthesis of other chemical products. For example, benzene-, phenol- and naphthalene-sulphonates are utilised in the synthesis of high volume chemical products including dyes, surfactants, and optical brighteners (Riediker *et al.*, 2000a). It is estimated that the annual global production of linear alkylbenzenesulphonates for use in synthetic detergents is 2.4 million tonnes (de Almeida, 1994). The chemical structures of a number of aromatic sulphonates and their derivative compounds are shown in Figure 1.1.

The absence of naturally occurring analogues for aromatic sulphonates defines them as xenobiotic. Xenobiotic compounds may be defined as organic compounds that are not the product of biosynthesis (Alexander, 1994a). Their release into the environment may be deliberate or inadvertent as a result of their use in industry and agriculture. The greater the difference in the structure of the xenobiotic from naturally occurring compounds, the less the likelihood of its biodegradation. Therefore, these compounds have the potential to accumulate in the environment (Janssens *et al.*, 1997).

The presence of a sulphonate group in aromatic sulphonates molecules increases their solubility and dispersion properties (Leidner *et al.*, 1980) and renders them highly mobile in aquatic environments (Angelino *et al.*, 1999). It also confers a degree of resistance to biodegradation due to the thermodynamic stability of the carbon-sulphur bond (Leidner *et* al., 1980; Kertesz *et al.*1994a). Greim *et al.* (1994) examined the biodegradability of

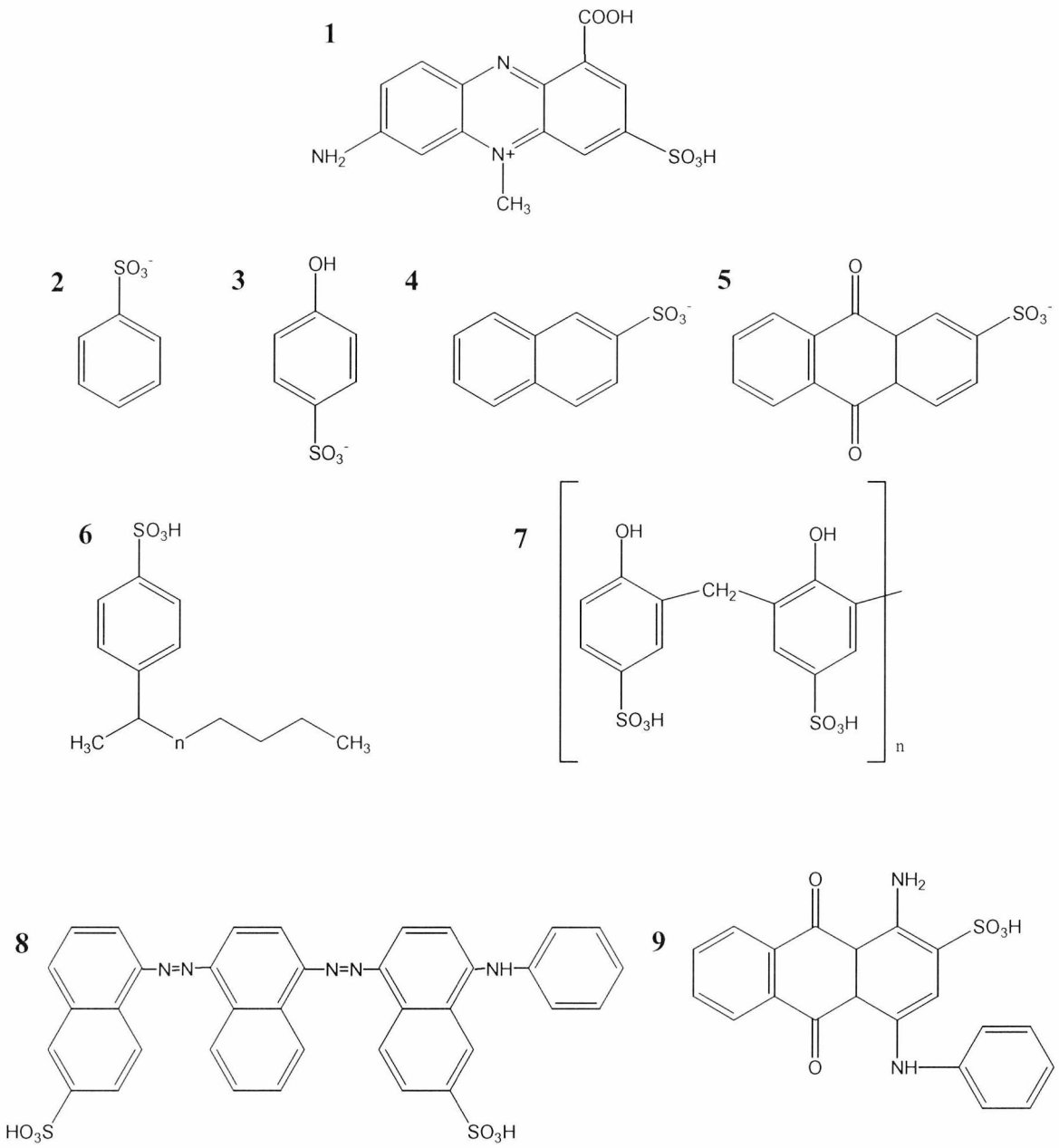

Figure 1.1. Structures of natural and synthetic aromatic sulphonates: 1 – aeruginosin B, a naturally occurring aromatic sulphonate; 2 – benzene sulphonate; 3 – phenol sulphonate; 4 – naphthalene-2-sulphonate; 5 – anthraquinone-2-sulphonate; 6 - linear alkylbenzene sulphonate (generic structure, n = 3 –7, Schwitzguebel *et al.*, 2002); 7 - phenol sulphonate-formaldehyde condensate polymer, used in synthetic tanning agents, optical brighteners etc. (M. Lancaster, personal communication); 8 - acid black 24, a naphthalene sulphonate-based dyestuff; 9 - acid blue 25, an anthraquinone sulphonate-based dye.

20 aromatic sulphonates and found that their biodegradability was highly variable and that many would be classified as recalcitrant. Due to their xenobiotic nature, mobility in aquatic environments and recalcitrance, aromatic sulphonates in industrial effluents are incompletely removed by conventional biological wastewater treatment. The effluents from biological wastewater treatment plants are considered to be a major source of aromatic sulphonates into the environment (Kölbener *et al.* 1994). The annual production of synthetic dyestuffs and pigments is in excess of 1 x 10^6 tonnes, of which approximately 280,000 tonnes are discharged per year into the environment in industrial effluents (Willets and Ashbolt, 2000). Additionally, benzene- and naphthalene sulphonates (Riediker *et al.*, 2000a), and *p*-toluene sulphonate have been detected in the leachates from landfill sites (Riediker *et al.*, 2000b). Naphthalene sulphonate has also been found in groundwater as a result of contamination of the site of a concrete factory (Crescenzi *et al.*, 2001).

The increasing discharge of these chemicals into the environment raises significant concerns for environmental regulatory bodies (Banat *et al.*, 1996). The European Community Directive on Integrated Pollution Prevention and Control (Anon, 1996) aims to control pollution arising from a number of industrial activities, such a chemical manufacture. The directive includes a list of main polluting substances for which emission limit values for aquatic discharge should be set, including persistent hydrocarbons. The Water Framework Directive (Anon, 2000) requires European member states to achieve 'good quality' water status by 2015, by reducing the emissions of polluting chemicals to the aquatic environment. The aim of this legislation is to reduce or eliminate pollution of the aquatic environment, arising from anthropogenic activity, i.e. to control the discharge of xenobiotic compounds, such as aromatic sulphonates.

1.2. Naphthalene sulphonates in the environment

One group of aromatic sulphonates, which are frequently found in the environment, are those based on naphthalene sulphonate. Naphthalene sulphonate and its substituted derivatives are of great importance in the manufacture of a number of industrial chemicals. Naphthalene intermediates represent 20% of the dye intermediates listed in

the Colour Index (Alonso & Barcélo, 1999). NSA is also utilised in the synthesis of synthetic tanning agents, plasticisers for concrete, optical brighteners and detergents (Alonso & Barcélo, 1999; Riediker *et al.*, 2000a).

Naphthalene sulphonates are characterised by a minimum of one sulphonate group and may also contain other substituents including hydroxyl-, methyl-, nitro- and amino groups (Riediker *et al.*, 2000a). They are highly polar molecules and will disassociate completely in water (Menzel *et al.*, 2002), and show a very low tendency to adsorb to organic material (Greim *et al.*, 1994). As such, naphthalene sulphonates would be expected to be highly mobile in aquatic environments.

Naphthalene sulphonates have been reported as contaminants in a number of aquatic environments, including landfill leachates, industrial effluents, the effluent from wastewater treatment plants, river water and seawater. Concentrations of between 7.7 and 170 μg L^{-1} of naphthalene sulphonate and four disulphonated naphthalene sulphonates were found in the leachate and in groundwater downstream, of a landfill site. Naphthalene-2-sulphonate (NSA) was found to be the most abundant contaminant at concentrations of up to 170 μg L^{-1} (Menzel *et al.*, 2002). Zerbinati *et al.* (1994) reported concentrations of naphthalene sulphonates of 76 – 1670 mg L^{-1} in a spring polluted by leachate from the waste dumps of a chemical plant manufacturing aromatic sulphonates. Naphthalene mono- and disulphonates, and 2-aminonaphthalene sulphonates were also detected in the leachates from four landfill sites and the groundwaters contaminated by leachates (Riediker *et al.*, 2000a).

Industrial effluents may also contain significant quantities of naphthalene sulphonates. Concentrations of 0.1 to 2.1 mg L^{-1} of mono- and disulphonated naphthalene sulphonates were detected in spent dyeing baths and the final effluent from a textile manufacturer (Storm *et al.*, 1999). Mono- and disulphonated naphthalene sulphonates were detected in the effluent from a tannery. The most abundant component, NSA, was present at a concentration of 30 mg L^{-1} (Z. Song, personal communication). Concentrations of NSA as high as 26.1 g L^{-1} were reported in an unspecified industrial effluent (Angelino *et al.*, 1999). Effluents from the tanning and cement industries were found to contain a number

of unsubstituted and substituted naphthalene mono- and disulphonates. The concentrations of these compounds ranged from 0.1 μg L^{-1} (1-amino-6-naphthalene sulphonate) to 1013.1 μg L^{-1} (NSA). These compounds were found to persist after primary and biological treatment and were, therefore, present in the final effluent discharged into the environment (Fichtner *et al.*, 1995; Alonso *et al.*, 1999; Castillo *et al.*, 2001; Farré *et al.*, 2002).

The mobility of naphthalene sulphonates in leachates, and their incomplete removal from industrial wastewaters by wastewater treatment systems means that these compounds are found in ground and surface waters. NSA has been detected in rivers in industrialised regions at concentrations ranging from ng L^{-1} to mg L^{-1} (Zerbinati, *et al.*, 1997; Kok *et al.*, 1998; Pocurull *et al.*, 1999; Liu & Ding, 2001). NSA was identified in all the samples of seawater taken from coastal water near a commercial port, at concentrations between 0.14 μg L^{-1} and 0.5 μg L^{-1} (Pocurull *et al.*, 1999; Gimeno *et* al., 2001; Alonso *et al.*, 2002). These compounds may ultimately be found in potable waters supplies. NSA was detected in the Italian rivers, Po and Sangone, at concentrations of between 8.9 and 220 ng L^{-1}. Concentrations of NSA of 6 – 21 ng L^{-1} were also detected in tap water abstracted from the river Po (Zerbinati *et al.,* 1999).

Due to their low toxicity and ecotoxicity, naphthalene sulphonates are not regarded as priority pollutants. However, their poor degradability and subsequent persistence in aquatic environments means that they may represent an ecotoxicological hazard (Riediker *et* al., 2000a). The increasing stringency of environmental legislation regarding the discharge of industrial effluents, may also lead to remediation of naphthalene sulphonate compounds assuming greater importance in the future (Banat *et al.*, 1996; Schwitzguébel *et al.*, 2002).

Greim *et al.* (1994) examined the toxicity, ecotoxicity and biodegradability of a number of naphthalene sulphonates. The data are shown in Table 1.1 and it can be seen that the naphthalene sulphonates displayed low mammalian toxicity and ecotoxicity. The majority of these compounds were also found to be poorly biodegradable or entirely recalcitrant, under the given test conditions. In contrast to the majority of the compounds

Name	Acute toxicity Rat LD_{50} (mg kg^{-1} body weight)	Ecotoxicity at different trophic levels (all values mg L^{-1})				Degradability
		Bacteria	Algae	Daphnia	Fish	
2-amino-1-naphthalene sulphonic acid	> 5000	EC_{50} > 10000	no data	no data	LC_0 96 h – 5000 LC_{100} 96 h - 7071	no degradation – OECD test
7-amino-4-hydroxy-2-naphthalene sulphonic acid	> 5000	EC_0 24 h – 1000	no data	no data	LC_0 96 h - 1000	no degradation – closed bottle test
6-amino-4-hydroxy-2-naphthalene sulphonic acid	> 5000	EC_0 24 h – 500	no data	no data	LC_0 48 h - 1000	no degradation – closed bottle test
6-amino-1,3-naphthalene disulphonic acid	2000	EC_0 24 h – 1000	no data	no data	LC_0 96 h - 1000	no degradation – BOD_{30} test
7-hydroxy-1,3-naphthalene disulphonic acid	> 5000	EC_0 24 h –10000	no data	no data	LC_0 72 h - 1000	no degradation – closed bottle test
4-amino-5-hydroxy-2,7-naphthalene disulphonic acid	> 5000	EC_0 24 h –1000	no data	no data	LC_0 96 h - 1000	< 10 % degradation- closed bottle test
Naphthalene sulphonic acid	1400	EC_{50} 17 h –1000 EC_{50} – 133	EC_{10} 96 h – 73.3 EC_{50} 96 h – 54.3	EC_{50} 24 h – 85 EC_{50} 48 h – 34	LC_{50} 96 h – 100 - 500	> 60 % (moderate) degradation – OECD test

Table 1.1. Mammalian toxicity, ecotoxicity and biodegradability values of a number of unsubstituted and substituted naphthalene mono- and disulphonates (reproduced from Greim *et al.*, 1994).

examined, unsubstituted naphthalene sulphonic acids, napthalene-1-sulphonic acid and naphthalene-2-sulphonic acid, were moderately (>60%) biodegradable. These compounds were also found to have a greater potential for ecotoxic effects, albeit at concentrations in excess of those at which they have been reported to occur in the environment.

However, the presence of naphthalene-1-sulphonic acid or naphthalene-2-sulphonic acid at concentrations of greater than 100 μg L^{-1} have also been reported to synergistically increase the toxicity of other environmental pollutants such as linear alkylbenzene sulphonates and nonionic polyethoxylated surfactants (Castillo & Barceló, 1999; Castillo *et al.,* 2001). Naphthalene sulphonates have also been described as having acute toxic effects on bacteria at concentrations of approximately 30 mg L^{-1} (Castillo *et al.,* 1999). Therefore, it is possible that shock loads of these compounds in industrial effluents could adversely affect the activity of biological wastewater treatment plants.

Despite showing the highest degree of biodegradability of the naphthalene sulphonates, NSA was also found to be the most abundant naphthalene sulphonate in the previously described aquatic environments, and to be incompletely removed by biological treatment (Alonso & Barcélo, 1999; Zerbinati *et al.*, 1999; Loos *et al.*, 2000; Riediker *et al.*, 2000a; Gimeno *et al.*, 2001; Castillo *et al.*, 2001; Farré *et al.*,2002; Menzel *et al.*, 2002; Z. Song, personal communication). As such, the prevalence of NSA in the environment, and its potential for direct and indirect toxic effects would suggest that NSA would be the priority for remediation strategies directed at the naphthalene sulphonates.

1.3. Naphthalene sulphonate-formaldehyde condensates

NSA is used extensively in the synthesis of condensation polymers with formaldehyde (Figure 1.2). These sulphonated naphthalene-formaldehyde condensates (SNFC) have a number of industrial applications such as plasticisers for the production of high performance concretes (Piotte *et* al., 1995), and as synthetic tanning agents (syntans) in the manufacture of leather (Reemtsma & Jekel, 1994). The functionality of these compounds is thought to be dependent upon the degree of polymerisation (Piotte *et al.*,

1995). Polymerisation of up to 90-mer has been reported (Miller, 1985). SNFC are included in the OECD list of high production chemicals with approximately 300,000 tonnes per annum used as concrete additives and dispersants for dye stuffs (Crescenzi *et al.*, 2001). All of these products contain NSA monomers as an impurity (Ash & Ash, 1991). Therefore, these compounds represent an important source of NSA in the environment (Menzel *et al.*, 2002).

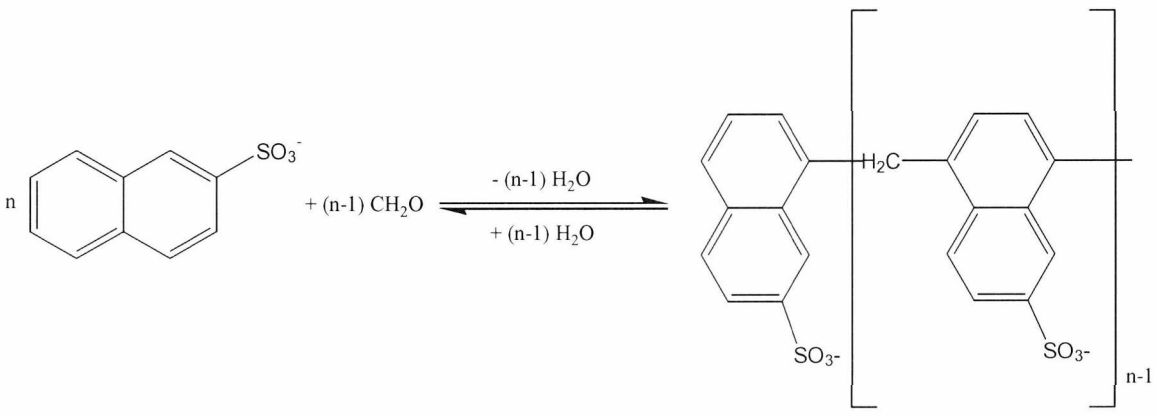

Figure 1.2. The synthesis of SNFC. The reaction is a 'one pot' reaction, with sulphonation of naphthalene with concentrated sulphuric acid at 90 – 160 °C and subsequently, condensation effected with formaldehyde at 30 – 70 °C in acid or alkaline media.

In common with the NSA precursor, SNFC are highly water soluble and mobile in aquatic environments. These compounds have also been reported as environmental contaminants. Little is known about the constitution of SNFC, particularly with regard to the degree of polymerisation in individual products (Crescenzi *et al.*, 2001) and their analysis in environmental samples is hampered by an absence of reference materials (Redín *et al.*, 1999; Crescenzi *et al.*, 2001). Redín *et al.* (1999) synthesised three SNFC dimers and peaks with comparable UV-profiles and retention times to the synthesised dimers were found in a proprietary SNFC-based syntan. The synthesised dimers were then used as reference compounds for the detection and quantification of SNFC dimers in environmental samples. SNFC dimers were detected in industrial effluents from a tannery, a paper manufacturer and a manufacturer of SNFC, public sewage and samples from two rivers. The concentrations of SNFC detected ranged from 20 ng L^{-1} in river water samples to 110 μg L^{-1} in the effluent from the manufacturer of SNFC.

SNFC were also detected and quantified in environmental samples with HPLC-MS by extracting the ion chromatographic profile from the total chromatographic profile of the sample and using a synthesised SNFC dimer as a reference compound. Using this method, SNFC dimers and trimers were detected in river water samples (10 – 180 ng L^{-1} total SNFC) and the effluent from a concrete factory (7.5 μg L^{-1} total SNFC). SNFC dimers were found in a well and in groundwater (73 ng L^{-1} total SNFC) near the concrete factory, demonstrating the mobility of these compounds and their potential for contamination of potable water supplies (Crescenzi *et al.*, 2001).

Similarly, SNFC of 4-mer or less were detected in groundwater (10.85 μg L^{-1} total SNFC) contaminated by leachate from concrete used in the construction of a tunnel (Ruckstuhl *et al.*, 2002). SNFC dimers were also identified in groundwater contaminated by leachate from a landfill site (Menzel *et al.*, 2002). Significant concentrations of SNFC-based syntans have also been detected in industrial effluents. Syntan concentrations of 41 mg L^{-1} and 34 mg L^{-1} were detected in untreated effluent and effluent subjected to combined anaerobic and aerobic biological treatment, respectively (Reemtsma & Jekel, 1994). SNFC dimers were also detected in a tannery effluent at a concentration equivalent to that measured in a 100 mg L^{-1} solution of a proprietary syntan, indicating a significant concentration of SNFC-based syntan contamination in the effluent (Z. Song, personal communication).

The concentrations of SNFC detected varied considerably with the type of sample analysed. In those samples where the size of the SNFC molecules were determined, the oligomer size did not exceed 4-mer, suggesting that the larger oligomers were adsorbed and thus, rendered immobile (Redín *et al.*, 1999; Crescenzi *et al.*, 2001; Menzel *et al.*, 2002; Ruckstuhl *et al.*, 2002; Z. Song, personal communication). However, short SNFC oligomers have been shown to be highly mobile in aquatic environments and this, coupled with their potential to contaminate drinking water sources, has led to increasing concerns regarding the fate and quantity of SNFC in the environment (Crescenzi *et al.*, 2001; Ruckstuhl *et al.*, 2002).

1.4. Aerobic bacterial transformation of aromatic sulphonates

Bacterial isolates and consortia capable of degrading aromatic sulphonates have been isolated. *Pseudomonas testosteroni* T2 was reported to completely mineralise *p*-toluene sulphonate (Locher *et al.*, 1989) and a four-member consortium, capable of mineralising LAS, was isolated from activated sludge (Jiménez *et al.*, 1991). Bacterial communities capable of degrading aminonaphthalene-2-sulphonates and hydroxynaphthalene sulphonates have been isolated from the industrially polluted river Rhine (Nortemann *et al.*, 1986). Isolation of a number of bacterial strains capable of degrading NSA has also been reported (Brilon *et al.*, 1981a; Nortemann *et al.*, 1986; Ohe *et al.*, 1990; Balashov & Boronin, 1996).

Aerobic transformations of aromatic sulphonates are formalised into two routes: i) utilisation as a source of carbon with excretion of the sulphonate moiety, with degradation largely regulated by an inducible catabolic pathway and independent of a supply of sulphur for growth; or ii) utilisation as a source of sulphur with excretion of the carbon moiety, and regulated by sulphur scavenging systems (Cook *et al.*, 1998). Bacterial utilisation of aromatic sulphonates as a source of carbon is characterised by a narrow substrate spectrum (Balashov & Boronin, 1996; King & Quinn, 1997).

Aerobic desulphonation of aromatic sulphonates can take place before ring cleavage, during ring cleavage or after ring cleavage. Desulphonation before ring cleavage occurs via oxygenation of the aromatic ring by a multimeric dioxygenase, giving rise to an unstable cis-diol which undergoes spontaneous rearomatisation with concurrent elimination of sulphonate as sulphite (Figure 1.3 a; Locher *et al.*, 1991). Desulphonation during ring cleavage has been reported during the degradation by *Alcaligenes* sp. O-1 of 2-aminobenzenesulphonic acid as a source of carbon. The reaction was initiated by a multimeric dioxygenase with ring cleavage thought to occur due to the spontaneous decay of an unstable sulphonated intermediate (Figure 1.3 b; Junker *et al.*, 1994). Aerobic desulphonation after ring cleavage is also known. A syntropic culture of *Hydrogenophaga palleroni* S1 and *Agrobacterium radiobacter* S2 was found to deaminate 4-aminobenzenesulphonic acid, supplied as a source of carbon, nitrogen and

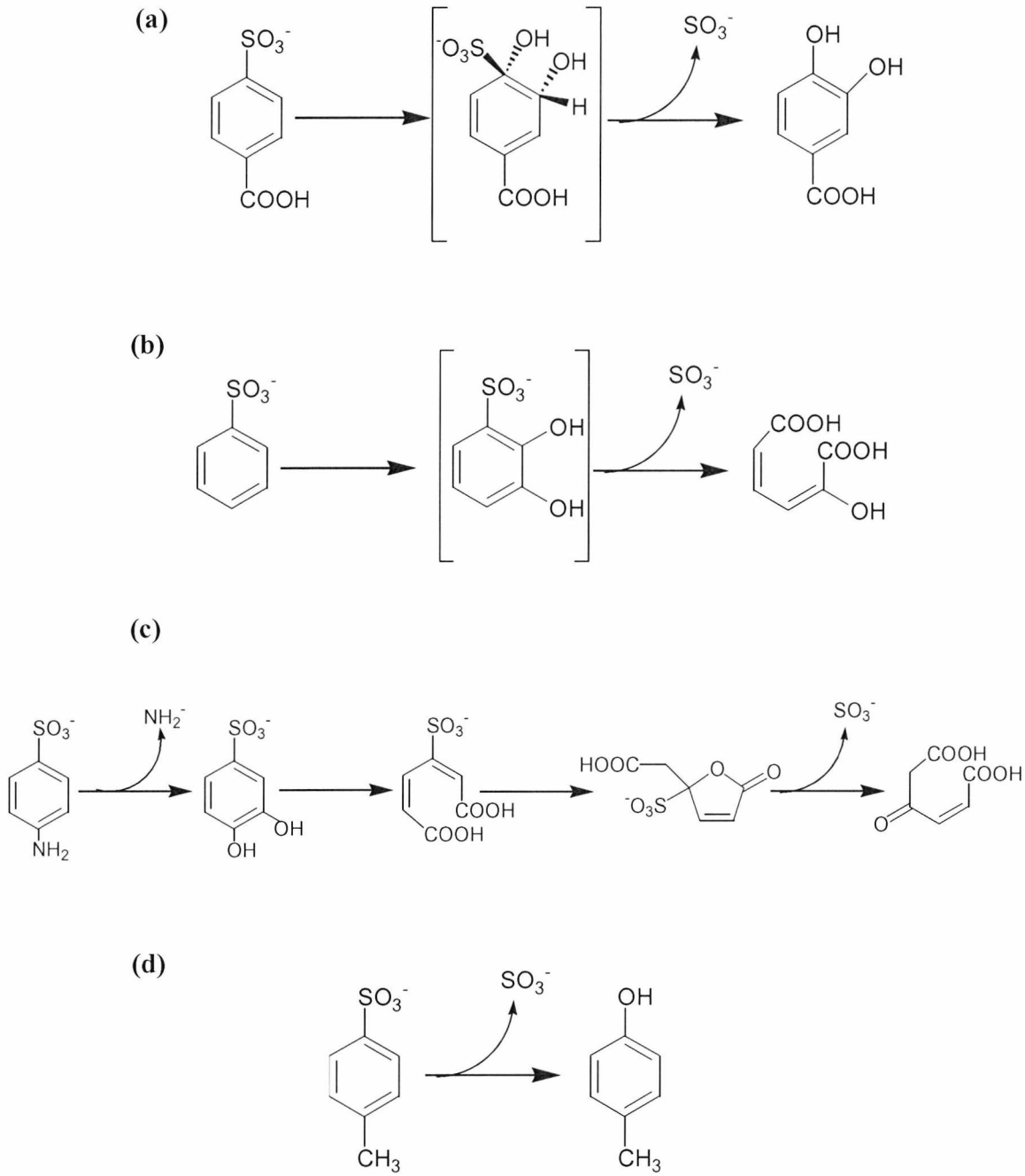

Figure 1.3. Four pathways for the desulphonation of aromatic sulphonates. Desulphonation may occur prior to ring cleavage ((a) and (d)), concurrent with ring cleavage (b), or after opening of the aromatic ring (c). Pathways (a) and (b) were observed when the sulphonate was supplied as a source of carbon, pathway (d) when it was supplied as a source of sulphur, and pathway (c) when supplied as a source of carbon, nitrogen and sulphur.

energy, with subsequent ring cleavage prior to desulphonation (Figure 1.3 c; Fiegel & Knackmuss, 1993). This pathway has also been reported in a mixed culture degrading benzene-1,3-disulphonate (Contzen *et al.*, 1996) and in the degradation of 4-carboxy-4'-sulphoazobenzene, a model sulphonated azo compound (Blümel *et al.*, 1997).

Typically, degradation of aromatic sulphonates is initiated by desulphonation before ring cleavage by a ring-hydroxylating dioxygenase and a huge diversity of these enzymes has been detected in both pristine and contaminated environments (Yeates *et al.*, 2000). Degradation of aromatic sulphonates without initial desulphonation has been reported to result in intermediates that inhibit bacterial growth (Kulla *et al.*, 1983).

1.5. Aerobic bacterial transformation of NSA

The catabolic pathway for the degradation of NSA is thought to be analogous to the catabolic pathway for the degradation of naphthalene. Degradation of NSA by *Pseudomonas* A3 and C22 was induced by pre-exposure to, and inhibited by the presence of, naphthalene, indicating that the degradation of NSA occurred through the same catabolic pathway as naphthalene. Degradation of NSA also led to an accumulation of gentisate and salicylate, the metabolic end products of the upper pathway of naphthalene degradation (Brilon *et al.*, 1981b). Degradation of 6-aminonaphthalene-2-sulphonic acid (6-ANS) to 6-aminosalicylate by *Pseudomonas* sp. BN6, was induced and reversibly inhibited by NSA, indicating a common pathway for the degradation of both compounds (Nörtemann *et al.*, 1986). Utilisation of NSA and naphthalene by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22, and 6-ANS and NSA by *Pseudomonas* sp. BN6, as growth substrates suggested that the dioxygenase initiating degradation had a relaxed substrate specificity (Brilon *et al.*, 1981b) and that both compounds were degraded through a common pathway. As such, the degradation of NSA via the catabolic pathway for naphthalene and initiated by naphthalene dioxygenase seems a valid hypothesis.

The catabolic pathway for naphthalene has been determined in *P. putida* G7. It is divided into upper and lower pathways and arranged in two polycistronic operons, designated

nah (upper) and *sal* (lower), on plasmid NAH7. The *nah* pathway encodes the catabolism of naphthalene to salicylate and pyruvate, and the *sal* pathway encodes the transformation of salicylates to catechol or gentisate and subsequent species dependent *ortho* or *meta* cleavage (Williams and Sayers, 1994). Expression of the two operons is regulated by a *nah*R, the activity of which is dependent upon the intracellular concentration of salicylate (Schell, 1985).

The initial step of the upper pathway is catalysed by naphthalene dioxygenase (NDO). NDO is a multimeric enzyme consisting of a reductase (encoded by *nah*Aa), a ferredoxin (*nah*Ab) and two sub-units of iron-sulphur protein (*nah*Ac and *nah*Ad). NDO catalyses the conversion of naphthalene to *cis*-naphthalene dihydrodiol. Further reactions involve a dehydrogenase (*nah*B), a dioxygenase (*nah*C), an isomerase (*nah*D), a hydratase-aldase (*nah*E) and a dehydrogenase (*nah*F), leading to the synthesis of salicylate and pyruvate (Eaton and Chapman, 1992; Eaton, 1994). Salicylate is converted to catechol and then catalysed via the *sal*-encoded lower pathway to pyruvate, and subsequently converted to acetyl-CoA by the multienzyme pyruvic dehydrogenase complex and passed into the tricarboxylic acid cycle (Dagher *et al.*, 1997; Conn & Stumpf, 1972). Alternatively, instead of catechol, salicylate may be converted to gentisate by salicylate 5-hydroxylase (Brilon *et al.*, 1981; Fuenmayor *et al.*, 1998). A schematic overview of the upper pathway is shown in Figure 1.4.

NDO catalyses the hydroxylation of naphthalene to *cis*-naphthalene dihydrodiol by the addition of both atoms of molecular oxygen to one of the aromatic rings in presence of NADH or NADPH (Figure 1.5). The enzyme is organised into two sub-units, α (encoded by *nah*Aa, -Ab and -Ac) and β (*nah*Ad). The α subunits contains a Rieske [2Fe-2S] cluster and non-heme iron (II) (Kauppi *et al.*, 1998). Electrons are transferred from NAD(P)H by the reductase and ferredoxin to the Rieske center of an α subunit of the oxygenase (Haigler & Gibson, 1990a; Haigler & Gibson, 1990b). The reduced Rieske center then transfers an electron to the mononuclear iron at the active site in the adjacent α subunit leading to oxidation of the aromatic nucleus (Kauppi *et al.*, 1998).

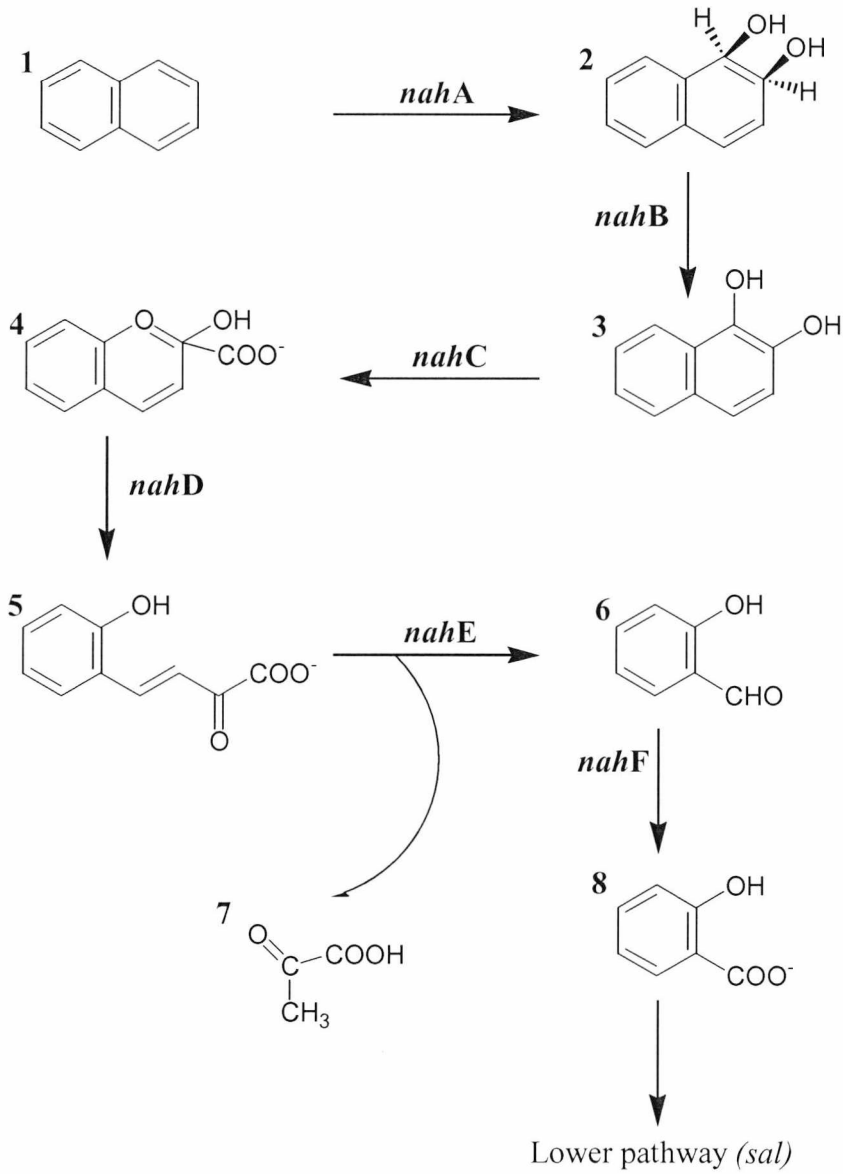

Figure 1.4. Schematic representation of the upper pathway for the catabolism of naphthalene. The metabolites are naphthalene (1), *cis*-naphthalene dihydrodiol (2), 1,2-dihydroxynaphthalene (3), 2-hydroxychromene-2-carboxylate (4), *cis-o*-hydroxybenzalpyruvate (5), salicylaldehyde (6), pyruvate, (7), salicylate (8). The enzymes are: *nah*A, naphthalene dioxygenase; *nah*B, naphthalene *cis*-dihydrodiol dehydrogenase; *nah*C, 1,2-dihydroxynaphthalene dioxygenase; *nah*D, 2-hydroxychromene-2-carboxylate dehydrogenase; *nah*E, 1,2-dihydroxybenzylpyruvate aldolase; *nah*F, salicylaldehyde dehydrogenase. Adapted from Bosch *et al.* (1999).

PAH-degrading genes characterised in other isolates are also organised in polycistronic operons, such as the *nah* operon on the PDTG1 plasmid of *P. putida* NCIB9816-4 (Harayama and Rekik, 1989; Simon *et al.*, 1993; Eaton, 1994), the *pah* operon in *P. putida* OUS82 (Takizawa *et al.*, 1994) and the *dox* operon in *Pseudomonas* sp strain C18 (Denome *et al.*, 1993). The nucleotide and amino acid sequences of the genes examined in the *nah, pah* and *dox* operons were found to be highly homologous.

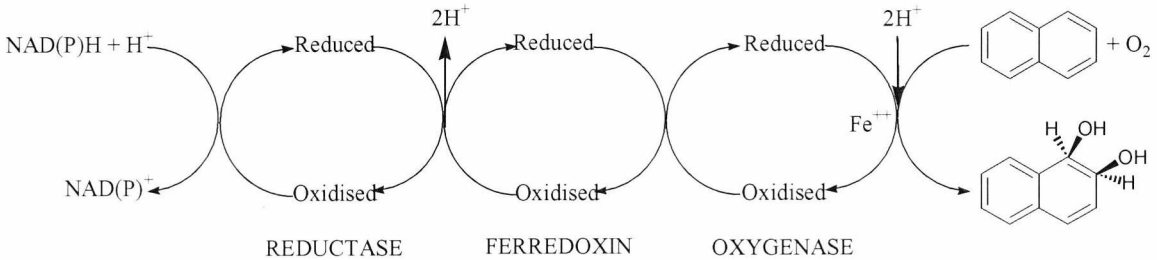

Figure 1.5. Oxidation of naphthalene to *cis*-naphthalene dihydrodiol by naphthalene dioxygenase. Electrons are transferred from NAD(P)H via a reductase (*nah*Aa) and a ferredoxin (*nah*Ab) to an oxygenase (*nah*Ac and *nah*Ad) which then catalyses the addition of molecular oxygen to the aromatic nucleus.

The genes encoding for the degradation of PAH by five environmental isolates were found to show 90 – 95% homology with identified *nah, pah* and *dox* sequences (Dagher *et al*, 1997). Similarly, genes for the degradation of PAH were detected in 17 of 20 PAH degrading isolates, using degenerate primers based on the sequence of *ndo*B (Hamann *et al.*, 1999). High degrees of similarity between analogous components encoding for the catabolism of naphthalene from different bacterial strains have been reported, indicating a common ancestor for these genes (Denome *et al.*, 1993, Suen & Gibson, 1993, Eaton, 1994, Takizawa *et al.*, 1994). More specifically, the α subunits of all Rieske non-heme iron oxygenases, such as those encoded for by *nah*Ac, have been shown to be related to each other. Four dioxygenase families, naphthalene, toluene/benzene, biphenyl and benzoate/toluene, were identified on the basis of amino acid sequencing of the oxygenase α subunits (Werlen *et al.*, 1996).

However, while dioxygenases show a high degree of homology, small changes in the α subunit have been reported to give rise to remarkable differences in substrate specificities

of those dioxygenases (Furukawa *et al.*, 1993, Gibson *et al.*, 1993). The α and β subunits of the biphenyl dioxygenases of strains KF707 and LB400 were similar, with only 20 and 1 amino acid differences, respectively, yet the enzymes were found to have very different specificities for polychlorinated biphenyl congeners.

It has been proposed that the initial dioxygenation of aromatic sulphonates will be mediated by unique aromatic sulphonate dioxygenases. *Pseudomonas (Comamonas) testosteroni* strain T-2 was reported as being able to degrade toluene sulphonate but not toluene (Locher *et al.*, 1989). The degradation of 6-ANS by *Pseudomonas* sp. BN6 was not induced by naphthalene, but was reversibly inhibited by NSA (Nörtemann *et al.*, 1986). Similarly, NSA-induced cells of *Pseudomonas* sp. BN6 were unable to degrade naphthalene (Kuhm *et al.*, 1991). The terminal amino acid sequence of the initial enzyme in the catabolism of NSA by *Pseudomonas* sp. BN6 was found to have only a 48% homology with the naphthalene dioxygenase from *P. putida* NAH7. The activity of enzyme was also dependent on an acidic functional group at the C2 position.

As such, the initial step in the degradation of NSA was suggested to be mediated by a unique NSA-dioxygenase. This enzyme oxygenates the aromatic ring, giving rise to unstable *cis*-dihydrodiol which then undergoes spontaneous rearomatisation to 1,2,-dihydronaphthalene with concurrent elimination of sulphonate as sulphite (Figure 1.6). 1,2,-Dihydronaphthalene would then be further catalysed through the naphthalene pathway (Figure 1.4; Kuhm *et al.*, 1991). While it did not degrade naphthalene, the degradation of NSA by *Pseudomonas* sp. BN6 was inhibited by naphthalene suggesting an affinity of the initial enzyme for this compound. *Pseudomonas* sp. BN6 was also found not to synthesise naphthalene *cis*-dihydrodiol dehydrogenase. This enzyme would be redundant in the proposed catabolic pathway as the formation of *cis*-naphthalene dihydrodiol is circumvented due to the spontaneous liberation of sulphite after the initial dioxygenation of NSA.

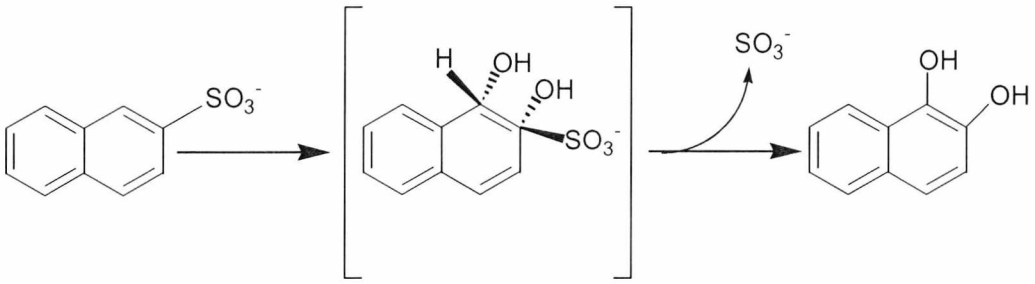

Figure 1.6. Proposed pathway for the initial dioxygenation of NSA. Dioxygenation of the sulphonated aromatic ring leads to the formation of an unstable cis-naphthalene dihydrodiol, followed by spontaneous rearomatisation to 1,2-dihydronaphthalene with release of sulphonate as sulphite.

1.6. Aerobic utilisation of aromatic sulphonates as a source of sulphur

Whereas bacteria usually organise catabolic enzymes in inducible operons, the utilisation of sulphur is regulated at the global level (Kertesz *et al.*, 1994a). Under sulphate-starvation conditions, many bacteria produce sulphate starvation induced (SSI) proteins (Kertesz *et al.*, 1993). Thirteen proteins, expressed during sulphate-starvation, were isolated from *Pseudomonas aeruginosa* PAO1 and included binding proteins, with affinity for sulphate and sulphonates, and enzymes involved in sulphonate metabolism (Quadroni *et al.*, 1999). Expression of these proteins facilitates scavenging of sulphur from non-sulphate sources. In contrast to their utilisation as a source of carbon, the utilisation of aromatic sulphonates is characterised by a wide spectrum of substrate utilisation. Under sulphur-limited conditions, *Pseudmonas putida* S-313 was found to desulphonate several hundred compounds (Zürrer *et al.*, 1987; Kertesz *et al.*, 1994b). Similar results have been reported by others (King & Quinn, 1997).

Desulphonation of aromatic sulphonates, as a source of sulphur, is catalysed by a monooxygenase, yielding the corresponding phenol (Zürrer *et al.*, 1987, Figure 1.3 d). Zürrer *et al.* (1987) isolated eight environmental isolates able to utilise a minimum of 16 different aromatic sulphonates (including NSA, aminonaphthalene sulphonates and naphthalene disulphonates) as a source of sulphur under sulphur-limited conditions. Desulphonation proceeded by oxygenolytic cleavage of the C-S bond, with release of the

sulphonate moiety and formation of the corresponding phenol. None of the isolates could utilise the compounds as a source of carbon. Similarly, *Comamonas acidovorans* I91 utilised *p*-toluene sulphonate as sole source of sulphur, under sulphur-limited conditions, but not as a source of carbon (Seitz *et al.*, 1993). Ruff e*t al.* (1999) isolated cultures capable of desulphonating 14 aromatic sulphonates, as a source of sulphur, to the corresponding phenol. The green alga, *Scenedesmus obliquus*, also utilised naphthalene-1-sulphonate as a source of sulphur under sulphur-limited conditions, resulting in the formation of 1-naphthol (Soeder *et al.*, 1987; Kniefel *et al.*, 1997).

However, sulphate has been shown to be used in preference to sulphonate as a source of sulphur (Seitz *et al.*, 1993, Uria-Nickelsen *et al.*, 1994, Chien *et al.*, 1999). When grown with one of cysteine or thiocyanate (group 1) and one of 4-toluene sulphonate, 4-nitrocatecholsulphate or ethanesulphonic acid (group 2), *Pseudomonas putida* S-313 was found to utilise group 1 before group 2. Therefore, the expression of SSI proteins would be repressed in the presence of inorganic or sulphate or simple organosulphur compounds (Beil *et al.*, 1996). As such, it would seem unlikely that desulphonation of aromatic sulphonates, as a source of sulphur, would represent a significant route for the degradation of these compounds in the environment.

1.7. Anaerobic utilisation of NSA

An isolate from anaerobic digester sludge was found to be capable of anaerobic desulphonation of 4-tolylsulphonate, *p*-sulphobenzoate, naphthalene-2,6-disulphonate and the dye, acid red 1, when supplied as sole source of sulphur (Denger *et al.*, 1996). However, utilisation of NSA by anaerobic bacteria only occurred when supplied as sole source of sulphur (Chien *et al.*, 1995). No biological transformation of NSA would be expected to occur under anaerobic conditions in waters that contain sufficient bioavailable sulphur (Cook *et al.*, 1998), and the detection and persistence of aromatic sulphonates, including NSA, in anaerobic environments such as landfill leachates, demonstrates that these compounds would be recalcitrant to microbial degradation in environmental anaerobic conditions (Giger *et al.*, 1989; Kim *et al.*, 1990; Menzel *et al*, 2002).

Utilisation of organosulphonates as terminal electron acceptors in anaerobic respiration has been reported. The marine sediment isolate, *Desulfovibrio desulfuricans* IC1, utilised the sulphonate moiety of isothionate as terminal electron acceptor, while the isothionate carbon moiety acted as an electron donor, leading to the reduction of the sulphonate sulphur to sulphide (Lie *et al.*, 1996). However, aromatic sulphonates do not appear to be utilised in this manner (Laue *et al.*, 1997). Evaluation of the use of sulphonates as electron donors in anaerobic respiration indicated that only a very narrow range of naturally occurring alkylsulphonates, e.g. taurine, could be utilised (Denger *et al.*, 1997). On the basis of the available evidence, anaerobic degradation of NSA would not represent a significant route for the elimination of NSA and other aromatic sulphonates from the environment.

1.8. Biological degradation of naphthalene sulphonate formaldehyde condensates (SNFC)

Despite the extensive use of SNFC in industry, very little is known about their distribution and behaviour in the environment. The detection of these compounds in environmental samples has only recently been made possible with the development of reliable and sensitive analytical equipment and methods for polar organic polymers (Menzel *et al.*, 2002). As previously discussed (section 1.3), SNFC have been detected in industrial effluents (Redín *et al.*, 1999), landfill leachates and aquifers (Menzel *et al.*, 2002), river water and drinking water sources (Crescenzi *et al.*, 2001). The potential for short SNFC oligomers to pollute water sources that are abstracted for human consumption, gives cause for concern about the persistence of these compounds in the aquatic environment.

Little is known about the potential for the biodegradation of SNFC. SNFC, used as syntans in the manufacture of leather, were found to persist in tannery effluent subjected to combined anaerobic and aerobic biological treatment (Reemstma & Jekel, 1994). SNFC leached from concrete during the construction of a tunnel, were found to persist in groundwater, even when monomeric naphthalene sulphonates were biologically degraded. Similarly, biodegradation experiments, carried out using material from a

piezometer used to sample the same groundwater site and an activated sludge adapted to SNFC, did not measure any degradation of SNFC. In contrast, monomeric naphthalene sulphonates were completely degraded (Ruckstuhl *et al.*, 2002). The available evidence suggests that SNFC would be undegradable in soil and aquatic environments.

1.9. Bioaugmentation for the bioremediation of aromatic pollutants

Although bacterial isolates with the metabolic capacity for the degradation of aromatic sulphonates have been isolated from environmental samples (Brilon *et al.*, 1981; Nortemann *et al.*, 1986; Locher *et al.*, 1989; Ohe *et al.*, 1990; Jiménez *et al.*, 1991; Balashov & Boronin, 1996), these compounds are still found as pollutants (section 1.2). Biological removal of aromatic pollutants in the environment may proceed relatively slowly due to insufficient degradation capacity and slow adaptation of the indigenous microorganisms to a xenobiotic substrate (Tchelet *et al.*, 1999).

The degradation of pollutants may be facilitated by: the stimulation of the metabolically competent indigenous population following the addition of nitrogen, potassium, phosphate or organic carbon; altering the environmental pH; or aeration or irrigation (Burns and Stach, 2002). Biostimulation of indigenous microorganisms, by the addition inorganic nutrients, resulted in increased degradation of oil in microcosm experiments simulating a contaminated beach (Röling *et al.*, 2002). However, the success of biostimulation is dependent upon the presence of indigenous bacteria with the appropriate catabolic faculty. Biostimulation of indigenous microorganisms in diesel-contaminated soil did not increase either the rate or extent of degradation of the contaminant diesel (Seklemova *et al.*, 2001). Additionally, biostimulation was found to dramatically reduce the microbial diversity of the treated environment (Röling *et al.*, 2002). Speculatively, in addition to not stimulating degradation of the target xenobiotic, targeted biostimulation of an effluent treatment plant may decrease microbial diversity and compromise the efficiency with which the total organic content of the effluent is removed.

Bioaugmentation (i.e. the inoculation of an environment with a specifically enriched microorganism(s), to facilitate or increase the degradation of a target compound) may enhance the degradation of pollutants such as aromatic sulphonates (Megharaj *et al.*, 1997; Selvaratnam *et al.*, 1997; Watanabe *et al.*, 1998). Bioaugmentation provides a mechanism to reduce the acclimation period of competent indigenous microorganisms or, where competent indigenous organisms are absent, to introduce the desired catabolic activity into the contaminated environment (Alexander, 1994b).

Several reports have been published describing successful studies of bioaugmentation of biotreatment plants. Bioaugmentation with the dehydroabietic acid (DhA)-degrading isolate, *Zooglea resiniphilia* DhA-35, restored DhA removal in high and low pH–stressed biomass of aerated lagoons treating paper mill effluent. *Zooglea resiniphilia* DhA-35 was found to persist in the indigenous biomass and showed a 100-fold increase in cellular rRNA : rDNA ratio, indicating growth and metabolic activity, during DhA removal (Yu & Mohn, 2002). Inoculation of reactors, fed peptone and phenol, with two phenol-degrading organisms shortened the start-up period for phenol removal (Watanabe *et al.*, 1996). Inoculation of membrane separation reactors (MBR) with 3-chlorobenzoate (3CBA)-degrading *Pseudomonas putida* BN210 increased the resistance of the MBR to shock-loading with 3CBA (Ghyoot *et al.*, 2000). Addition of the 2-chlorophenol-degrading isolate (2-CP), *Pseudomonas putida* CP1, to a commercial mixed culture enhanced the degradation of 2-chlorophenol. The augmented mixed culture degraded 2-CP via an *ortho*-cleavage pathway, whereas the non-augmented culture utilised a *meta*-cleavage pathway, resulting in the accumulation of undegradable dead-end products (Farrell & Quilty, 2002). Inoculation of granular activated carbon (GAC) columns treating s-triazine herbicide contaminated water with the s-triazine-degrading isolates, *Rhodococcus rhodochrous* strain SL1 and *Acinetobacter junii* strain WT1, reduced the concentration of s-triazines in the column effluent during start up and prior to adaptation of the indigenous biomass, and significantly increased the biodegradation of the s-triazines herbicides adsorbed to the GAC columns compared with uninoculated columns (Feakin *et al.*, 1995).

Bioaugmentation to facilitate the degradation of aromatic sulphonates has also been reported. Increasing rates of *p*-toluene sulphonate (*p*TS) removal were measured with increasing inoculum size when a *p*TS-degrading isolate, *Comamonas testosteroni* T-2, was added to domestic activated sludge (Bokhamy *et al.*, 1997). Inoculation of an aerobic reactor with a sulphanilic acid-degrading culture allowed for the complete biodegradation of Mordant Yellow 10, an azo dye, by combined anaerobic and aerobic biological treatment (Tan *et al.*, 2000).

A number of properties desirable in an isolate which may be utilised in bioaugmentation strategies (as appropriate to the inoculated environment), have been proposed (Burns & Stach, 2002) and include:

- soil, biofilm and rhizosphere 'competence' genes
- does not disrupt beneficial microbial processes
- can degrade the target pollutant at both high and low concentrations
- is resistant to heavy metal pollutants
- increases bioavailability by producing surfactants
- functions as a donor in the horizontal transfer of catabolic plasmids
- can be detected and tracked once released
- serves as a reporter of contaminant concentration and their location

However, introduction of the desired catabolic capacity into the contaminated environment, may not always result in enhanced degradation of the target compound. Inoculated strains may fail to survive, or lose their desired catabolic activity in mixed highly competitive microbial ecosystems (Watanabe *et al*, 1998; Tchelet *et al.*, 1999). Application of competent isolates for bioaugmentation may fail for a number of reasons including;

- inability to adapt to the prevailing environmental conditions, including physicochemical factors, such as the presence or absence of oxygen, sub optimal pH or temperature,
- predation by protozoa,
- competition with the indigenous microbial population,
- low substrate availability and,

- repression or irreversible loss of the desired catabolic function.

The failure of bioaugmentation has been reported. Inoculation of a nitrifying batch reactor with an aerobic nitrifying isolate, *Microvigula aerodenitrificans*, did not improve nitrification. Additionally, a second, massive inoculation led to a breakdown in nitrification in the reactor. The size of the second inoculation was thought to result in the breakdown in nitrification by stimulating the population of predatory protozoa, resulting in a catastrophic crash in the indigenous nitrifying bacterial population (Bouchez *et al.*, 2000).

The presence of 1,2,4-TCB provided no selective advantage for a 1,2,4-TCB-degrading strain, *Pseudomonas* sp strain P51, inoculated into an activated sludge community, and after 24 h, the inoculated cell numbers were reduced to less than 1 % of the initial population. No degradation of 1,2,4-TCB by the inoculated sludge was observed. In contrast, the same isolate was able to establish itself throughout a soil column resulting in degradation of 1,2,4-TCB to below detectable levels. The failure of the organism to survive in the activated sludge was thought to be due to its inability to successfully compete with the indigenous biomass for micronutrients, specifically, iron (Tchelet *et al.* 1999). Similarly, the capacity of a mixed culture from soil for the removal of butane and transformation of 1,1,1-trichloroethane, was lost after bioaugmentation into butane-contaminated groundwater (Jitnuyanont *et al.*, 2001). The loss of activity was attributed to a deficiency of mineral nutrients in the groundwater, relative to the initial enrichment medium. Reductions in substrate availability to the inoculated biomass, due to 'ageing' (increasing resistance of adsorbed PAH to extraction and degradation) of the substrate, limited the bioremediation of soil contaminated with phenanthrene (Schwartz & Scow, 2001).

Isolates, which are able to incorporate successfully into the indigenous community, and have ready access to the target compound, may still fail to achieve remediation due to catabolite repression. Catabolite repression is the regulatory mechanism by which the expression of genes required for the utilisation of secondary carbon sources is suppressed in the presence of a preferred substrate. In the presence of a range of carbon sources,

bacteria preferentially utilise the source that allows for the highest growth rate and may only synthesise the enzymes required to utilise that source (Goldstein *et al.*, 1985; Stülke & Hillen, 1999). As such, it may be that when exposed to the target compound at environmental concentrations, the catabolic pathways for the degradation of the target compound are repressed in favour of more abundant or more readily degradable carbon sources. Deutz *et al.* (1994) reported repression of toluene utilisation by *Pseudomonas putida* in the presence of succinate and glucose. The expression of styrene monooxygenase, the first enzyme in the styrene degradation pathway, was also repressed in the presence of succinate (O'Leary *et al.*, 2002). Phenol utilisation by *Ralstonia eutrophia* 335 was repressed in the presence of acetate (Ampé *et al.*, 1998) and the degradation of NSA and BSA by *Pseudomonas maltophila* BSA6, was almost entirely repressed by substrates, such as acetate, allowing for rapid growth (Lee & Clark, 1993).

The catabolic activity of the isolate may also be lost under unfavourable environmental conditions. Most catabolic genes are carried by wide host range, conjugative or mobilisable plasmids. Plasmid encoded pathways for the degradation of a number of environmentally significant compounds have been reported including, 2,4-D (Ka *et al.*, 1994), carbofuran (Feng *et al.*, 1997), nitrobenzene (Park & Kim, 2000) and fenitrothion (Hayatsu *et al.*, 2000). The catabolic genes for naphthalene are generally found on self-transmissible plasmids (Sayler *et al.*, 1990). Plasmid-carried catabolic genes may enable the proliferation of these genes in the environment, due to horizontal transfer coupled with selective pressure from xenobiotic compounds (Van der Meer *et al.*, 1992). Horizontal transfer of a catabolic plasmid from *Pseudomonas putida* HC1 conferred the ability to degrade crude oil hydrocarbons upon marine bacteria (Latha & Lalithakumari, 2001). The plasmid pJP4, which encodes for the partial degradation of 2,4-D, was detected throughout the entire length of a soil column and in a number of indigenous transconjugants, following inoculation with donor *E. coli* D11 (Newby & Pepper, 2002).

However, plasmids are dispensable as their function is only of benefit to the cell in a limited set of environmental conditions. Plasmid loss may, therefore, occur spontaneously in the absence of the appropriate environmental selection pressure (Parker, 2000). The loss of catabolic genes, under non-selective conditions has been reported

(Seitz *et al.*, 1993; Dabrock *et al.*, 1994; Kulakova *et al.*, 1995; Feng *et al.*, 1997; Coleman *et al.*, 2002). Isolates capable of degradation of xenobiotic compounds, are typically isolated by application of a strong selection pressure, with the xenobiotic compound as a sole source of one or more biogenic elements, such as carbon, nitrogen or sulphur (Harder & Dijkhuizen, 1982). Inoculation of the isolate into an environment, where the pressure to maintain the selected catabolic activity is considerably reduced by both the presence of alternative substrates and lower concentrations of the target compound, may lead to repression, and subsequent loss of the catabolic activity, with the consequence that the bioaugmentation may fail.

Bioaugmentation is a relatively new approach to bioremediation and, due to the difficulties associated with predicting the success of an inoculated organism, the success rate of bioaugmentation has been mixed (Alexander, 1994b). However, it may also offer an effective and inexpensive route for the *in situ* bioremediation of recalcitrant, xenobiotic contaminants (Mesarch *et al.*, 2000; Gianfreda & Nannipieri, 2001). Further research can only help to rationalise the development and increase the success rate of bioaugmentation strategies.

1.10. Biomarkers for monitoring the survival of isolates used for bioaugmentation

Stephens & Stephens (1992) proposed that the success or failure of bioaugmentation depends on two conditions: the survival of the inoculated strain(s) and the demonstration of the desired degradative activity. The degradative activity can be most readily determined by measuring the concentration of the target compound in the inoculated environment. However, distinction of an inoculated microorganism in a complex microbial community requires that a specific and unique identifier is available for the introduced organism. Identification and enumeration of an inoculated isolate may provide an insight into the reasons for bioaugmentation failure, e.g. loss of the inoculated isolate from the system.

A number of techniques have been applied to the detection and quantification of inoculated isolates including, selective plate counts (Tchelet *et al.*, 1999; Ghyoot *et al.*,

2000), amplification and quantification of 16S rRNA and rDNA with strain-specific primers (Tchelet *et al.*, 1999; Muttray *et al.*, 2001; Yu & Mohn, 2002), fluorescent *in situ* hybridisation (FISH) with strain-specific probes (Bouchez *et al.*, 2000), and labelling of the inoculated organism with the fluorescent and luminescent markers, green fluorescent protein (*gfp*) (Valdivia *et al.*, 1996; Eberl *et al.*,1997; Boon *et al.*, 2000) and *lux* or *luc* (Shaw *et al.*, 1999; Unge *et al.*, 1999; Xing *et al.*, 2000), respectively.

Some of the techniques may be subject to a lack of specificity. Plate counts may be confounded by the presence of indigenous organisms able to grow on the selective media, e.g. by utilising the target compound when selection is based on catabolic activity, or tolerating antibiotics that the introduced organism is resistant to and which may be used as a selection pressure. The sensitivity of plate counts may also be low. Tchelet *et al.* (1999) were unable to enumerate the inoculated isolate, *Pseudomonas putida* P51, in activated sludge by selective plating, after the inoculated population dropped below 1 x 10^6 cells ml^{-1}. The stresses placed on laboratory cultured strains when inoculated into a contaminated environment may also render them unculturable (Jansson *et al.*, 2000).

Sensitive enumeration of *P. putida* P51 (10^3 – 10^4 cells ml^{-1} in an indigenous population of 10^9 cells ml^{-1}) was achieved by PCR amplification of a strain specific region of the 16S rDNA. However, specific amplification of *P. putida* P51 was not possible and background levels of 10^4 cells ml^{-1} were detected in uninoculated activated sludge samples. These background levels were probably due to the presence of related bacteria (Snaidr *et al.*, 1997). The use of strain-specific FISH probes, based on the 16S rDNA of the isolate, may be subject to the same interferences as PCR amplification with 16S rDNA-specific primers. Additionally, enumeration of FISH-probed communities, by confocal laser scanning microscopy, correlates only with the microscopic field viewed and may not correspond to the numerical abundance of the probed bacteria (Bouchez *et al.*, 2000).

Isolates may also be monitored by the use of biomarkers or marker genes. A biomarker or marker gene is defined as a DNA sequence, introduced to an organism, which confers a

distinct genotype or phenotype (Jansson *et al.*, 2000). Alternatively, it may be a unique DNA segment that can be tracked with DNA probes or by PCR amplification (Jansson, 1995) to enable monitoring in a given environment. The use of the bacterial luciferase gene (*lux),* the firefly luciferase gene (*luc*) or the green fluorescent protein gene (*gfp)* (from the jellyfish, *Aequorea victoria*) provides unique markers for the detection of isolates. The expression of *lux* or *luc* by a microorganism is manifested as luminescence. The main advantage of luminescent markers is the ability to directly monitor light output without cultivation of the cells (Rattray *et al.,* 1990). Similarly, microorganisms expressing *gfp* will fluoresce, again allowing for direct monitoring of the organism (Unge *et al.*, 1999). The use of the eukaryote-derived *luc* and *gfp* genes as markers, also avoids the possibility of false-positives from the indigenous population, which may occur with the bacterially derived *lux* system (Tombolini *et al.*, 1997) and some of the previously described techniques.

Tagging with *luc* and *lux* to monitor the survival of isolates used for bioaugmentation has been reported. Tagging with *luc* was used to determine the fate of two gasoline-degrading isolates, after inoculation into gasoline-contaminated soil (Jansson *et al.*, 2000). Shaw *et al.* (1999) inserted promoterless *lux* into the chromosome of the 2,4-dichlorophenol-degrading isolate, *Burkholderia* sp. RASC c2, leading to constitutive light output, with a view to using the diminishing luminescence of the isolate as a biosensor for toxic concentrations of 2,4-DCP during its bioremediation.

The light output of *lux* and *luc*-marked isolates is an indicator of their metabolic status as the activity of luciferase, and hence luminescence, is dependent on cellular reserves of ATP (*luc*) or $FMNH_2$ (*lux*). In growing cells, light output is proportional to bacterial numbers. However, in cells that have been starved or stressed, as may be the case for bacteria in the environment, light production may diminish in response to changes in the cellular energy status (Duncan *et al.,* 1990: Meikle *et al.*, 1992). Low levels of the target pollutant may also stimulate the luminescence of lux. Metabolic uncoupling by 2,4-DCP led to increased luminescence by *Burkholderia* sp. RASC c2 (Shaw *et al.*, 1999). Therefore, luminescence, due to tagging with *luc* or *lux*, may not be an accurate marker of bacterial cell numbers in starved, stressed or stimulated isolates.

Gfp is a useful marker for monitoring microbial populations, as it fluoresces when exposed to near-UV or blue light and requires no substrate for fluorescence. *Gfp* fluorescence is therefore, independent of cellular energy reserves (Chalfie *et al.*, 1994) and can be used as marker for total cell numbers, regardless of their metabolic status (Unge *et al.*,1999). *Gfp* also persists after exposure to heat and extremes of pH (Kremer *et al.*, 1995, Valdivia *et al.*, 1996).

Tagging with *gfp* has been applied successfully to ecological study of the fate of *Pseudomonas putida* KT2442 (Eberl *et al.*, 1997) and the success of bioaugmentation with *Comamonas testosteroni* I2*gfp* (Boon *et al.*, 2000), after inoculation into activated sludges, and the survival of *Arthrobacter chlorophenolicus* A6 after inoculation into soil (Elväng *et al.*, 2001). However, although *gfp*-tagged cells have been shown to fluoresce under a range of growth conditions, including starvation, the *gfp* fluorescence phenotype does not indicate the metabolic status of the cells and estimates of cell numbers surviving in the inoculated environment may be skewed by dead yet still fluorescent cells (Unge *et al.*, 1999).

The choice of biomarker for an inoculated strain will depend on the experimental requirements and the environmental conditions into which the isolate is introduced. Where the metabolic status of the cells is required *lux* or *luc* would be the marker of choice. However, where bacterial numbers are required, *gfp* would be the marker of choice. Isolates have been marked with a dual *gfp-lux* marker system, allowing for determination of both parameters (Unge *et al.*, 1999).

1.11. Aims of the study

The overall aim of the study was to evaluate bioaugmentation as a route for the remediation of NSA- and SNFC-contaminated effluents. This aim was to be satisfied by the achievement of the following objectives:

1. Determination of NSA concentrations in industrial effluents from plants utilising: i) NSA in the production of SNFC; and ii) SNFC in the manufacture of leather
2. Isolation of bacterial isolates capable of degrading NSA under aerobic conditions and elucidation of kinetic parameters relevant to their application in bioaugmentation strategies
3. Identification of the isolates and determination of the catabolic pathway for the degradation of NSA
4. Molecular marking of the isolates to allow their detection and quantification in mixed microbial communities
5. Evaluation of the use of the isolates for the bioaugmentation of activated sludge communities treating NSA-contaminated effluents
6. Evaluation of the use of the isolates for the degradation of SNFC-based compounds in isolation or, if necessary, in combination with physicochemical pretreatment.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Microbial Sources

The microorganisms used in this study were obtained from four sludges:

1. The solid media from a pilot-scale reed bed plant (Northampton, Northamptonshire), treating primary treated effluent containing syntans from a leather finishing plant.
2. Activated sludge from a municipal wastewater treatment plant (Beverly, North Yorkshire) treating domestic effluent and the primary treated effluent from a chemical manufacturing plant producing a range of products including naphthalene sulphonate-based compounds.
3. Activated sludge from a municipal treatment plant (Canterbury, Kent) treating industrial and domestic effluent.
4. Activated sludge from a municipal treatment plant, located in Northamptonshire, treating industrial and domestic effluent.

2.1.2 Maintenance and storage of bacterial strains

The bacterial strains used in this study are shown in Table 2.1. All strains were stored by cryopreservation at -70°C in Protect vials (Technical Service Consultants Ltd, Heywood, UK). *Escherichia coli* S17/1λpir (pUT-miniTn5*gfp*km) was supplied courtesy of Nico Boon (Ghent University, Belgium) and maintained on Luria Bertani agar containing 50 mg L^{-1} of ampicillin (Sigma Chemical Co., Poole, UK) and 50 mg L^{-1} kanamycin (Sigma Chemical Co., UK).

Table 2.1 Bacterial strains used in this study. Provisional identification shown on the basis of partial 16S rDNA sequences (section 2.10.6)

Strains	Sludge
Sphingomonas sp. RBN	This study – source 1 (section 2.3)
Comamonas testosteroni BAS	This study – source 2 (section 2.3)
Sphingomonas sp. strain RBN*gfp*	This study (section 2.11)
Escherichia coli S17/1λpir (pUT-miniTn5*gfp*km)	Boon *et al.*, 2000

2.1.3 Chemicals

Naphthalene-2-sulphonic acid, sodium salt, (NSA) (>98% purity) and phenol sulphonic acid, sodium salt dihydrate, (PSA) (99% purity) were obtained from Sigma Chemical Co. (Poole, UK). A commercially available naphthalene sulphonate-based syntan, designated compound #22, and a commercially available phenol sulphonate-based syntan, designated compound #21, were supplied by Hodgson Chemicals (Beverley, UK). For reasons of commercial confidentiality, the exact compositions of #21 and #22 were not known. All other chemicals used (except those specifically mentioned in the text) were Analar grade and supplied by Sigma-Aldrich Co. Ltd. (UK), Difco Laboratories (Detroit, USA), Oxoid Ltd (Basingstoke, UK), BDH Laboratory Supplies (Poole, UK) or Acros Chemicals Ltd (Loughborough, UK). Effluent samples were obtained from the untreated effluent of chemical plant manufacturing naphthalene sulphonate compounds and the biologically treated effluent of a tannery using naphthalene sulphonate compounds for leather manufacture.

2.1.4 Primer Sets

Three primer sets were used for PCR amplification of specified regions of DNA during this study. A fragment of the V4 and V5 regions of eubacterial 16S rDNA was amplified with Com1 (forward) (5'-CAGCAGCCGCGGTAATAC-3') and Com2 (reverse) (5'-CCGTCAATTCCTTTGAGTTT-3'). A phosphorylated version of the forward primer Com1

was also used to facilitate digestion of a single strand of the amplicon for single strand conformation polymorphism (SSCP) analysis (section 2.10.9) (Schwieger and Tebbe, 1998).
A region of the gene encoding for *nah*Ac was amplified with Ac114F (forward) (5'-CTGGC(T/A)(T/A)TT(T/C)CTCAC(T/C)CAT-3') and Ac596R (reverse) (5'-C(G/A)GGTG(C/T)CTTCCAGTTG-3') (Wilson *et al.*, 1999).
A region of the gene encoding for green fluorescent protein (*gfp*) was amplified with *gfp*F (forward) (5'-CCATGGCCACACTTGTCAC-3') and *gfp*R (reverse) (5'-CTTTCGAAAGGGCAGATTGT-3') (Boon *et al.*, 2000).

The primers were synthesised by MWG AG Biotech (Ebersberg, Germany) and dissolved in PCR-grade water to a concentration of 100 pmol μl^{-1}. Stock solutions of primers were prepared by adding 10 µl of both the forward and reverse primer to 80 µl of PCR-grade water, to a final concentration of 10 pmol μl^{-1} of each primer. Primer stocks were stored at -20°C.

2.1.5 Culture media

A minimal salts medium (MSM), containing an excess of inorganic nitrogen, phosphate, vitamins and trace elements, was used for the majority of the enrichments and growth experiments in this study, and supplemented as required. The MSM contained 10 ml of mineral solution (NaCl, 80 g; NH_4Cl, 100 g; KCl, 10 g; KH_2PO_4, 10 g; $MgSO_4$, 20 g; $CaCl_2$, 4 g per litre), 10 ml of vitamin solution (pyridoxine-HCl, 10 mg; thiamine-HCl, 5 mg; riboflavin, 5 mg; calcium pantothenate, 5 mg; thioctic acid, 5 mg; *p*-aminobenzoic acid, 5 mg; nicotinic acid, 5 mg; vitamin B12, 5 mg; mercaptoethanesulphonic acid, 5 mg; biotin, 2 mg; folic acid, 2 mg per litre) and 1 ml of trace metal solution (Nitriloacetic acid adjusted to pH 6.0, 2 g; $MnSO_4.H_2O$, 1 g; $Fe(NH_4)_2(SO_4)_2.6H2O$, 0.8 g; $CoCl_2.6H2O$, 0.2 g; $ZnSO_4.7H_2O$, 0.2 g; $CuCl_2.2H_2O$, 0.02 g; $NiCl_2.6H_2O$, 0.02 g; $Na_2MoO_4.2H_2O$, 0.02 g; Na_2SeO_4, 0.02g; Na_2WO_4, 0.02 per litre) (Tanner, 1997). The media was buffered with 0.5 g L^{-1} of K_2HPO_4 and the pH adjusted to 7.0 with 1M NaOH. The MSM was used as a solid medium by adding 15 g L^{-1} of Technical Agar No. 3 (Oxoid) and supplementing as required.

2.2 Methods

2.2.1 Analytical methods

The experimental work for this project was carried out at two sites, the University of Kent at Canterbury (UKC) and BLC, Leather Technology Centre, Northampton (BLC). There were differences in the analytical equipment available at either site, and therefore methods were evaluated for each site as necessary. Site-based variations in equipment are indicated by either (UKC) or (BLC).

2.2.1.1 Chemical Oxygen Demand (COD) Analysis

COD analysis was carried out using either a Dr Lange Lasa 50 spectrophotometer and Dr Lange LCK514 COD vials with an analytical range of 100 – 2000 mg L^{-1} COD (Dr Lange, Dusseldorf, Germany) (UKC) or a HACH DR2000 spectrophotometer with HACH high range COD vials with an analytical range of 0 – 1500 mg L^{-1} COD (HACH Company, Loveland, USA) (BLC). Prior to analysis, samples were filtered through Minisart 0.2 μm filters (Sartorius, Göttingen, Germany). A 2 ml aliquot of the sample was pipetted into a COD vial, inverted to mix and incubated at 150°C for 2 h. After incubation, the vials were cooled and the COD measured spectrophotometrically at 620 nm. The spectrophotometers were zeroed against distilled water and COD quantification was determined against standard curves supplied by the manufacturer (Dr Lange or HACH Company).

2.2.1.2 Biological Oxygen Demand analysis

The 5-day biological oxygen demand (BOD_5) of effluent samples, NSA and compound #22 were determined using Oxitop IS6 BOD_5 instruments (Wissenschaftlich-Technische Werkstätten GmbH, Weilhem, Germany). The COD of the effluent samples was determined prior to BOD_5 analysis and the samples diluted with distilled water to give a COD value of 100 – 200 mg L^{-1}, to ensure that the BOD_5 values were within the analytical range of the equipment. Effluent samples were used as received whereas solutions of NSA or compound

#22 were supplemented with 10 ml L^{-1} of vitamins and mineral solutions, and 1 ml of trace metal solution (section 2.1.5). The pH of the samples was adjusted to 7.0 with 5 M HCl or NaOH as required, and 250 ml of the samples were placed in the Oxitop bottles and inoculated with 2 ml of a 0.5% solution of activated sludge from sludge 4 (section 2.1.1). Two sodium hydroxide pellets were placed in the neck of the bottles and the Oxitop measuring system was firmly screwed onto the bottles. The samples were then incubated, with mixing using a magnetic stirrer, at 20°C for 5 days. A blank was also incubated, containing distilled water (and vitamins minerals and trace metals as required) and the inoculant only. After incubation, the BOD_5 were read from the Oxitop measuring system and the true BOD_5 of the samples calculated using the following equations:

$$BOD_5 = \frac{M_S \times F \times (V_B + V_S)}{V_S} - BW$$

Where

$$BW = \frac{M_B \times F \times V_B}{V_S}$$

Where M_S = the measured value of the sample

V_S = the volume of original sample in the diluted sample

V_B = the volume of dilution water in the diluted sample

F = the factor to correct for volume

BW = the correction value that accounts for the contribution of to the apparent COD by the blank

2.2.1.3 Solid Phase Extraction of Effluents

NSA, PSA and syntans were extracted from effluents by solid phase extraction (SPE). Effluent samples (50ml) were filtered under vacuum through Whatman 0.45 µm cellulose acetate membrane filter papers (Whatman Plc, Maidstone, UK). The pH of the sample was then adjusted to 6.5, with either 1 M HCl or 1 M NaOH, and 5 ml of 20 mM tetrabutylammonium bromide (TBABr) added. Prior to addition of the sample, a Supelco Supelclean LC-18 SPE cartridge (6ml capacity, 500 mg adsorbent packing material)

(Supelco Inc., Bellefonte, USA) was primed with sequential additions of 10 ml of methanol, 5 ml of HPLC grade water, and 5 ml of 1 mM TBABr. The sample was passed under vacuum through the cartridge. The cartridge was dried briefly and then eluted with 3 ml HPLC grade methanol and 3 ml HPLC grade acetonitrile. The eluents were pooled, dried under a stream of nitrogen and the residue redissolved in 5 ml HPLC grade water (Reemstma & Jekel, 1994) for analysis by HPLC (section 2.2.1.4).

2.2.1.4 High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) analysis was carried out at BLC using a HPLC system consisting of two Gilson 306 pumps (Gilson, Villers Le Bel, France), a Jasco AS-950 autosampler (Jasco UK Ltd, UK) and a 250 mm x 4.6 mm Hichrom RPB C18 (5µm) reverse phase HPLC column (Hichrom, Theale, UK). The chromatography was carried out using a mobile phase consisting of solvent A: 40 µl orthophosphoric acid (85%), 24 mM sodium dihydrogen phosphate, 2 mM tetrabutyl ammonium bromide (ion pair reagent) and 1 l of HPLC grade water; and solvent B: 80 µl orthophosphoric acid, 12 mM sodium dihydrogen phosphate, 2 mM tetrabutyl ammonium bromide, 200 ml of HPLC grade water and 800 ml of HPLC grade methanol. The mobile phase was supplied at 1 ml min^{-1} on a linear gradient from 45% B to 57% B over 20 min, held at 57% B for 4 min, run back to 45% B over 3 minutes and equilibrated for 3 min (total run time 30 min) (Reemstma & Jekel, 1994). The column temperature was maintained at 35°C with an Eppendorf CH-30 column oven (Eppendorf AG, Hamburg, Germany).

A 20 µl sample was injected onto the column for analysis and the UV-spectra of the analytes were measured between 200 and 350 nm using a Jasco MD-910 UV-diode array detector (Jasco UK Ltd, UK). Spectral data collection and peak integration were carried out using Jasco DP-910/915 software (Jasco UK Ltd, UK).

HPLC analysis was carried out at UKC on a HPLC system consisting of a Kontron 422 pump, a Kontron 465 autosampler (Biotek Kontron Instruments, Watford, UK) and a 250 mm x 4.6 mm Econosil C18 (5µm) reverse phase HPLC column (Alltech Associates Inc.,

Deerfield, USA). The chromatography was carried out using an isocratic mobile phase of methanol, water and orthophosphoric acid (85%) in a ratio of 50:49.7:0.3 (v/v) and pumped at a flow rate of 0.6 ml min^{-1} (Keck *et al*, 1997). The mobile phase was degassed with a Kontron DEG 103 degasser (Biotek Kontron Instruments) and the column temperature was maintained at 35°C with an Alltech 530 column heater (Alltech Associates Inc., USA).

A 20 µl sample was injected onto the column for analysis and the UV-spectra of the analytes were measured between 200 and 300 nm with a Kontron 440 UV-diode array detector (Biotek Kontron). Spectra data collection and peak integration were carried out using Kromasystem 2000 software (Biotek Kontron). Peak integration was carried out at 280 nm for NSA, and 271 nm for PSA.

2.3. Isolation and Characterisation of naphthalene sulphonate-degrading bacteria

2.3.1 Isolation of naphthalene sulphonate -degrading bacteria

Sludge 1 and sludge 2 (section 2.1.1), were used to isolate NSA-degrading isolates by enrichment in MSM (section 2.1.4) supplemented with 500 mg L^{-1} NSA. Approximately 1 g (wet weight) of each material was added to separate 250 ml Erlenmeyer flasks containing 100 ml of MSM plus NSA and shaken at 150 rpm and 25°C in an orbital shaker (Gallenkamp, Loughborough, UK). The enrichment media was assessed visually and when turbidity was observed, 1 ml was subcultured to fresh MSM plus 500 mg L^{-1} NSA and shaken as before.

After a series of subcultures, the enriched cultures were streaked on to MSM agar containing 500 mg L^{-1} NSA (section 2.1.4) and incubated at 25°C. Individual colonies growing on the MSM:NSA agar were aseptically picked and reinoculated into MSM plus 500 mg L^{-1} NSA. Flasks showing signs of turbidity were subcultured and these cultures used in subsequent procedures.

2.3.2 The utilisation by the isolates, of naphthalene sulphonate, phenol sulphonic acid and compound #22, in batch culture

The degradation of NSA, PSA and compound #22, and growth of the isolates (section 2.3.1) was assessed in batch culture by inoculating 1 ml of a 48 h growth culture into 100 ml of MSM plus 500 mg L^{-1} NSA, PSA or compound #22 in 250 Erlenmeyer flasks. The flasks were then shaken in an orbital shaker at 25°C and 150 rpm for 96 h. All enrichments were carried out in triplicate. Each flask was sampled (3 ml/flask) for HPLC measurement of NSA, PSA or compound #22 and turbidity, as a measure of microbial growth, after 0, 6, 24, 48, 72 and 96 h. Flasks of non-inoculated MSM plus 500 mg L^{-1} NSA or PSA were incubated as negative controls.

Growth cultures were sampled for HPLC analysis by aliquoting 2 ml of medium into 2 ml microfuge tubes. The aliquots were centrifuged at 13000 x g for 10 min and 1 ml of the supernate pipetted into HPLC vials. Samples for HPLC analysis were kept at 4°C prior to analysis. The concentration of the NSA, PSA or compound #22 in the samples was measured as previously described (section 2.2.1.4).

The turbidity of the enrichment cultures was measured by pipetting 1 ml of the culture into a 2 ml disposable cuvette and measuring the optical density at 600 nm (OD_{600}) with a Unicam 5625 UV/vis spectrophotometer (Unicam, Cambridge, UK).

2.4 Determination of growth kinetics

2.4.1 Determination of cell concentrations

Biomass dry weights (BDW) were determined by centrifuging (13000 x g) batch cultures of the isolates and washing the resuspended pellets twice in phosphate-buffered saline (PBS). The pellets were then dried at 105°C for 16 h, cooled in a desiccator at room temperature and weighed. A standard curve of BDW vs. OD_{600} was plotted and used for routine determinations.

2.4.2 Specific growth rate

The specific growth rate (μ) of the individual isolates was calculated according to the equation:

$$\mu = \frac{\ln (x_t/x_0)}{t}$$

where; x_0 and x_t = the OD_{600} during early and late exponential growth respectively and; t = the time elapsed between those measurements (Pirt, 1975).

2.4.3 Maximum specific growth rate and half-saturation constant

The maximum specific growth rate (μ_{max}) and the half saturation constant (K_s) of NSA were determined at a range of concentrations (10 – 1000 mg L^{-1}). K_s is the concentration of growth limiting substrate at which μ is equal to 0.5 μ_{max}. Both K_s and μ_{max} were calculated using Lineweaver-Burk plots (1/s vs 1/μ) derived from the Monod equation:

$$\mu = \frac{\mu_{max} \times s}{K_s + s}$$

The best fit line for the plots was determined using least-squares analysis.

2.4.4 Biomass yield coefficient

The biomass yield coefficient, Y, (g of biomass produced g^{-1} of substrate consumed) was calculated by dividing the increase in BDW by the amount of substrate (NSA) consumed over a given time period (Rittmann & McCarty, 1980). BDW was estimated from the OD_{600} (section 2.4.1) and NSA removal was measured by HPLC (section 2.2.1.4)

2.5 Utilisation of naphthalene sulphonate or compound #22 as a sole source of sulphur or carbon, energy and sulphur

The ability of the isolates to utilise NSA as either a sole source of sulphur or a sole source of carbon and sulphur was investigated. A sulphate-free medium was used containing K_2HPO_4, 0.6 g; NaCl, 0.8 g; NH_4Cl, 1 g; KCl, 0.1 g; $CaCl_2$, 0.04 g per litre. The medium was supplemented with: 500 mg L^{-1} NSA, as a source of carbon and sulphur; 2.0 g glucose as a source of carbon without sulphur or; 500 mg L^{-1} NSA plus 2.0 g L^{-1} glucose, as sources of carbon and sulphur. Positive growth controls were made by adding 0.2g L^{-1} $MgSO_4.7H_2O$ to the medium. The media was supplemented as required and the pH adjusted to 7.0 with 1M NaOH. The growth cultures were carried out and sampled as previously described (section 2.3.2).

2.6 Utilisation of Naphthalene as a sole source of carbon

Growth of the isolates with naphthalene as sole source of carbon and energy was investigated by adding 50 mg of naphthalene to 100 ml (500 mg L^{-1}) of MSM in 250 ml Erlenmeyer flasks. The flasks were inoculated with 1 ml of a 48 h culture of the isolates, incubated and sampled for turbidity (OD_{600}) as previously described (section 2.3.2).

2.7 Utilisation of naphthalene sulphonate in the presence of alternative carbon sources

The utilisation of NSA as a source of carbon in the presence of alternative carbon sources was investigated using a medium containing: K_2HPO_4, 1 g; oxalic acid dihydrate, 133.4 mg; glucose, 400 mg, sodium acetate trihydrate, 100 mg; sodium proprionate, 26.7 mg; methanol, 10 µl (Selvaratnam *et al*, 1995); plus 10 ml of vitamin solution, 10 ml of mineral solution and 1 ml of trace metal solution (section 2.1.5) per litre of water (CM) and supplemented with 500 mg L^{-1} (equivalent to 52% of the total carbon) or 50 mg L^{-1} (equivalent to 10.9% of the total carbon) of NSA. Flasks containing 100 ml of media were inoculated with 1 ml of 48 h culture of each isolates and shaken on an orbital shaker at 150 rpm and 25°C for 96 h.

The flasks were sampled for HPLC analysis and turbidity as previously described (section 2.3.2).

2.8 Plasmid curing of the isolates

The locations of the NSA catabolic genes of isolates were determined by plasmid curing. One hundred microlitres of 48 h growth cultures of the isolates were inoculated into 5 ml Luria-Bertani (LB) medium in 15 ml falcon tubes. The inoculated medium was shaken for 24 h at either 25°C or 37°C. After incubation, 0.1 ml of the medium from each tube was subcultured into fresh LB media. This procedure was repeated over 3 cycles of subculturing (Feng *et al*, 1997).

After the final subculture, the medium was centrifuged at 10000 x g and the LB media aseptically removed. The microbial pellet was then washed 3 times with 5 ml sterile phosphate buffered saline (PBS) and resuspended in 5 ml PBS. The turbidities of the resuspended isolates were measured and the isolates diluted with PBS to the equivalent OD_{600} of a 48 hour growth culture of the isolates in MSM:NSA. One millilitre of the washed and diluted isolates were inoculated, in triplicate, into 250 ml Erlenmeyer flasks containing 100 ml MSM plus 500 mg L^{-1} NSA. The flasks were incubated and sampled as previously described (section 2.3.2).

2.9 Detection of dioxygenase activity by transformation of indole to indigo

The isolates were streaked on tryptone soya agar plates and incubated at 25°C. Indole (Acros, UK) was supplied to the plates in the vapour phase, by attaching several crystals of indole to the inner surface of the plate lid with masking tape. The plates were sealed and periodically inspected for colour development (Dagher *et al.*, 1997).

2.10 Molecular techniques

2.10.1 Extraction of DNA from isolates and activated sludges

DNA was extracted from the isolates by centrifuging aliquots (4 – 6 ml) of 48 hour cultures at 13000 x g. The DNA was extracted from the pelleted isolates using a Qiagen DNA-easy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturers instructions.

DNA was extracted from activated sludge by pipetting 2 ml into a bead beating tube and centrifuging (13000 x g) for 5 min. The supernate was discarded and 0.5 ml of extraction buffer (equal volumes of 10% (w/v) hexadecyltrimethylammonium bromide (CTAB) in 0.7 M NaCl and 240 mM KH_2PO_4 buffer (pH 8)) and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1)) added to each tube. Sterilised glass beads, 0.5 g, (0.6 – 1.0 mm diameter) were added to each tube and beaten at 4200 rpm for 2 min on a Mini-beadbeater (Biospec Products Inc, Bartlesville, USA) to lyse the cells.

The tubes were then centrifuged (13000 x g) for 5 min at 4°C, and the aqueous phase removed and pipetted into clean 1.5 ml microfuge tubes. An equal volume of chloroform-isoamyl alcohol (24:1) was added to each tube and the tubes centrifuged (13000 x g) for 5 minutes at 4°C. The aqueous phase was removed and pipetted into a clean 1.5 ml microfuge tube and an equal volume of isopropanol added. The tubes were left for 2 h at 20°C to allow precipitation of the nucleic acids. After precipitation, the nucleic acids were pelleted by centrifugation (13000 x g) for 10 minutes at 4°C. The supernate was removed by aspiration and the pellets washed in 0.5 ml of ice cold 70% ethanol. The washed pellets were then centrifuged (13000 x g) for 5 minutes at 4°C, aspirated to remove the ethanol, and left to air dry in a laminar flow hood. After drying, the pellets were redissolved in 50 µl PCR grade water and stored at -20°C (Griffiths *et al.*, 2000). The presence of DNA was determined by gel electrophoresis (section 2.10.2).

2.10.2. Agarose gel electrophoresis

Agarose gels were prepared by dissolving molecular grade agarose (Bio-Rad Laboratories Inc, Hercules, USA) in TAE buffer, (4.84 g Tris base, 1.142 g glacial acetic acid, 2 ml 0.5 M EDTA (pH8.0) per litre) (Sambrook *et al.*, 1989), with 1µl ml^{-1} (v/v) of 500 mg L^{-1} ethidium bromide. For separation of DNA extracts, a 0.8% agarose gel (w/v) was used; for separation of PCR amplicons, a 2.0% agarose gel (w/v) was used. The gels were run in a Horizon 58 horizontal gel electrophoresis tank (Life Technologies, Gaithersburg, USA) with TAE plus 1µl ml^{-1} (v/v) 500 mg L^{-1} ethidium bromide as the running buffer. Electrophoresis of DNA extracts was carried out at 50 volts; for PCR amplicons, electrophoresis was carried out at 120 volts.

Five microlitres of each DNA sample was mixed with 1 µl of 5x loading buffer, (Ficoll type 400 polymer, 15%, bromophenol blue, 0.25% and xylene cyanol, 0.25% in distilled water) and 4 µl loaded onto the gel. The size of PCR amplicons was determined by comparison with a Bio-Rad EZ-load 100 base pair molecular ruler (Bio-Rad Laboratories Inc., USA). After electrophoresis, the DNA was visualised with UV-light on a Gel-Doc 2000 (Bio-Rad Laboratories Inc., USA) and the images analysed using Multianalyst 1.1 software (Bio-Rad Laboratories Inc., USA).

2.10.3 Quantification of DNA

The DNA in extracts was quantified using a Beckman DU 640 spectrophotometer (Beckman Coulter Ltd, High Wycombe, England). Diluted samples were measured at 260 nm in order to determine the total DNA concentration, and at 280 nm in order to determine the level of protein contamination. An absorbance ratio of 260 nm: 280 nm of greater than 1.7 indicated that the sample was free of protein and suitable for subsequent PCR (Yeates *et al.*, 1998).

2.10.4 Amplification of selected regions of DNA by PCR

A single PCR protocol was used in this study for the amplification of selected regions of DNA. DNA fragments were amplified in 50 µl of a PCR mix containing: 50 – 100 ng of DNA template, 4 µM of forward primer (section 2.1.4), 4 µM of reverse primer (section 2.1.4), 400 mg ml^{-1} of bovine serum albumin (BSA), 400 µM of each dNTP (Roche Diagnostic Corporation, Indianapolis, USA), 1.0 mM $MgCl_2$, 5 µl of x 10 reaction buffer (15 mM $MgCl_2$, pH 8.7), 1 U of Hotstar Taq DNA polymerase (Qiagen, Germany) and PCR water to a total volume of 50 µl. A negative control, containing no DNA and made to volume with PCR-grade water, was included in each series of amplifications.

PCR amplification was performed on a Techne Elite thermal cycler using a 2 step touchdown programme: step 1, an initial denaturation at 95°C for 15 min followed by 23 cycles of denaturation at 94°C for 1 minute, primer annealing for 45 sec, decreasing with each cycle from an initial annealing temperature of 72°C to a final temperature of 42°C, and extension at 72°C for 1 min and; step 2, 17 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 45 sec and extension at 72°C for 1 min with a final extension time of 10 min at 72°C (Roux, 1995).

2.10.5 Confirmation of the presence of a napthalene dioxygenase by *nah*Ac-PCR

The presence of a dioxygenase, as indicated by colour formation with indole (section 2.9), was confirmed by PCR of the extracted DNA (section 2.10.4) with *nah*Ac primers (section 2.1.4), and subsequent cloning and sequencing of amplicons (section 2.10.7). The sequence data was then analysed as described in section 2.10.8.

2.10.6 Identification of the isolates by partial 16S rDNA-PCR

After purification, the isolates were identified provisionally using PCR of a fragment of the V4 and V5 regions of eubacterial 16S rDNA (Schwieger and Tebbe, 1998). DNA was

extracted from the isolates (section 2.10.1) and amplified by PCR (section 2.10.4) with Com1/Com2 primers (section 2.1.4). The Com1/Com2 PCR amplicons were then cloned and sequenced (section 2.10.7) and the isolates provisionally identified by sequence analysis using BLAST and nucleotide distance analysis using CLUSTAL W (section 2.10.8).

2.10.7 Cloning and sequencing of PCR amplicons

PCR amplicons were cloned for sequencing using the Promega pGEM-T Easy Vector system (Promega Corp., Madison, USA) according to the manufacturers instructions. Two microlitres of PCR product was ligated into the pre-cut pGEM-T vector and transformed into *Escherichia coli* JMP109 High Efficiency Competent Cells (Promega Corp., Madison, USA). The pGEM-T vector encodes for the enzyme β-galactosidase. Succesful insertion of the amplicon into the vector causes insertional disruption of the α-peptide encoding region of β-galactosidase.

Transformation of the competent cells was achieved by heat shocking the cells at 42°C with the ligated vector. The transformed cells were then incubated by shaking at 37°C for 90 min in SOC medium. SOC medium contained 2.0 g tryptone, 0.5 g yeast extract, 1ml 1 M NaCl, 0.25 ml 1 M KCl, 1 ml 2 M Mg^{2}+ (from stock solution of 20.33 g $MgCl_2$ and 24.65 g $MgSO_4.7H_2O$ in 100 ml distilled water, filter sterilised) and 1 ml 2 M glucose in 100ml sterile distilled water. One hundred microlitres of the transformed cells were plated onto duplicate LB/ampicillin/isopropyl-beta-D-thiogalactoside (IPTG)/ 5-bromo-chloro-3-indoyl-β-D-galactoside (X-Gal) agar plates (LB agar plus 100 μg ml^{-1} ampicillin and spread with 100 μl 100 mM IPTG and 20 μl 500 mg ml^{-1} X-Gal) and incubated for 24 h at 37°C.

Colonies containing the re-ligated vector without an insert, were able to express β-galactosidase and transform X-Gal into an insoluble blue dye causing a blue colouration in the colony. Colonies containing the insert were unable to express β-galactosidase and were white. Blue-white screening of the colonies was used to determine those colonies containing the insert. White colonies were picked off each plate and inoculated into LB medium containing 50 mg L^{-1} ampicillin and incubated at 37°C for 24 h. After incubation, 2 ml of the

LB-ampicillin medium was pipetted into a microfuge tube and the cells pelleted by centrifugation (13000 x g) for 10 minutes. The vector was extracted from the cells with a Qiaprep Spin Miniprep kit (Qiagen, Germany) according to the manufacturers instructions, lyophilised and sent, with the appropriate primer sets, to MWG AG Biotech (Ebersberg, Germany) for sequencing.

2.10.8 Sequence analysis

Sequence data was analysed using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). BLAST uses a set of similarity search programs to explore all available sequence databases. The BLAST programs have been designed for speed, with a minimal sacrifice of sensitivity to distant sequence relationships. The scores assigned in a BLAST search have a well-defined statistical interpretation, making real matches easier to distinguish from random background hits. BLAST uses a heuristic algorithm which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences which share only isolated regions of similarity (Altschul *et al.*, 1990). Using BLAST, the sequence data was compared to a number of protein and DNA databases.

The isolate sequences, 16S rDNA and *nah*Ac, and closely related sequences retrieved from Genbank using BLAST, were aligned with MEGALIGN (DNAStar Inc., Madison, USA) and CLUSTALW (Thompson *et al.*, 1994). The nucleotide distance matrix was calculated using DNADIST from the PHYLIP package. Phylogenetic relationships were inferred by UPGMA distance analysis with Kimura two-parameter genetic distances (DNADIST 2:1 transition to transversion ratio). In order to determine the confidence values for each branch of the phylogenetic tree, bootstrap analysis was carried out on 100 pseudoreplicates of the distance matrix using SEQBOOT.

2.10.9 Single Strand Conformation Polymorphism (SSCP)

Samples for SSCP were amplified from extracted DNA (section 2.10.4) and 6.25 µl PCR mix was added to 4 µl denaturing loading buffer containing 0.25% xylene cyanol, 0.25% bromophenol blue and 10 mM NaOH in 95% formamide solution (Schwieger and Tebbe, 1998). The samples were prepared for gel electrophoresis by denaturing at 95°C for 2 min and snap-cooling on wet ice.

The complexity of diverse community samples was reduced by digesting one strand of the DNA with a λ-exonuclease (Schwieger and Tebbe, 1998). PCR amplicons (50 µl) amplified with Com1ph and Com2 primers (section 2.1.4) were made up to 100 µl with PCR water. The DNA was precipitated and cleaned with phenol:chloroform:isoamyl alcohol and subsequent chloroform:isoamyl alcohol treatment (section 2.10.1), vacuum-dried and resuspended in 10 µl PCR water.

Digestion of the phosphorylated DNA strand was achieved by mixing 10 µl of the DNA sample with 2 µl λ-exonuclease (5000 u ml^{-1}), 2.5 µl x 10 reaction buffer (enzyme and buffer, New England Biolabs Inc., Beverly, USA) and 10.5 µl of ultrapure water and incubation at 37°C for 2 h (Schwieger and Tebbe, 1998). After digestion, the DNA was precipitated and cleaned, and resuspended in 12.5 µl PCR water. The samples were then denatured as described above.

Gel electrophoresis of denatured DNA samples was carried out on non-denaturing 0.5 x MDE (National Diagnostics, Atlanta, USA) polyacrylamide gels with 1.0 x TBE, (10.8 g Tris base, 5.5 g boric acid and 4 ml 0.5 M EDTA (pH 8.0) per litre) (Sambrook *et al*, 1989), as the running buffer. The gels were run on Hoefer SQ3 sequencing equipment using a 0.4 mm comb and spacer set (Amersham Pharmacia Biotech, Little Chalfont, England). Denatured samples (4 µl) were loaded onto gels and run at 600 volts and 20°C for 20 h.

After electrophoresis, the gels were fixed by shaking for 20 minutes in 10% glacial acetic acid, washed 3 x in ultrapure water, and stained by shaking in 0.1% $AgNO_3$ solution for 30 min. After staining, the gels were rinsed briefly in ultrapure water and placed in a 3% Na_2CO_3 solution to develop the template bands. Once the bands were developed, the gels were rinsed in 10% acetic acid for 2 – 3 min, rinsed twice in ultrapure water and left overnight to dry. After drying, the gels were scanned on a Hewlett Packard C7190A flatbed scanner and the profiles examined visually.

2.11 Labelling of isolates with green fluorescent protein

2.11.1 Transconjugative labeling of isolates with green fluorescent protein

The NSA-degrading isolates were labelled with green fluorescent protein (*gfp*) by plate-mating the isolates with *Escherichia coli* strain S17/1λpir (pUT-miniTn5*gfp*Km) (Boon *et al.*, 2000). The plasmid pUT-miniTn5*gfp*Km encodes for *gfp* and kanamycin-resistance (figure 2.1).

A single colony of *Escherichia coli* strain S17/1λpir (pUT-miniTn5*gfp*Km) was aseptically inoculated from agar into 10 ml of LB media plus 50 mg L^{-1} kanamycin, and incubated in an orbital shaker at 25°C and 150 rpm for 20 h. The recipient strains, RBN and BAS were harvested from a 24 h culture in MSM:NSA. After incubation, the cells of both *Escherichia coli* strain S17/1λpir (pUT-miniTn5*gfp*Km) and the recipient strains, were harvested by centrifugation (10000 x g) for 5 min, washed in PBS (3 x 1ml PBS, 10000 x g for 3 min per wash) and resuspended in 1 ml LB medium. The recipient strain, either RBN or BAS, was mixed with *Escherichia coli* strain S17/1λpir (pUT-miniTn5*gfp*Km) in a ratio of 4:1 (v/v), The cells were harvested by centrifugation (10000 x g) for 30 seconds, and resuspended in 50 µl of LB media. The suspension was then pipetted on to a 13 mm diam., 0.2 µm nitrocellulose filter paper (Whatman Plc, UK) resting on a TSA plate containing 500 mg L^{-1} NSA. The plates were incubated overnight at 25°C.

After incubation, the mated cells were resuspended by vortexing the filter paper in 10 ml $1/10^{th}$ strength LB media. Transconjugant isolates were selected by streaking the resuspended cells to MSM agar containing 500 mg L^{-1} NSA and 50 mg L^{-1} kanamycin and incubated at 25°C (Timms-Wilson & Bailey, 2001). Colonies growing on the selective agar were inoculated into 100 ml of MSM plus 500 mg L^{-1} NSA and 50 mg L^{-1} kanamycin, and shaken in an orbital shaker at 25°C and 150 rpm. Control inoculations were also incubated and inoculated with either *Escherichia coli* strain S17/1λpir (pUT-miniTn5*gfp*Km) or one of the wild-type strains.

Gfp-labelled isolates were identified by epifluorescent microscopy (section 2.11.2), PCR of the DNA with *gfp* primers (sections 2.10.1, section 2.1.4) and the ability to degrade NSA (section 2.3.2) in the presence of kanamycin. The catabolic activity of the *gfp*-labelled isolates was assessed by inoculation into MSM plus 500 mg L^{-1} NSA (section 2.3.2).

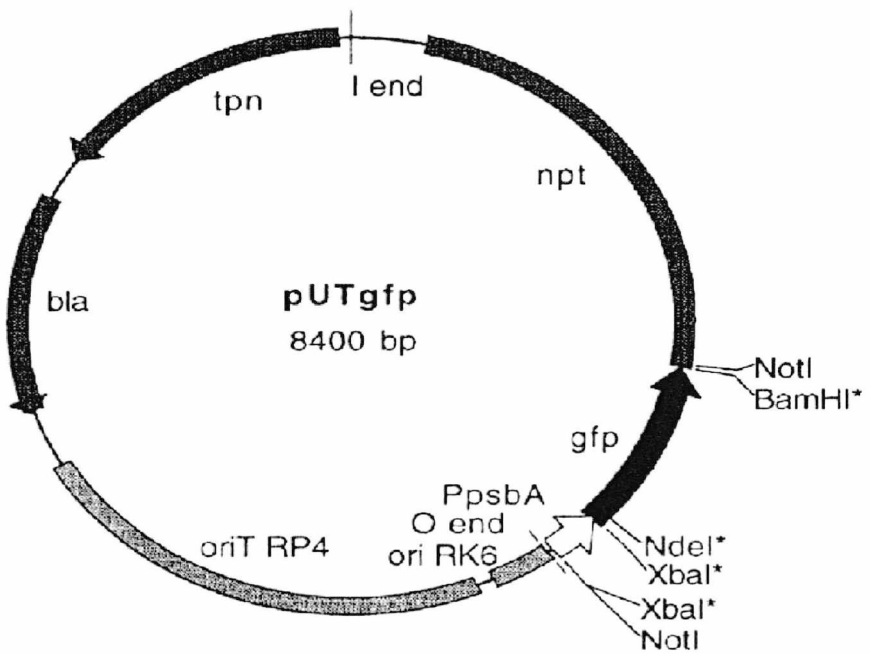

Figure 2.1 – Bacterial plasmid vector, pUT-miniTn5*gfp*Km, used for the transconjugative labelling of isolates with *gfp*. Enzyme restriction sites are shown (reproduced from Tombolini *et al.*, 1997).

2.11.2 Epifluorescent microscopy of *gfp*-labelled transconjugants

Transconjugant cells, 2 ml, grown in MSM plus 500 mg L^{-1} NSA and 50 mg L^{-1} kanamycin, were centrifuged (10000 x g) in a microfuge tube for 3 minutes. The volume of medium was reduced to 0.2 ml and the cell pellet resuspended by flicking the tube. The suspension was pipetted onto a microscope slide, covered with a coverslip and viewed on a Nikon epifluorescent microscope at 100 x magnification with blue light excitation. Unlabelled wild-type isolates were viewed as a control.

2.12 Reactor configurations

2.12.1 Bioaugmentation with *Sphingomonas* sp. strain RBN of a suspended floc reactor receiving a multi-component feed containing NSA

A suspended floc reactor, consisting of an aeration tank and a settling tank (working volumes of 5 litres and 1.75 litres respectively) was fabricated in perspex (Figure 2.2). At start up, the aeration tank was inoculated with 50 ml of activated sludge from sludge 4 (section 2.1.1) with a mixed liquor suspended solids (MLSS) content of 17500 mg L^{-1} (dry weight of solids) to give a MLSS in the aeration tank of 175 mg L^{-1}. MLSS was measured by filtering 50 ml of the aeration tank medium through Whatman Grade 4 filter paper (Whatman, UK) that had been oven-dried at 100°C and weighed on a four figure microbalance (Avery Berkel, UK). After filtration, the filter papers were oven-dried at 100°C, cooled in a desiccator and weighed.

The reactor was operated with a hydraulic retention time (HRT) of 16 h and the mixed liquor MLSS was maintained at approximately 175 mg L^{-1} by recycling of the settled MLSS from the settling tank into the aeration tank. The reactor was fed with CM (section 2.7) at 5.4 ml/minute and the sludge recycled to the aeration tank at 1.6 ml/min, by two Flow Inducer peristaltic pumps (Watson-Marlow Inc., Falmouth, UK). The medium in the reactor was aerated with a Dymax 2 pump (Charles Austen Pumps Ltd, West Byfleet, UK). The relative dissolved oxygen was measured with an Ingold polarographic oxygen electrode (Mettler

Toledo Process Analytical Inc., Woburn, USA), a Uniprobe 501A polargraphic electrode preamplifier (Uniprobe Instruments Ltd., Llanfyrnach, UK) and a Braun Biolab CP oxygen meter (B. Braun Diessel Biotech GmbH, Melsungen, Germany). Prior to inoculation of the reactor, the oxygen electrode was calibrated by sparging the medium in the culture vessel with nitrogen, to determine 0% O_2, and with air to determine 100% O_2 at the requisite flow rate.

The reactor was operated as described above and the COD (section 2.2.1.1) of the influent and effluent, and the MLSS in the aeration tank measured. After 17 d of operation, 50 mg L^{-1} of each of NSA, phenol sulphonic acid (PSA) and compound #21 were added to the reactor feed. In addition to the measurements of COD and MLSS, the concentrations of NSA, PSA and compound #21 in the influent and effluent were measured by HPLC (section 2.2.1.4).

After addition of the NSA, PSA and compound #21 to the reactor feed, the reactor was equilibrated for 5 days and then inoculated with 1 litre of a 48 h growth culture of strain RBN in MSM plus 500 mg L^{-1} NSA. Subsequent to inoculation, the reactor was sampled at 0, 1, 2, 3, 4, 5, 6 and 24 h and COD, MLSS and HPLC analysis performed as described previously. The COD and MLSS data were used to derive k_{COD}, the maximum organic substrate removal rate, using the equation:

$$k = So(So\text{-}Se)/XvT$$

where So = COD of the influent (mg L^{-1})

Se = COD of the effluent (mg L^{-1})

Xv = MLSS in the aeration tank (mg L^{-1})

T = HRT (d^{-1})

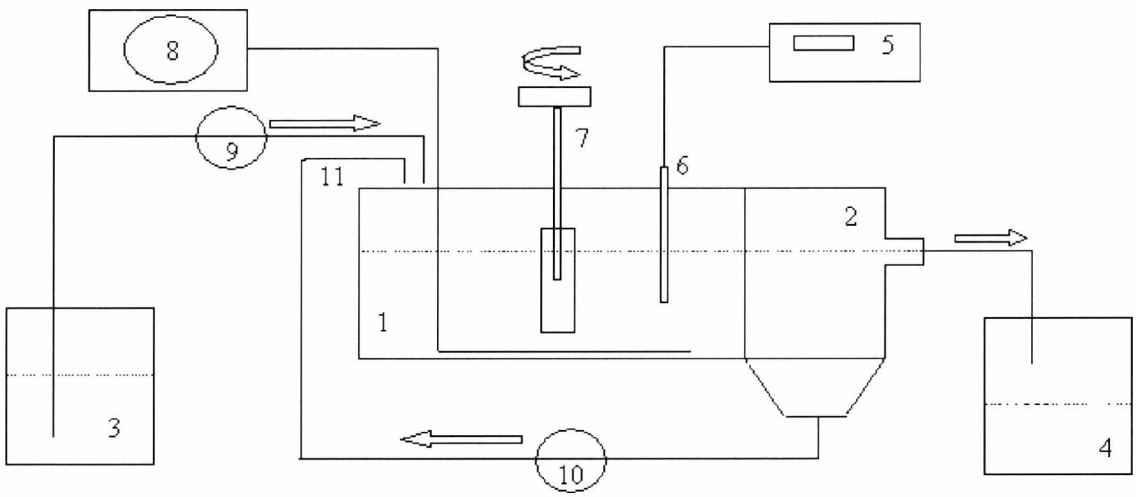

Figure 2.2. - The configuration of the suspended floc reactor: 1- Aeration tank; 2 - Settling tank; 3 - Feed reservoir; 4 - Effluent reservoir; 5 - Oxygen meter; 6 - Oxygen probe; 7 - Stirrer; 8 - Aeration pump; 9 - Peristaltic pump, feed line; 10 - Peristaltic pump, sludge return line; 11 - sludge return.

2.12.2 Chemostat reactor

Enrichments were carried out with chemostat equipment (B. Braun Diessel Biotech GMBH, Germany) consisting of an oxygen meter, stirrer speed controller, temperature controller, pH meter and pH controller, and a 2 litre culture vessel (Figure 2.3). For all the procedures described, the working volume of media in the culture vessel was 1 litre. Aeration was supplied at 1 l h^{-1} with a LH Fermentation 500 Series III aerator (L.H. Engineering Ltd., Stoke Poges, UK) through a sparger at the bottom of the culture vessel. The relative dissolved oxygen was measured with an Ingold polarographic oxygen electrode (Mettler Toledo Process Analytical Inc., USA) and a Uniprobe 501A polargraphic electrode preamplifier (Uniprobe Instruments Ltd., UK). Prior to inoculation of the chemostat, the oxygen electrode was calibrated by sparging the medium in the culture vessel with nitrogen, to determine 0% O_2, and with air, to determine 100% O_2 at the requisite flow rate. The medium was stirred at 250 rpm with 2 six-blade impellers and maintained at 25°C with a heater cartridge. The pH in the culture vessel was measured with a Mettler Toledo 465-35-

K9 pH probe (Mettler Toledo, USA) and automatically corrected to pH 7.0 (± 0.05) with 1M HCl and 1M NaOH. Aseptic inoculation and sampling of the medium was achieved through ports in the lid of the culture vessel. Prior to operation, 1 litre of media was added to the culture vessel and autoclaved for 2 h at 121°C.

The chemostat was run as both a batch reactor and a continuous culture reactor. In continuous culture format, fresh medium was supplied to the reactor by a Watson-Marlow MHRE 7 H.R. Flow Inducer peristaltic pump (Watson-Marlow Ltd, UK) from an autoclaved 20 l demi-john. The flow rate of the pump was calibrated with a measuring cylinder and the dilution rate (d) and HRT were determined. The working volume was maintained at 1 litre by pumping medium out of the reactor with a Watson-Marlow 101U Mk 2 peristaltic pump (Watson-Marlow Ltd, UK) at the same flow rate as the influent medium.

2.12.3 Batch enrichment and continuous culture of *Sphingomonas* sp. strain RBN with naphthalene sulphonate

Batch growth of the isolates was carried out in the chemostat reactor as described (section 2.12.2). One litre of MSM plus 500 mg L^{-1} NSA was inoculated with 10 ml of a 48 h culture of *Sphingomonas* sp. strain RBN. The medium was sampled for HPLC and turbidity for 48 h (section 2.3.2), before the reactor was switched to continuous culture format. MSM plus 500 mg L^{-1} NSA was supplied at a varying dilution rate of 0.16 – 0.02 (HRT of 6 – 48 h). The reactor was sampled for HPLC and turbidity, as described previously.

2.12.4 Batch growth of *Sphingomonas* sp. strain RBN with compound #22

Batch growth of *Sphingomonas* sp. strain RBN was carried out in the chemostat reactor as described (section 2.12.2). One litre of MSM plus 500 mg L^{-1} compound #22, was inoculated with 10 ml of a 48 hour culture of *Sphingomonas* sp. strain RBN. The medium was sampled for HPLC and turbidity, as previously described, until the turbidity in the media reached a steady state. At this point, NSA was added to the reactor to a final concentration of 500 mg L^{-1}. Sampling of the reactor then continued as previously described.

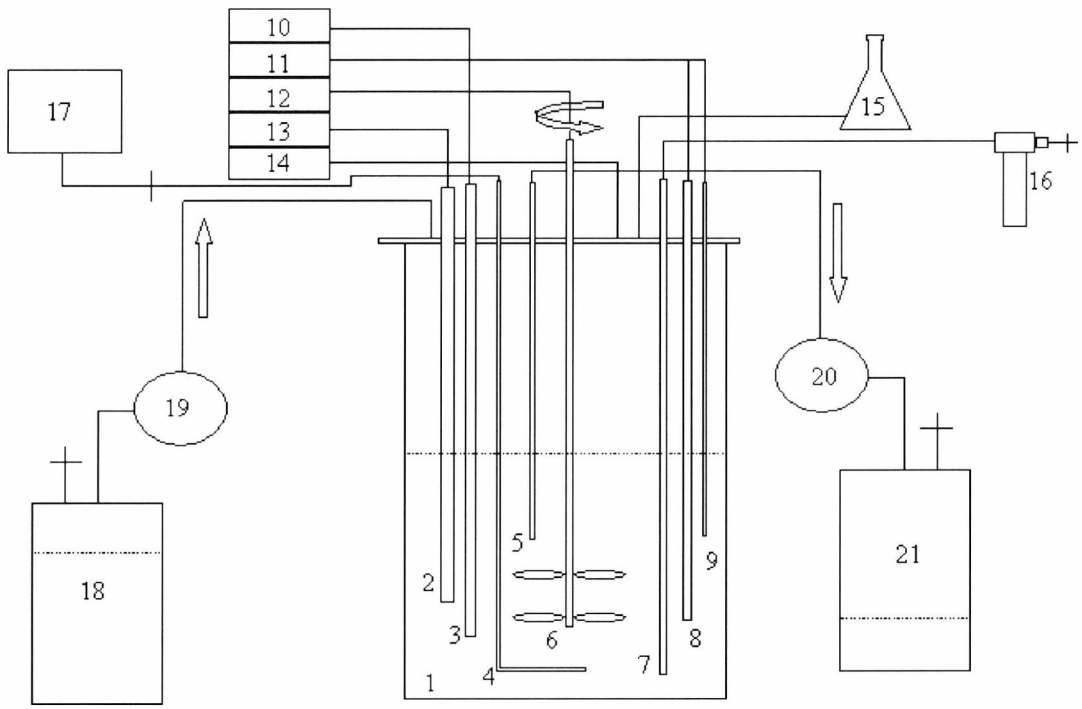

Figure 2.3. Configuration of the chemostat reactor: 1 – culture vessel; 2 – pH probe; 3 – oxygen probe; 4 – sparger; 5 – media outflow; 6 – impeller; 7 – sample port; 8 – heater cartridge; 9 – temperature probe; 10 –oxygen meter; 11 – temperature meter and controller; 12 –impeller speed controller; 13 – pH meter; 14 – pH controller; 15 – inoculation port; 16 – sample collection bottle; 17 – aerator; 18 – feed reservoir; 19 – peristaltic pump, media in; 20 – peristaltic pump, media out; 21 – effluent reservoir. Air filters (0.2 μm) are indicated by +. For batch operation, the reactor was operated without the pumps.

2.12.5 Bioaugmentation of a mixed bacterial community with *Sphingomonas* sp. strain RBN*gfp* for the enhanced degradation of naphthalene sulphonate in a mixed carbon source media

One litre of CM medium (section 2.7) was added to each of two matched chemostat reactors (section 2.12.2). The medium was supplemented with 45.9 mg L^{-1} NSA, equivalent to 10% of the total organic carbon in the medium. Two 15 ml samples of activated sludge (sludge 3) were washed in 2 x 15 ml of PBS for 10 min at 6000 x g, resuspended in 15 ml PBS and 15

ml inoculated into each of the reactors. After inoculation, the inoculation port of each reactor was flushed with 15 ml PBS. The reactors were operated in batch format for 48 h. After 48 h, the reactors were switched to continuous culture format at a dilution rate of 0.04 (HRT = 24 h). Both reactors were fed from a single feed containing CM medium plus 45.8 mg L^{-1} NSA.

The reactors were sampled for HPLC (section 2.1.4.1), DNA extraction (section 2.10.1) and plate counts. For plate counts, a 2 ml sample of the biomass from each reactor was pipetted into a bead-beating tube with 0.2 ml of 50 mM sodium pyrophosphate, and deflocculated by beating at 2500 rpm for 2 min on a Mini-beadbeater (Biospec Products Inc., USA). The deflocculated samples were then serially diluted in PBS and 0.2 ml plated in triplicate on TSA and TSA plus 50 mg L^{-1} kanamycin. The plates were incubated at 25°C for 48 h and the colonies counted.

After 28 d, the reactors were inoculated with 10 ml of 48 h growth cultures of either strain RBN*gfp* or plasmid-cured strain RBN*gfp*C. Strain RBN*gfp*C was plasmid cured by serial subcultures in 10% LB media. The loss of NSA degrading catabolic genes was assessed by subculture of cured cells into MSM plus 500 mg L^{-1} NSA, and PCR of the DNA with *nah*Ac primers (section 2.1.4). Ten millilitres of each inoculum was washed twice 10 ml of PBS, pelleted by centrifugation (5000 x g) for 10 min and resuspended in PBS to an OD_{600} of approximately 0.45, equivalent to the OD_{600} of a 48 h growth culture of RBN*gpf* in MSM plus 500 mg L^{-1} NSA. The reactors were sampled for HPLC, DNA extraction and plate counts before and after inoculation, as previously described. Eight days after inoculation with the *gfp*-labelled isolates, the reactors were shock-loaded by the addition of 250 mg of NSA and sampled for a further 5 days.

The DNA samples were amplified by PCR (section 2.10.4.) with Com1ph/Com2 and *gfp* primer sets (section 2.1.4). The *gfp* amplicons were then quantified by spectrophotometry (section 2.10.3) and the Com1ph/Com2 amplicons analysed by SSCP (section 2.10.9).

2.13 Influence of physicochemical treatment with UV and Fentons reagent on the biodegradability of compound #22

2.13.1 Physicochemical treatment of compound #22 with hydrogen peroxide and UV-light

A 500 mg L^{-1} aqueous solution of compound #22 (250 mg #22 in 500 ml distilled water) was sampled, in triplicate, for COD analysis (section 2.2.1.1, without filtration) and HPLC analysis (section 2.2.1.4). The solution was treated with 5.64 ml of 9% hydrogen peroxide (H_2O_2), to a final H_2O_2 concentration of 1016 mg L^{-1}. The addition of H_2O_2 was calculated from the theoretical oxygen demand (ThOD) for complete oxidation of the putative naphthalene sulphonate-formaldehyde subunit of compound #22 as shown;

Stoichiometric oxidation of 1 sub-unit of compound #22 ($C_{11}H_9O_3S$) by H_2O_2:

$$C_{11}H_9O_3S + 13.25\ H_2O_2 \longrightarrow 11CO_2 + 4.5\ H_2O + SO_3^- + 26.5H^+$$

ThOD of 1 M of naphthalene sulphonate-formaldehyde subunit = 13.25M of H_2O_2 for complete oxidation.

500 mg of naphthalene sulphonate-formaldehyde subunit (Mwt 221) = 0.5/221 = 0.00226M

ThOD of 0.00226M as H_2O_2 = 0.00226M x 13.25 = 0.0299M H_2O_2

Mass of H_2O_2 (Mwt 34) required for ThOD of 500 mg naphthalene sulphonate-formaldehyde subunit = 0.0299 x 34 = 1016 mg

Solutions of #22 (500 mg L^{-1}) were treated with concentrations of H_2O_2 to the equivalent of 0%, 10%, 25%, 50% and 100% of ThOD, with or without iron (III) chloride as a catalyst. The treated solution was then placed in a Heraeus Suntest Accelerated Exposure machine (W.C. Heraeus GmbH, Hanau, Germany) and treated with UV-A & UV-B light (280 – 380 nm) for 2 h. The solution was sampled for COD measurement and HPLC analysis at 0,

30,60, 90 and 120 min. Immediately after sampling, 5 ml of 5 M KH_2PO_4 buffer was added to the solutions, the pH was adjusted to 7.0 with 1M NaOH and residual H_2O_2 removed by treatment with catalase (crystalline suspension of bovine liver catalase, Sigma Chemical Co., UK). Catalase treatment was carried out by adding 28 U of catalase, in 0.05M phosphate buffer at pH 7.0, to each sample and incubation for 30 minutes at 37°C in a Clifton NE5-28 shaking waterbath (Nickel Electro Ltd, Weston Super Mare, UK).

The oxidation products of Fenton-treated compound #22 were determined by GC-MS. Solutions of compound #22 treated with photo-Fentons reagents for 120 min, at 0%, 10%, 25% and 50% of ThOD, were adjusted to pH 2.0 with 1 M HCl and extracted (50 ml) with two changes (20 ml each) of diethyl ether. The diethyl ether extracts were pooled and dried with a stream of dry nitrogen. The residue was dissolved in 15 ml of acetone and derivatised with 5 ml of iodomethane and heating over steam bath for 20 min. The derivatised samples were evaporated under a stream of dry nitrogen, redissolved in 2 ml of hexane and analysed by GC-MS. A 2 µl sample was injected onto a Saturn 2000 GC (Varian Corp., Walnut Creek, USA) with a 60 m (0.25 µm internal diameter, 0.1 µm film thickness) Chrompak CP-Sil 5 CB low bleed GC-column (VMR International Ltd, Poole, Dorset). Analyte detection was carried out with a Saturn 3800 MS-detector (Varian Corp., Walnut Creek, USA). The chromatography conditions were: injector temperature, 250 °C; column oven temperature gradient, 80 °C held for 1 min, to 280 °C (at 10 °C min^{-1}), held at 280 °C for 5 min; total run time, 26 min. Underivatised samples were also analysed. The analytes were identified by comparison of their mass spectra with the NIST 98 (version 1.6) mass spectral library (National Institute of Standards and Technology, Gaithersburg, USA).

2.13.2 Physicochemical treatment and subsequent biological treatment of compound #22

Solutions of compound #22 (500 mg L^{-1}) were sampled for COD analysis (section 2.2.1.1) and HPLC analysis (section 2.2.1.4). The solutions were treated with 9% H_2O_2 to 0%, 10%, 25% and 50% of ThOD and exposed to UV-light for 2 h in a Heraeus Suntest machine (W.C. Heraeus GmbH, Germany).

After the UV-light treatment, 5 ml of 5 M KH_2PO_4 buffer was added to the solutions, the pH was adjusted to pH 7.0 with 1M NaOH and residual H_2O_2 removed by treatment with catalase. Catalase treatment was carried out by adding 28 U of catalase, in 0.05M phosphate buffer at pH 7.0, to each sample, and incubating the samples for 30 minutes at 37°C in a Clifton NE5-28 shaking waterbath (Nickel Electro Ltd, Weston Super Mare, UK).

The treated solutions were amended with 4 ml of vitamin and mineral solutions and 0.4 ml of trace metals solution (section 2.1.5) and sampled for COD and HPLC analysis. One hundred ml of each of the amended solutions was aliquoted into three Erlenmeyer flasks and each flask inoculated with 2.5 ml of a 48 hour culture of *Sphingomonas* sp strain RBN in MSM plus 500 mg L^{-1} NSA. The flasks were sampled for turbidity (OD_{600}), measured spectrophotometrically on a Pye Unicam SP8-100 ultraviolet spectrophotometer (Pye Unicam, England) and then shaken on an Edmund Bühler Type KL2 orbital shaker (Johanna Otto GmbH, Hechingen, Germany) at 150 rpm at 20°C for 96 h. After shaking, the flasks were sampled for COD, HPLC and OD_{600}. The BOD_5 of compound #22, treated with Fentons reagent at 0%, 10%, 25% and 50% of ThOD were also determined (section 2.2.1.2).

2.14 Statistical Analysis

All data points shown are the mean of at least triplicate samples. The standard deviation (σ) of the mean was calculated using the equation;

$$\sigma = \sqrt{\frac{n\sum x^2 - \left(\sum x\right)^2}{n(n-1)}}$$

The standard error of the mean (S.E.M) was calculated from σ using;

$$\text{S.E.M} = \frac{\sigma}{\sqrt{n}}$$

The significance of the difference between two points within a treatment was assessed using the paired student t-test as shown;

$$t = \frac{\bar{x} - \bar{y}}{\hat{o}\sqrt{\frac{1}{n_x - 1} + \frac{1}{n_y - 1}}}$$

where x is the mean of sample x, n_x is the sample number in x and ô is the pooled estimate of variance. The same principles are applied to y. The data were compared with statistical tables at n-1 degrees of freedom. The pooled estimate of variance was calculated as shown below;

$$\hat{o} = \sqrt{\frac{n_x - 1\,\sigma_x^2 + n_y - 1\,\sigma_y^2}{n_x + n_y - 2}}$$

The significance of the difference between two treatments was assessed using the unpaired student t-test as shown;

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{\frac{\sigma^2 x}{n_x - 1} + \frac{\sigma^2 y}{n_y - 1}}}$$

The data were compared with statistical tables applying degrees of freedom;

$$n_x - 1 + n_y - 1$$

CHAPTER 3

DETECTION AND QUANTIFICATION OF NAPHTHALENE SULPHONATE AND NAPHTHALENE SULPHONATE-FORMALDEHYDE CONDENSATES IN INDUSTRIAL EFFLUENTS

Chapter 3

DETECTION AND QUANTIFICATION OF NAPHTHALENE SULPHONATE AND NAPHTHALENE SULPHONATE-FORMALDEHYDE CONDENSATES IN INDUSTRIAL EFFLUENTS

3.1. Evaluation of High Performance Liquid Chromatography for the detection and quantification of naphthalene sulphonate and a naphthalene sulphonate-formaldehyde condensate, compound #22

The experimental work for this project was carried out at two sites, the University of Kent at Canterbury (UKC) and BLC, Leather Technology Centre (BLC). There were differences in the HPLC analytical equipment available at either site and, therefore, two methods were evaluated for the detection and quantification of NSA and compound #22, a synthetic tanning (syntan) material based on naphthalene sulphonate-formaldehyde condensate polymers (SNFC). SNFC are typically a mixture of mono- and disulphonated monomers and oligomers of up to 20-mer (Piotte *et al.*, 1995). As such, reference compounds are not available for SNFC (Redín *et al.*, 1999). Therefore, #22 was chosen as for use as a reference compound.

An ion-pair HPLC (IP-HPLC) method (Reemstma & Jekel, 1994) was evaluated for use at BLC (section 2.2.1.4). Standards curves of NSA and #22 were analysed over a concentration range of 1 – 500 mg L^{-1}. The results are shown in Figures 3.1 and 3.3. The UV-profile of NSA was characterised by a single peak (Figure 3.2) with dual λ_{max} of 227 nm and 273 nm and a retention time (t_R) of 15.5 min (Appendix 1.1). The linearity of the standard curve at 227 nm was poor at concentrations of greater than 50 mg L^{-1}. However, a good fit was achieved at 280 nm (Figure 3.1) with a quantification limit of 1 mg L^{-1}.

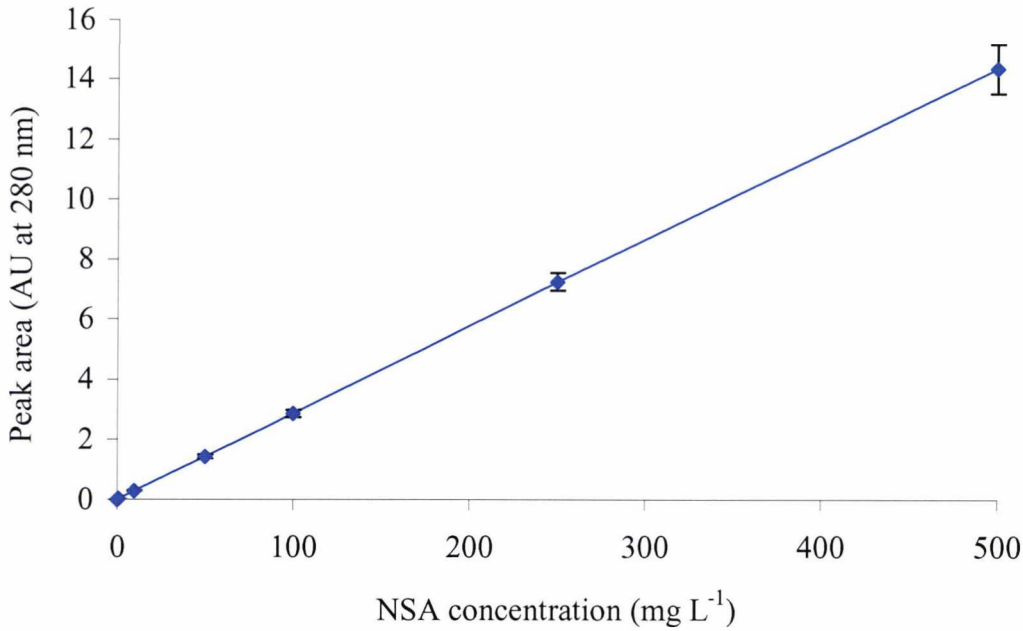

Figure 3.1. Standard curve of NSA determined by IP-HPLC at 280 nm. r^2 = 1.000. Vertical bars indicate S.E.M (n = 11, except 1 mg L^{-1}, n = 7); for symbols without bars, the limits of error were within the confines of the symbol.

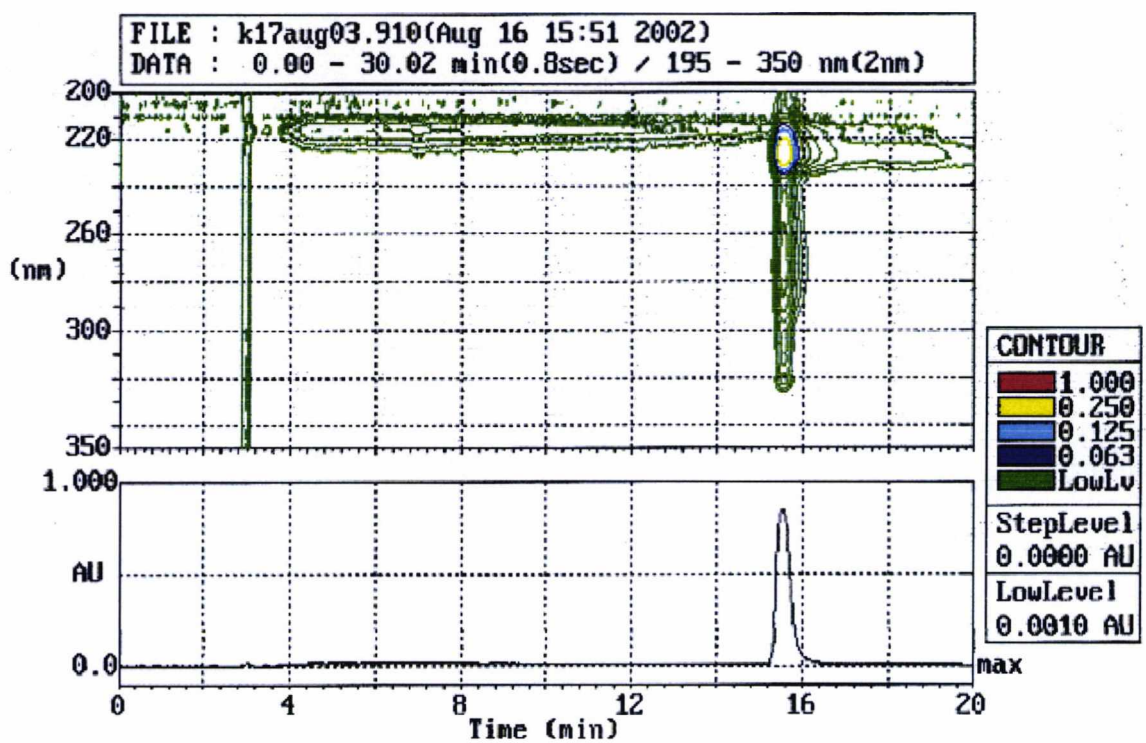

Figure 3.2. IP-HPLC-UV chromatogram of NSA (50 mg L^{-1}), showing dual λ_{max} at 227 and 273 nm and t_R of 15.5 min.

Compound #22 was comprised of four detectable components with t_R of 6.2 min, 8.3 min, 14.1 min and 15.4 min, respectively (Figure 3.4). The peaks of the four components showed dual λ_{max} of 229 and 279 nm, 229 and 281 nm, 223 and 283 nm, and 227 and 273 nm, respectively (Appendix 1.2). In contrast to the standard curve for NSA, the absorbance at 280 nm was weak and, consequently, the quantification of #22 was carried out at 227 nm. Each peak showed a linear relationship between concentration of #22 and peak area (Appendix 1.3). The sum of the peaks was found to give a linear curve (r^2 = 0.997) and, as the relative concentrations of the individual components could not be determined, the quantification of #22 was based on the sum of peaks. A quantification limit of 10 mg L^{-1} was determined. The peak detected in compound #22 at t_R 15.4 min had the same t_R and peak profile as NSA (Appendix 1.1). This peak was therefore, assumed to be NSA. The NSA component of #22 was quantified using the previously determined NSA standard curve and comprised 7.90 % of #22 (39.48 ± 0.68 mg NSA L^{-1} in 500 mg #22 L^{-1}). The standard curve was recalculated without NSA to allow for quantification of the unknown components of #22. The sum of peaks gave a linear curve (r^2 = 0.999) and a quantification limit of 25 mg L^{-1}.

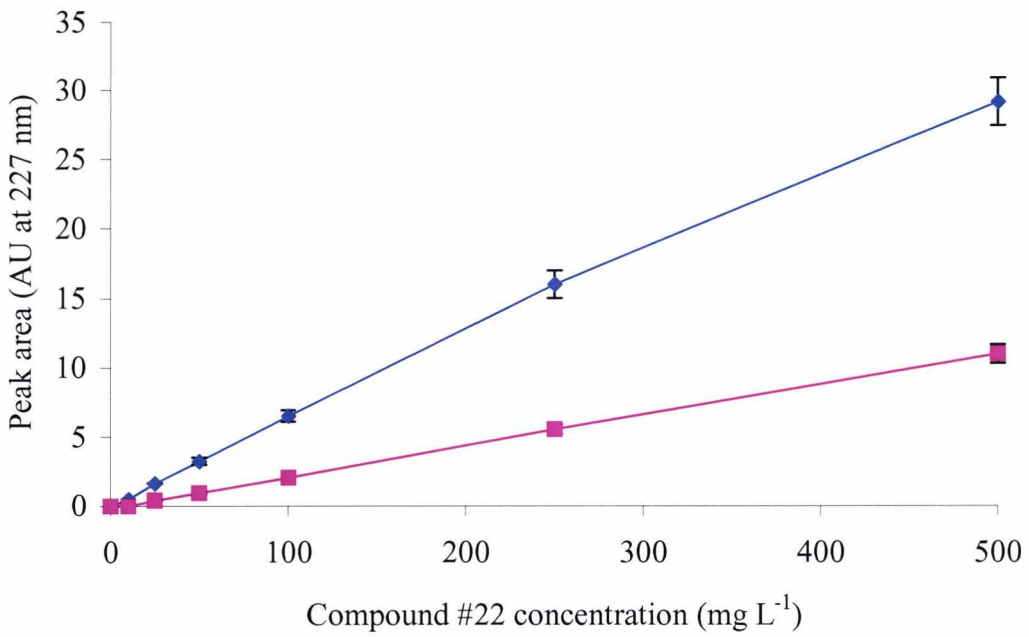

Figure 3.3. Standard curves of compound #22 determined by IP-HPLC at 227 nm; ♦ - sum of peaks plus NSA, ■ – sum of peaks minus NSA. r^2 = 0.997 and 0.999, respectively. Vertical bars indicate S.E.M (n = 6); for symbols without bars, the limits of error were within the confines of the symbol.

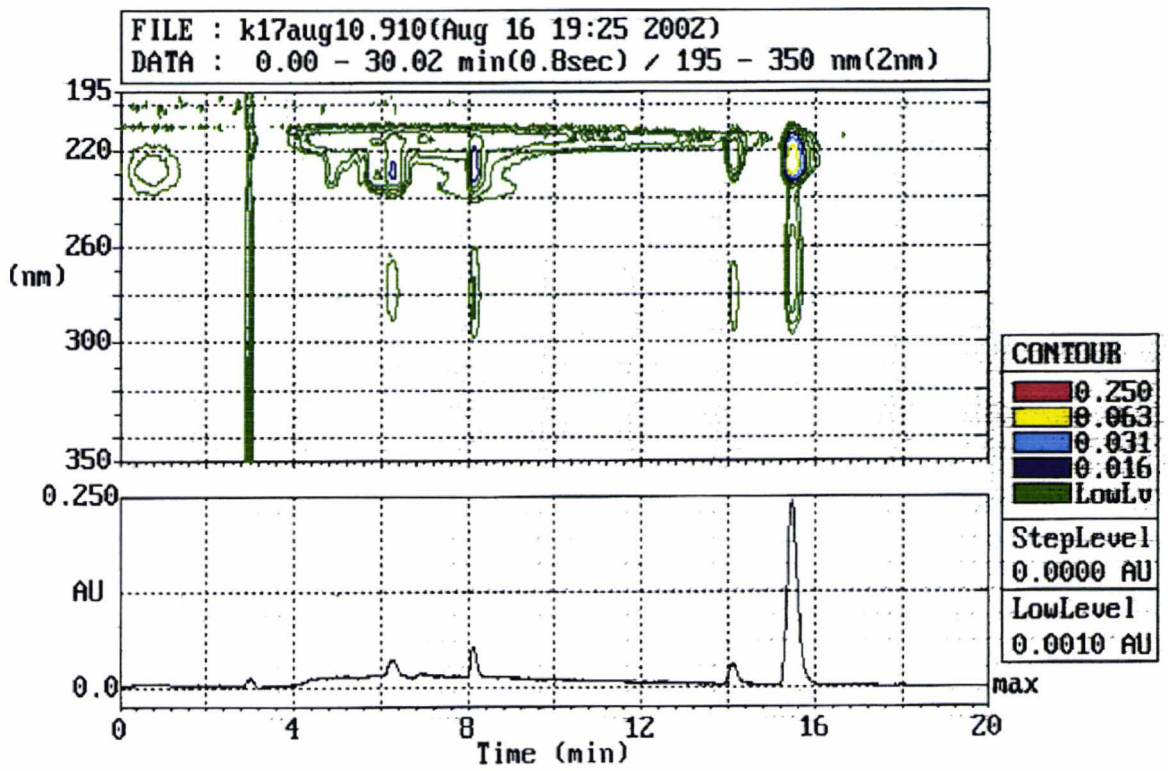

Figure 3.4. IP-HPLC-UV chromatogram of compound #22 (100 mg L^{-1}), showing four components with dual λ_{max} of 229 and 279 nm, 229 and 281 nm, 223 and 283 nm, and 227 and 273 nm and t_R of 6.2 min, 8.3 min, 14.1 min and 15.4 min, respectively.

A second HPLC method (Keck *et al.*, 1997) was evaluated for use at UKC (section 2.2.1.4). Standards curves of NSA and #22 were analysed over a concentration range of 1 – 500 mg L^{-1}. The results are shown in Figures 3.5 and 3.6. Again, the UV-profile of NSA was characterised by a single peak with dual λ_{max} of 227 nm and 273 nm, but at a t_R of 4.9 min (Appendix 1.4). The standard curve showed good linearity at 280 nm (r^2 = 0.999) and a quantification limit of 1 mg L^{-1} was determined. Compound #22 was characterised by a single peak with a t_R 3.5 min and dual λ_{max} of 223 nm and 275 nm (Appendix 1.4). The standard curve showed good linearity at 280 nm (r^2 = 0.999) and a quantification limit of 10 mg L^{-1} was determined. NSA was again detected in #22 but was found to comprise 11.36 % of #22 (56.82 ± 0.49 mg NSA L^{-1} in 500 mg #22 L^{-1}) using this method. Both methods were shown to be reliable and reproducible.

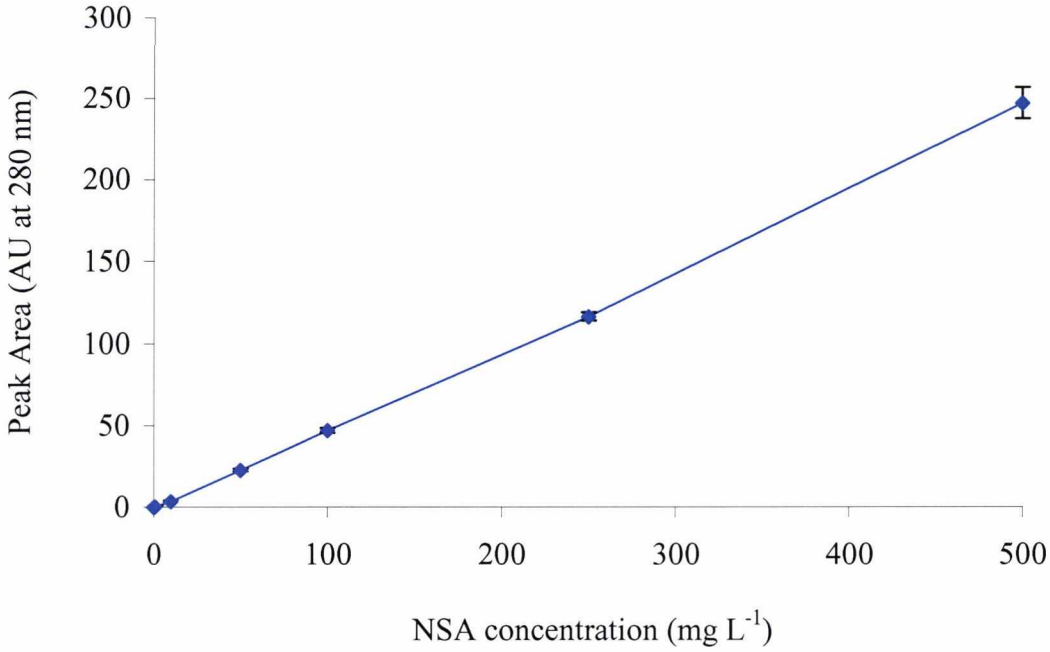

Figure 3.5. Standard curve of NSA determined by HPLC at 280 nm. $r^2 = 1.000$. Vertical bars indicate S.E.M (n = 8); for symbols without bars, the limits of error were within the confines of the symbol.

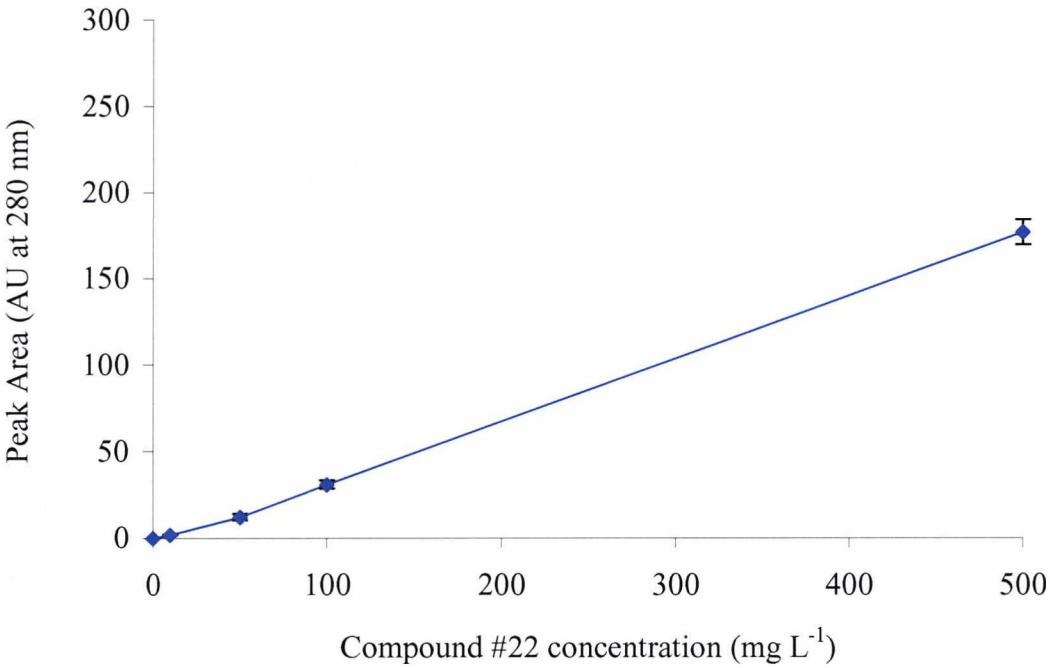

Figure 3.6. Standard curve of compound #22 determined by HPLC at 280 nm. $r^2 = 1.000$. Vertical bars indicate S.E.M (n = 8); for symbols without bars, the limits of error were within the confines of the symbol.

3.2. Determination of naphthalene sulphonate and sulphonated naphthalene-formaldehyde condensates (SNFC) in two industrial effluents

The presence of NSA and SNFC has been reported in groundwater (Riediker *et al.*, 2000; Ruckstuhl *et al.*, 2002), landfill leachate (Riediker *et al.*, 2000; Menzel *et al.*, 2002), industrial effluents (Alonso & Barceló, 1999; Storm *et al.*, 1999) and the effluent from wastewater treatment plants (Alonso & Barceló, 1999; Farré *et al.*, 2002). Two industrial effluents, from a chemical plant manufacturing SNFC including syntans and a tannery using syntans during leather manufacture, were analysed for NSA and SNFC. The effluent from the chemical plant (CE) was subject to primary treatment only prior to discharge to the local municipal sewage treatment works. The tannery effluent (TE) had been biologically treated (secondary treatment) in an on-site conventional activated sludge plant.

Prior to extraction of the effluents, the recovery rates of the SPE extraction (section 2.2.1.3) were determined by extracting 10 mg L^{-1} solutions of NSA and #22. The recovery rates of NSA and #22 were 103 % (± 1.85) and 100 % (± 3.94), respectively. Breakthrough of the retained components, as a result of overloading the SPE cartridges, was assessed by diluting the effluents 1:5 and 1:10 prior to extraction.

The effluents were analysed by IP-HPLC (section 2.2.1.4) after separation of the polar fraction, including NSA and SNFC, by SPE. The polar fraction was eluted, dried and redissolved in HPLC grade water prior to analysis. The precision of the method was measured by repeated analyses (n = 4) of 250 mg L^{-1} of NSA or compound #22 (Di Corcia *et al.*, 1991). Relative standard deviations of 0.97 % and 2.20 % were calculated for NSA and compound #22, respectively. This equated to a theoretical precision of ± 0.01 mg L^{-1} and ± 0.22 mg L^{-1}, at the quantification limit of 1 mg L^{-1} and 10 mg L^{-1} for NSA and compound #22, respectively.The concentrations of NSA and compound #22 in samples were determined by intergration of the peak area and calculation of the analyte using the equation of the line of a concurrently measured standard curve. Significant figures (S.E.M.) are given as a measure of the intra-group variation in the analyte concentrations measured in individual samples. All measured concentrations should be considered within the precision limitations of the analytical method. The results of the analysis are shown in Figures 3.7 and 3.8.

Both effluents contained a number of polar components. The effluent from CE (Figure 3.8) gave rise to a larger number of peaks than the effluent from TE (Figure 3.7), indicating a greater degree of complexity in the polar fraction of that effluent. However, the four largest peaks in both effluents were found to have similar retention times and peak profiles (Appendix 1.5 & 1.6). The retention times and λ_{max} were also similar to those of the four peaks observed in #22 (Appendix 1.2). As with #22, the peak detected at a t_R of approximately 15.4 min was similar to NSA (Appendix 1.1) and this peak was used for quantification of NSA in the effluents. An approximation of the syntan component of the effluents was made, based on the sum of the three peaks common to both effluents and, in the absence of reference standards, using the sum of the same 3 peaks in #22 as a standard curve for quantification (section 3.1, Figure 3.2). The results are shown in table 3.1.

There was a significant difference in the NSA concentrations measured between the diluted and undiluted samples, with the mean concentration measured in the most dilute sample (1 : 10) of TE and CE significantly lower than the mean values of the other dilutions (ANOVA, $F = 9.62$ and 16.66, $p = 0.007$ and 0.001, for TE and CE, respectively). The data indicate that breakthrough of NSA on the SPE cartridge had not occurred but that the measurements had become unreliable in the most dilute samples (1 : 10). Values from these samples were therefore, disregarded when calculating the mean concentrations of NSA in the effluents. Mean NSA concentrations of 61.78 mg L^{-1} ($\pm$ 0.67) and 25.29 mg L^{-1} ($\pm$ 0.06) were measured in the effluent from TE and CE, respectively.

The concentration of syntan measured in the undiluted sample of TE was significantly lower than that measured in the diluted samples indicating that breakthrough of syntan components on the SPE column had occurred (ANOVA, $F = 9.12$, $p = 0.007$). Values from these samples were therefore, disregarded when calculating the mean concentrations of putative syntan in TE. The first peak (t_R 6.8 min) in the undiluted sample from CE was not sufficiently resolved to allow for accurate quantification of the peak area and consequently, the apparent concentration of putative syntan components in the undiluted sample was reduced. Hence, these values were not used to calculate the mean putative syntan concentration in CE. Using the standard curve for sum of peaks in #22, excluding

NSA, mean values for putative syntan components of 394.04 mg L^{-1} (± 3.56) and 838.77 mg L^{-1} (± 11.71) were measured in TE and CE, respectively.

Table 3.1. Concentration of NSA and putative syntan components in two industrial effluents, determined by SPE-IP-HPLC. SEM are shown in brackets (n = 4, except mean values, n = 8), n / a – data not available. The values shown are those in the effluent after adjustment to account for pre-SPE dilution. Values for NSA and syntan shown in italics, were disregarded for calculations of mean concentrations due to the influence of dilution and SPE column, respectively.

Pre-SPE sample dilution	Tannery biological treatment plant effluent		Chemical plant effluent	
	NSA (mg L^{-1})	Syntan (mg L^{-1})	NSA (mg L^{-1})	Syntan (mg L^{-1})
Undiluted	61.45 (± 1.97)	*369.06 (± 2.29)*	25.38 (± 0.08)	n / a
1 : 5	61.95 (± 0.67)	392.78 (± 5.67)	25.19 (± 0.09)	839.00 (± 3.88)
1 : 10	*54.73 (± 1.79)*	395.30 (± 5.09)	*23.87 (± 0.32)*	838.55 (± 24.99)
Mean	**61.78 (± 0.67)**	**394.04 (± 3.56)**	**25.29 (± 0.06)**	**838.77 (± 11.71)**

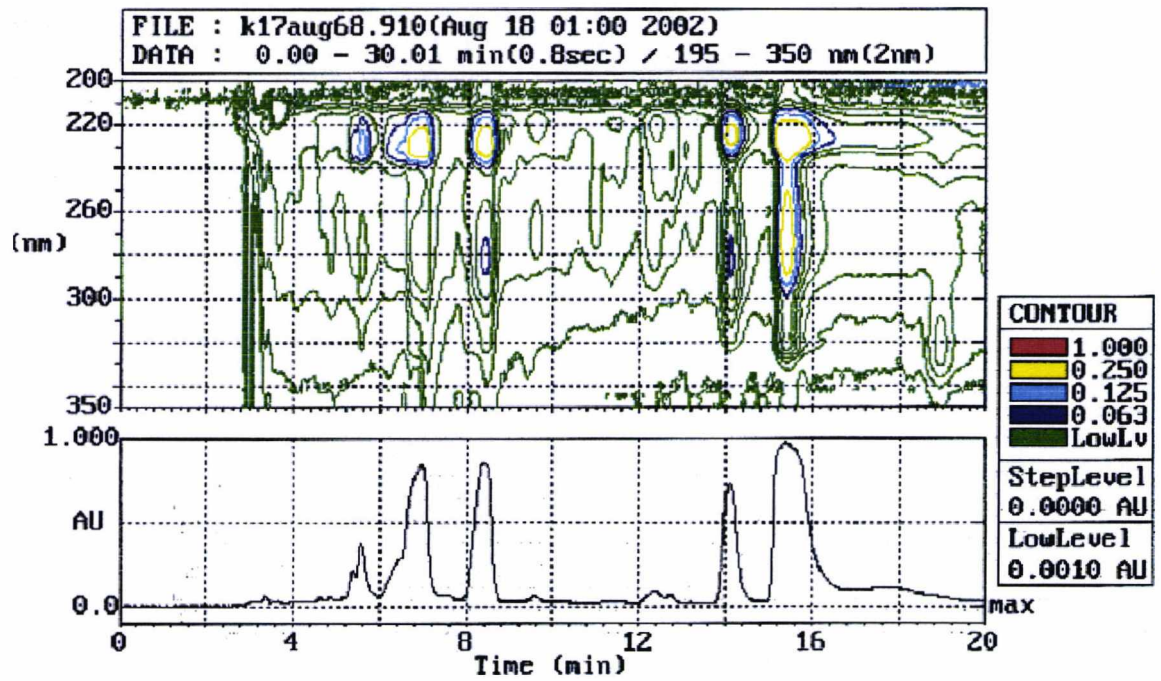

Figure 3.7. SPE-IP-UV-HPLC chromatogram of undiluted tannery biological treatment plant effluent, showing peaks associated with the polar organic components (see Appendix 1.5 for detail).

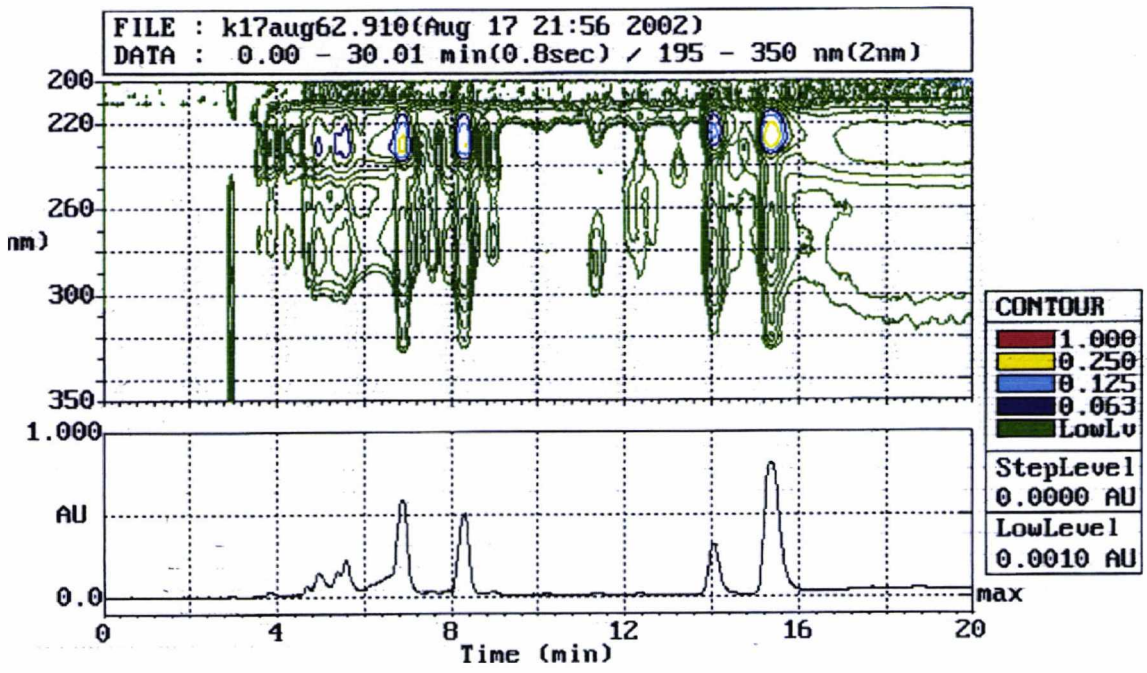

Figure 3.8. SPE-IP-UV-HPLC chromatogram of diluted (1:10) chemical plant effluent, showing peaks associated with the polar organic components (see Appendix 1.6 for detail).

3.3. Estimation of the influence of NSA and SNFC on the chemical oxygen demand and biological oxygen demand of effluents containing NSA and SNFC

The presence of recalcitrant organic compounds will increase the chemical oxygen demand (COD) and reduce the biodegradability of effluents. As a consequence, the ratio of biological oxygen demand (BOD) to COD of effluents will be reduced, decreasing their relative susceptibility to biological degradation. Therefore, the influence of NSA and SNFC (compound #22) on the biodegradability of the effluents was examined.

The ratio of COD to concentration of NSA and #22 was determined (section 2.2.1.1). The results are shown in Figure 3.9. A linear relationship between COD and concentration of each compound was observed. The COD contribution coefficients (*f*), were determined from the slope of the curves by least square regression. Values of *f* of 1.91 (NSA) and 1.20 (#22) were calculated.

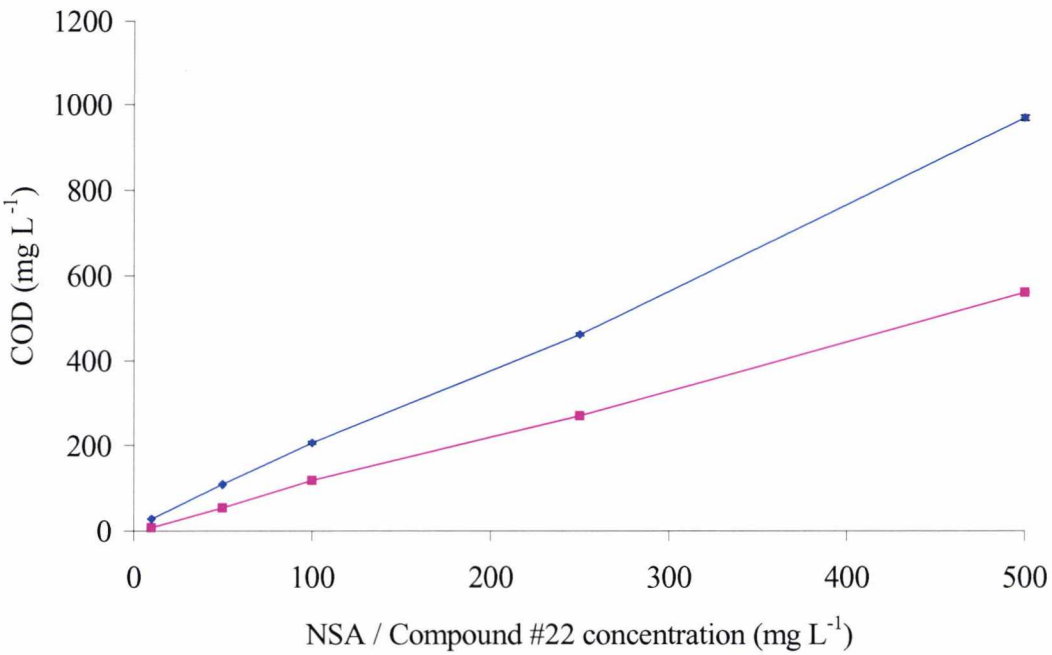

Figure 3.9. Relationship between concentration and COD contribution of NSA (♦) and compound #22 (■), r^2 = 0.999 and 0.999, respectively. Vertical bars indicate S.E.M (n = 3); for symbols without bars, the limits of error were within the confines of the symbol.

The COD of the industrial effluents, TE and CE, were measured as 1788 mg L^{-1} (± 104, n = 3) and 2183 mg L^{-1} (± 54, n = 3), respectively. The proportion of COD in the effluents due to the presence of NSA and SNFC was calculated using the measured concentrations of NSA and SNFC in the effluents (section 3.2) and the *f* values. The results are shown in Table 3.2. NSA and SNFC components were calculated to contribute 33.03 % of the total COD of TE, and 48.31 % of the total COD of CE. NSA was found to contribute 6.59 % (117.99 mg L^{-1}) and 2.21 % (48.30 mg L^{-1}) of the COD of TE and CE, respectively. The contribution of SNFC to the effluent COD was considerably larger than that of NSA, accounting for 26.44 % (472.85 mg L^{-1}) and 46.10 % (1006.52 mg L^{-1}) of TE and CE, respectively. The results indicated that NSA and SNFC were significant components in the COD of both effluents.

The BOD_5 of solutions of NSA, compound #22 and the industrial effluents, with COD values of approximately 200 mg L^{-1}, was determined (section 2.2.1.2). The ratio of BOD to COD was calculated to determine the relative biodegradability. The residual COD of the samples was calculated using the BOD : COD and the initial COD values. The results are shown in table 3.3.

The BOD : COD of NSA (0.28) indicated that limited biodegradation had occurred under the test conditions. The residual COD was estimated from the BOD_5 : COD as a measure of the proportion of COD in that sample that would be deemed to be undegradable under the test conditions. The results indicated that 72 % of the initial concentration of NSA remained in the sample at the end of the test period. The absence of a measurable BOD_5 for #22 suggested that it was entirely undegradable. The BOD_5 : COD measured for TE and CE were 0.73 and 0.41, respectively. On this basis, the residual COD for TE and CE was calculated as 481.1 mg L^{-1} and 1290.83 mg L^{-1}. These figures are similar to the estimated total contribution of NSA and SNFC to the COD of the effluents (table 3.2), suggesting that NSA and SNFC were responsible for a significant proportion of the residual COD in both effluents.

Table 3.2. Calculated values for contribution to the total COD of tannery biological treatment plant effluent (TE) and a chemical plant effluent (CE) due to the presence of NSA and SNFC.

	TE		CE	
	NSA (mg L^{-1})	Syntan (mg L^{-1})	NSA (mg L^{-1})	Syntan (mg L^{-1})
Mean concentration (C) in effluent (mg L^{-1})	61.78	394.04	25.29	838.77
f	1.91	1.20	1.91	1.20
COD (*f* x C) (mg L^{-1})	117.99	472.85	48.30	1006.52
Mean COD of effluent (mg L^{-1})	1788	1788	2183.33	2183.33
Contribution to effluent COD (%)	6.59	26.44	2.21	46.10
Total contribution to effluent COD (%) / mg L^{-1}	33.03 / 590.84		48.31 / 1054.82	

Table 3.3. Determination of the BOD_5 and BOD : COD values for NSA, compound #22, tannery biological treatment plant effluent (TE) and a chemical plant effluent (CE). SEM shown in brackets (n = 3).

Mean samples values	NSA	Compound #22	TE	CE
COD (mg L^{-1})	205.46 (± 3.78)	187.87 (± 1.15)	1788 (± 60)	2183.33 (± 54)
BOD_5 (mg L^{-1})	58.33 (± 2.04)	0	1306.9 (± 30.48)	892.5 (± 0)
BOD_5 : COD	0.28 (± 0.01)	0	0.73 (± 0.03)	0.41 (± 0.01)
Residual COD (mg L^{-1})	147.5	187	481.1	1290.83

Discussion

A reliable method for detection and quantification of target compounds is a prerequisite to the study of effluent remediation. For this study, a reliable and reproducible technique was required for the analysis of NSA and NSA-formaldehyde condensate polymers (SNFC). Several methods for the detection and quantification of NSA in water samples have been reported, including LC-MS (Alonso & Barcelo, 1999), HPLC with fluorescence detection (Redín *et al.*, 1999) and capillary electrophoresis (CE) with laser-induced fluorescence detection (Cugat *et al.*, 2001). As neither LC-MS nor CE analysis were readily available during the course of this study, GC-MS and HPLC techniques were considered. Aromatic sulphonate compounds, such as NSA, have been reported as being very difficult to derivatise (Alonso & Barceló, 1999) and attempts to make both NSA and SNFC suitable for GC-MS analysis, by derivatisation of the sulphonate group with thionyl chloride and trifluoroethanol (Ding *et al.*, 1998), were unsuccessful. Therefore, two methods using HPLC with UV-detection were evaluated: method 1, an IP-HPLC method, and method 2, a HPLC method without IP-reagent (section 2.2.1.4).

Both methods were found to have quantification limits for NSA of 1 mg L^{-1} (section 3.1). A 10-fold concentration of NSA on SPE cartridges prior to analysis of effluent samples (section 2..2.1.3) suggested an effective quantification limit for NSA of 100 µg L^{-1}. Concentrations of between 1 mg L^{-1} (Alonso & Barceló, 1999) and 30 mg L^{-1} (Z. Song, personal communication) of NSA, have been detected in tannery effluents using HPLC with electrospray mass spectrometry (LC-MS). Consequently, both methods were deemed to be sufficiently sensitive for the detection and quantification of NSA in this study.

The analysis of SNFC in industrial wastewaters is hampered by an absence of reference compounds (Redín *et al.,* 1999; Crescenzi *et al.*, 2001). SNFC are used in a variety of industrially applied products and are typically a mixture of components, including mono- and di-sulphonated monomers and oligomers of up to 20-mer (Piotte *et al.*, 1995; Redín *et al.,* 1999; Crescenzi *et al.*, 2001). Redín *et al* (1999) achieved a degree of quantification and identification of the naphthalene sulphonate polymers present in industrial wastewaters by synthesis of defined SNFC oligomers using controlled condensation reactions with formaldehyde. Three dimers were synthesised and used as

reference compounds for the identification and quantification by HPLC, of unidentified compounds in two river water samples. Both the river water samples contained components with peak shapes and retention times that correlated well with the synthesised reference compounds, indicating that those compounds were present in the samples tested. All three compounds were also found in the effluents of a company producing naphthalene sulphonate compounds, of a tannery, a paper manufacturer and domestic effluent. A high degree of similarity was found between the synthesised reference peaks and those in a proprietary syntan. The peak profile of the syntan also matched the peak profiles found in the river water samples, indicating that the syntan components could be used as markers of contamination of environmental samples by SNFC.

In this study a proprietary syntan, compound #22, was used as a reference compound for the detection of SNFC in effluents (section 3.2). The composition of #22 was elucidated by IP-HPLC with electrospray mass spectrometry and consists of 32.7 % monomers, 12.1 % dimers, 12.8 % trimers and 10.3 % tetramers with the remaining 32.1 % consisting of oligomers of 5 – 11-mer (Z. Song, personal communication). Only four peaks were resolved in #22 by method 1 (Figure 3.4). One of these peaks was identified as NSA, on the basis of retention time and peak shape. NSA has been detected in other SNFC-based compounds, such as dye dispersants (Piotte *et al.*, 1995) and specifically, as a component of #22 (Z. Song, personal communication). It is therefore, possible that the components of #22 were either coeluting in the three unidentified peaks or were below the detection limit of the method. As the ratio of peak area to concentration of the components was unknown, it was not possible to determine the concentration of the individual components in #22 and hence, quantification of #22 was based on the sum of the resolved peaks.

In contrast to method 1, only 2 peaks were resolved from #22 by method 2. One of these peaks was identified as NSA (section 3.1). The reduction in resolution efficiency can be attributed to the differences in the chromatographic conditions of both methods. Method 1utilised an ion-pairing reagent and gradient mobile phase whereas method 2 was based on an isocratic mobile phase without an ion-pairing reagent. Isocratic mobile phases rarely represent the optimum conditions for separation of complex mixtures. Additionally, the use of ion-pairing was shown to be critical in the resolution of the

components of SNFC. Analysis of a mixture of SNFC resolved only one peak when analysed by HPLC with pure water as the mobile phase, whereas no peaks were eluted when water containing tetrabutylammonium bromide (TBABr), as an ion-pairing agent, was used as the mobile phase. However, some resolution of the components of the SNFC mixture was achieved with an isocratic mobile phase of water and acetonitrile containing TBABr (Piotte *et al.*, 1995). It is therefore conceivable that, with the exception of NSA, all of the components of #22 were eluted in one peak during analysis with method 2.

Both methods gave reproducible linear standard curves for #22. The resolution of at least some of the components of #22 using method 1, suggested that it would be the more appropriate method for the analysis of environmental samples. This was because the peak shapes and retention times of the resolved components of #22 could be used for comparative identification of peaks resolved from environmental samples. This method has also previously been used for the quantification of syntans in biologically treated tannery effluent (Reemstma & Jekel, 1994). The single peak for #22 achieved with method 2 allowed for accurate quantification of #22. However, the lack of resolution with method 2 would have made it more susceptible to interference from coeluting peaks during the analysis of environmental samples. Consequently, this method was used only for the quantification of known SNFC during the growth experiments described in Chapter 7.

The NSA and SNFC concentrations in two industrial effluents, TE and CE, were determined by SPE and subsequent analysis with method 1 (section 3.2). NSA was expected be present in TE as a component of syntans used in the leather manufacturing process and the concentration in the effluent would be dependent on the efficiency of the biological treatment of the effluent prior to discharge. CE had not been subjected to any kind of treatment and hence, NSA would be present as unreacted raw material from the synthesis of syntans and other SNFC. Both effluents contained NSA at mean concentrations of 61.78 mg L^{-1} and 25.29 mg L^{-1}, for TE and CE, respectively (Table 3.1). Concentrations of NSA between 1 mg L^{-1} (Alonso & Barcelo, 1999) and 30 mg L^{-1} (Z.Song, personal communication) have been reported in tannery effluents.

The presence of NSA in TE demonstrated that NSA may be incompletely removed by biological treatment. Incomplete removal of NSA from tannery effluent by conventional

biological treatment (Castillo *et al.*, 2001; Farré *et al.*, 2002) and membrane biological reactors (Reemstma *et al.*, 2002) has been previously reported. The presence of NSA, as a result of industrial activity, has been detected in groundwater (Riediker *et al.*, 2000; Menzel *et al.*, 2002), river water (Redín *et al.*, 1999; Menzel *et al.*, 2002) and sea water (Gimeno *et al.*, 2001). As such, NSA may be an important component in the COD of industrial effluent and which may, ultimately, be discharged into the environment.

Several methods have been used to quantify SNFC in the environment but have been limited by an absence of reference standards for SNFC oligomers. Menzel *et al.* (2002) were able to detect and determine the distribution of SNFC oligomers in environmental samples with fluorescence detection (HPLC-FLD) but were unable to quantify them. Wolf *et al.* (2000) used HPLC-FLD to determine the quantities of SNFC in industrial effluents, riverwater and groundwater, after determining the elution order of the SNFC oligomers by LC-MS. A synthesised SNFC dimer was used as an external standard for calibration of all the detected oligomers as the degree of polymerisation was shown to have no influence on the fluorescence response factor of the different oligomers. This method was also used by Ruckstuhl *et al.* (2002) to quantify SNFC in groundwater samples. SNFC were also quantified by LC-MS by comparing the contribution of the ion chromatographic profile of an oligomer to the total chromatogram against an internal standard (Crescenzi *et al.*, 2001).

In this study, the analysis of SNFC in the effluents was restricted to HPLC-UV methods (section 2.2.1.4) with quantification by comparison with a reference SNFC, compound #22. With this method, syntan concentrations of 45 mg L^{-1} were previously detected in an aerobically treated tannery effluent by quantification against known concentrations of a proprietary SNFC product (Reemstma & Jekel, 1994). Analysis of both effluents for SNFC was carried out using compound #22 as a reference compound. Both effluents contained components with retention times and peak shapes that matched those resolved from #22 (section 3.2). The sum of these components was used to estimate the concentration of SNFC in the effluents against the standard curve of #22 (section 3.1). The use of the standard curve of #22 for quantification of SNFC in environmental samples was based an assumption of a similar range and ratio of components to compound #22 in the unknown SNFC. On this basis, mean values of SNFC of 394.04 mg L^{-1} and 838.77 mg L^{-1}, were measured in TE and CE, respectively.

Clearly, this is an arbitrary method for the quantification of SNFC in the effluent samples. It is clear from the chromatograms of #22 (Figure 3.4) and the effluents (Figures 3.5 and 3.6), that the ratio of the four quantified peaks was not consistent between the standard and the samples. A number of peaks with similar UV-spectra, but different retention times to the reference peaks, were also apparent in the effluent samples. The quantification of SNFC in the samples may lack precision due to the limitation of the available analytical technique. However, it is clear from the similarity in the retention time and the peak shape of the components in #22 and those detected in both effluents, that SNFC were present in the effluents. A similar correlation between the peak profiles of proprietary SNFC and effluents containing SNFC was reported previously (Redín *et al.*, 1999). Additionally, the sum of the peaks in both effluents indicated that SNFC were present in considerable quantities, albeit that the variation in ratios of the four peaks meant that accurate quantification using #22 as an external standard was not possible.

The proportion of the COD loading of the effluents due to the presence of NSA and SNFC was estimated. COD is an important parameter for the discharge of effluents and is often central to the setting of discharge limits and effluent charges. As such, the removal of components representing a significant proportion of the COD will be of benefit in achieving effluent consent limits and reducing discharge costs. The *f* values of NSA and #22 were determined by least squares regression analysis of the COD of both compounds at a range of concentrations (section 3.3). Values of 1.91 and 1.20 were calculated for NSA and #22, respectively. Values for *f* of between 1.0 (W. Bowden, personal communication) and 1.6 (Song *et al*, 2001) have been reported for SNFC syntans. The *f* values were used to calculate the proportion of COD in the effluents due to the presence of NSA and #22 (table 3.2). The calculated values for the contribution of NSA to the total COD of TE and CE were 6.59 % and 2.21 %, respectively. Due to the potential for inaccuracy in the quantification of SNFC in the effluents, the calculated values for the contribution of SNFC to the total COD of TE and CE, 26.44 % and 46.10 %, respectively, may be inaccurate. However, as SNFC are clearly present in considerable quantities in both effluents, it is reasonable to assume that their contribution to the COD will also be considerable. As such, the removal of NSA and SNFC from both effluents would reduce the COD of those effluents with the subsequent benefits with regard to their discharge.

Naphthalene sulphonate compounds, including NSA and SNFC, are poorly degradable (Wolf *et al.*, 2000). Poorly degradable compounds may contribute to a 'hard' fraction of the COD in effluents that is resistant to biological degradation and may, ultimately, be discharged into the environment (Klinkow *et al*, 1998). The BOD_5 of NSA, #22 and both effluents were determined and the BOD_5 : COD calculated as an indicator of relative biodegradability. (Table 3.3). The BOD_5 : COD of NSA and #22 were 0.28 and 0, respectively, indicating that only limited degradation of NSA occurred over the test period and that #22 was entirely undegradable. SNFC compounds have been reported to have BOD_5 : COD of less than 0.01 (Song *et al.*, 2001) and are therefore considered to be essentially undegradable.

The BOD_5 : COD of TE and CE were also calculated and from these figures, an estimation of the residual COD after the BOD_5 test was made (Table 3.3). These values were used as an indicator of the fraction of COD in the effluents that was not readily degradable. The estimated residual COD of 481.1 mg L^{-1} and 1290.83 mg L^{-1}, in TE and CE, respectively, was similar to the estimated total contribution of NSA and SNFC (590.84 mg L^{-1} and 1054.82 mg L^{-1}, in TE and CE, respectively) to the COD of the effluents (table 3.2). This suggests that NSA and SNFC may have been responsible for a significant proportion of the residual and undegradable COD in the effluents. This conclusion is supported by the limited biodegradability of NSA and total recalcitrance of #22, indicating that these compounds would form a fraction of the residual COD of biologically treated effluents, as shown by the presence of both NSA and SNFC in TE after biological treatment.

Conclusions

NSA and SNFC were detected in both effluents although the quantification of SNFC was limited by the lack of reference standards. The results indicated that NSA and SNFC made up a large proportion of the COD of both effluents. The poor degradability of both compounds suggested that they would be a significant part of the residual 'hard' COD in the effluents and would ultimately be discharged into the environment. As such, the presence of these compounds in effluents is undesirable. Therefore, the scope for increasing the efficacy of treatment of the effluents containing these compounds was investigated.

CHAPTER 4

ISOLATION AND GROWTH CHARACTERISTICS OF NAPHTHALENE SULPHONATE–DEGRADING BACTERIA

Chapter 4

ISOLATION AND GROWTH CHARACTERISTICS OF NAPHTHALENE SULPHONATE–DEGRADING BACTERIA

4.1. Isolation of bacteria degrading naphthalene sulphonate from acclimated activated sludges

Samples of activated sludges 1 and 2 (section 2.1.1) were added to MSM plus 500 mg NSA L^{-1} (section 2.3.1). Turbidity (OD at 600nm) was observed in flasks containing sludge 1 after 72 h, and in flasks containing of sludge 2 after 96h. In order to select for the fastest growing NSA utilising isolates, samples were subcultured into fresh liquid media and shaken for a further 96 h. Flasks showing dense growth were subcultured for a further 2 cycles and finally plated onto MSM agar supplemented with 500 mg NSA L^{-1} and incubated at 25°C. After 7 d, colonies from the subcultures of both sludges were visible on the agar plates but growth was poor.

Individual colonies were aseptically picked from the plates and inoculated into MSM medium plus 500 mg NSA L^{-1}. One morphotype from each of sludge grew in MSM supplemented with NSA. These isolates were designated RBN (sludge 1) and BAS (sludge 2).

4.2. Utilisation of naphthalene sulphonate by the isolates RBN and BAS

The utilisation of NSA by RBN and BAS was investigated (section 2.3.2) by measuring the loss of NSA and bacterial growth (OD_{600nm}) in batch culture (Figures 4.1 and 4.2). Six hours after inoculation with RBN, there was no significant change in the NSA concentration of the medium ($p > 0.05$). After 48 h, the NSA concentration had fallen to 37.1% (± 0.5%) of the 0 h concentration. The turbidity in the medium increased from 6 h to 48 h, to a mean OD_{600nm} of 0.449 (± 0.04). The increase in turbidity was concurrent with the fall in NSA concentration, indicating that RBN had transformed NSA as a source of carbon and energy. The absence of new peaks, indicative of metabolites, on the HPLC

chromatograms, indicated that the NSA had been mineralised. The concentration of NSA remained constant after 48 h ($p > 0.05$).

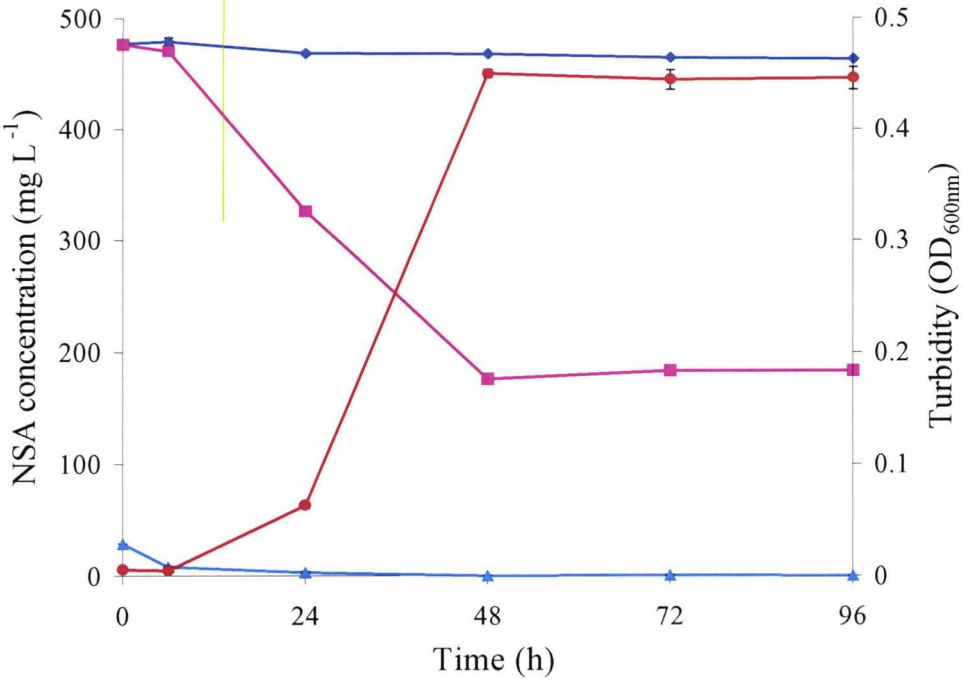

Figure 4.1. Utilisation of NSA and growth profile of isolate RBN. ■ – NSA concentration in RBN inoculated medium (mg L^{-1}), ● - turbidity in RBN inoculated medium, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

A significant decrease (5.9%, $p = 0.02$) in the concentration of NSA was measured six hours after inoculation of the medium with BAS. The concentration of NSA fell sharply from 6 – 48 h, to 34.6% (±0.7%) of the initial concentration. During this period, there was a linear increase in turbidity to a mean 48 h OD_{600nm} of 0.314 (± 0.04). Between 48 and 96 h, the concentration of NSA was constant although the turbidity increased to a mean OD_{600nm} of 0.348 (± 0.01) ($p > 0.05$). No new peaks were observed on the HPLC chromatograms when the concentration of NSA was measured.

After 96 h, the turbidity of the RBN-inoculated medium was significantly higher than that of the BAS-inoculated medium ($p = 0.003$). However, over the same period, the fall in

NSA concentration was significantly greater in the BAS inoculated medium than in the RBN inoculated medium (p = 0.002). After 96 h, the pH of the medium (inoculated with either RBN or BAS) had fallen to pH 4.0 (± 0.09).

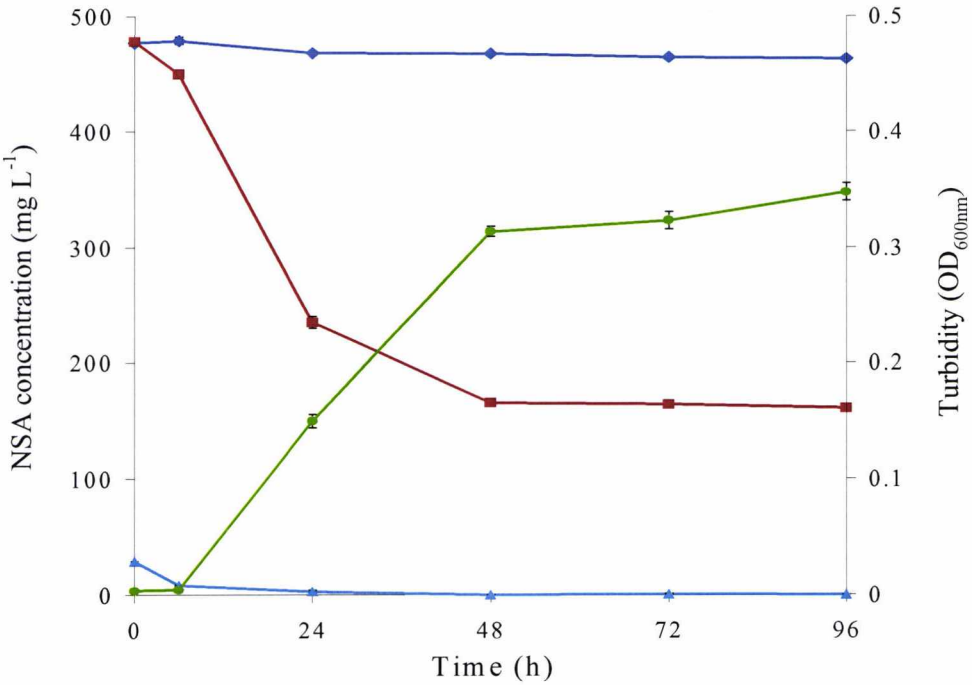

Figure 4.2. Utilisation of NSA and growth profile of isolate BAS. ■ - NSA concentration in BAS inoculated medium (mg L^{-1}), ● – turbidity in BAS inoculated medium, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

4.3. Growth kinetics of RBN and BAS with naphthalene sulphonate

The growth kinetics of the isolates with NSA were determined. Growth kinetics provide valuable insights into the effect of substrate concentration on the growth of the microorganism and its affinity for the substrate (Alexander, 1994a). Determination of growth kinetics will also assist in the selection of suitable organisms for the bioaugmentation of biotreatment systems. The effect of different concentrations of NSA (10 – 1000 mg L^{-1}) on the specific growth rates of both isolates was investigated (section 2.4.3). The data are shown as Lineweaver-Burk plots (Figure 4.3).

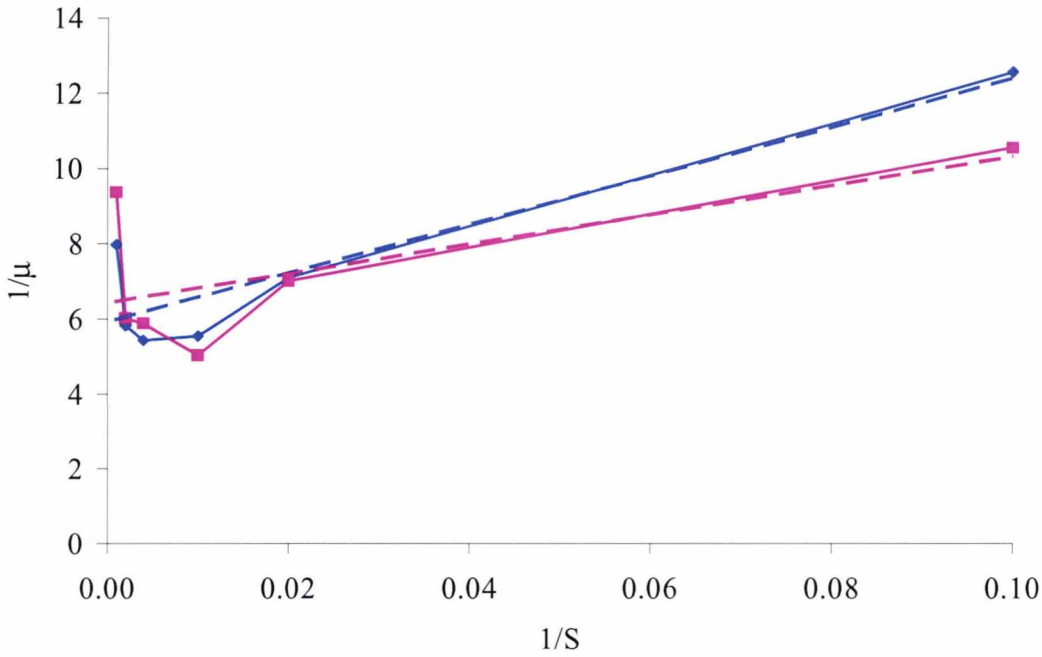

Figure 4.3. Lineweaver-Burk plots of specific growth rates (μ) of RBN (■) and BAS (♦) at varying concentrations (S) of NSA (10 - 1000 mg L^{-1}). Best fit lines (broken lines) were calculated by least squares regression analysis.

Inhibition was observed when the isolates were grown in medium containing 1000 mg L^{-1} NSA. As Monod kinetics assumes a linear relationship between 1/μ and 1/S, the inhibition of growth may have skewed the subsequent calculation of the growth kinetics of the isolates. Therefore, the Lineweaver-Burk plots and determinations of maximum specific growth rate (μ_{max}) and half saturation constant (K_s) were derived from the data from 10 - 500 mg L^{-1} NSA (Figure 4.4). The μ_{max} and K_s values (Table 4.1) were calculated by least squares regression and the biomass yield coefficient (Y) was calculated by dividing the increase in BDW by the amount of NSA consumed (section 2.4.4).

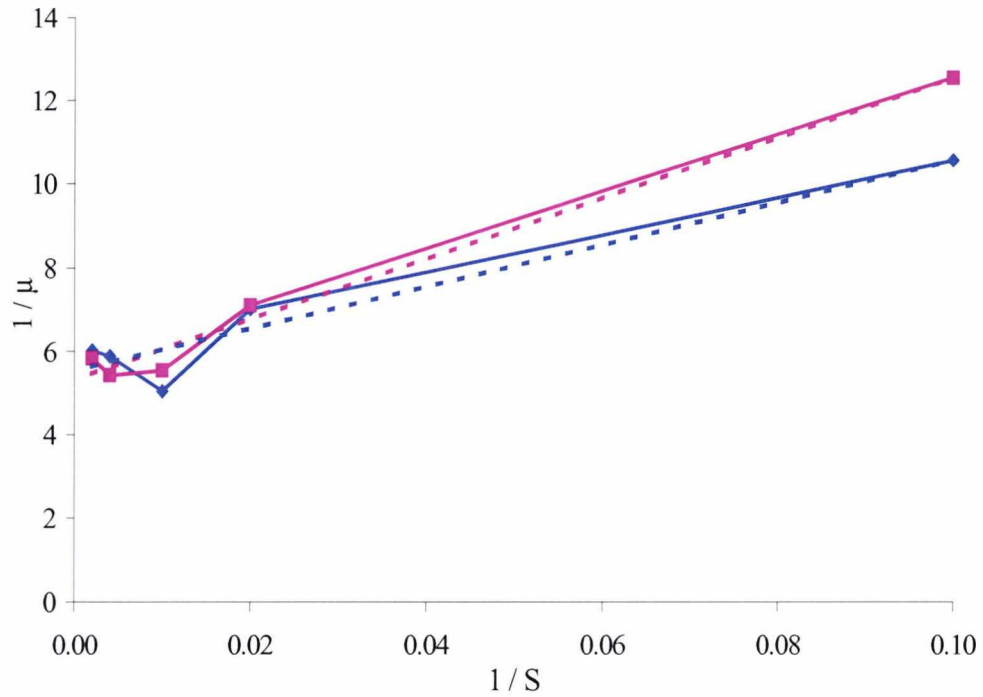

Figure 4.4. Lineweaver-Burk plots of specific growth rates (μ) of RBN (■) and BAS (♦) at varying concentrations (S) of NSA (10 - 500 mg L^{-1}). Best fit lines (---) were calculated by least squares regression analysis.

Table 4.1. Monod growth kinetics of isolates RBN and BAS using NSA as the rate limiting substrate (10 – 500 mg L^{-1} NSA)

Parameter	RBN	BAS
μ_{max} (h^{-1})	0.187	0.180
K_s (g L^{-1})	0.014	0.014
Y (g g^{-1})	0.204	0.144

The observed μ values were compared with theoretical μ values calculated using the Monod model (section 2.4.3). In order to test the assumption that the observed inhibition at 1000 mg L^{-1} would skew the calculated growth kinetics, theoretical values of μ were calculated from the data from 10 - 500 mg L^{-1} NSA and 10 - 1000 mg L^{-1} NSA. The observed and calculated μ values are shown in Table 4.2.

Table 4.2. Comparison of the observed specific growth rates for isolate RBN and BAS with the specific growth rates predicted by Monod kinetics from the growth data for NSA concentrations from 10 - 500 mg L^{-1} and 10 - 1000 mg L^{-1}.

NSA mg L^{-1}	Observed μ		Theoretical μ 10 - 500 mg L^{-1}		Theoretical μ 10 - 1000 mg L^{-1}	
	RBN	BAS	RBN	BAS	RBN	BAS
1000	0.125	0.107	0.185	0.178	0.129	0.154
500	0.171	0.165	0.183	0.176	0.128	0.153
250	0.184	0.169	0.178	0.171	0.126	0.151
100	0.180	0.198	0.165	0.158	0.122	0.147
50	0.141	0.143	0.148	0.141	0.114	0.139
10	0.080	0.090	0.079	0.076	0.078	0.100

Good agreement was found between the observed μ values and the theoretical μ values (10 - 500 mg L^{-1} NSA), indicating that the growth kinetics (Table 4.1) were reliable. The theoretical μ values calculated for isolate RBN from the data for 10 - 1000 mg L^{-1} NSA were considerably different to both the observed μ values, and the theoretical μ values based on the data from 10 - 500 mg L^{-1} NSA. This suggests that the inhibition of growth observed at 1000 mg L^{-1} NSA had skewed the values for the growth kinetics of isolate RBN. However, the theoretical μ values calculated for isolate BAS from the data for 10 - 1000 mg L^{-1} NSA were close to both the observed μ values and the μ theoretical values. This suggests that the growth of isolate BAS was less inhibited than the growth of isolate RBN.

4.4. Utilisation of naphthalene sulphonate by the isolates as a source of sulphur

Aerobic transformations of aromatic sulphonates are formalised into two routes; utilisation as a source of carbon with excretion of the sulphonate moiety, with the degradation largely regulated by an inducible catabolic pathway and independent of a supply of sulphur for growth or; utilisation as a source of sulphur with excretion of the carbon moiety, and regulated by sulphur scavenging systems (Cook *et al.*, 1998). Desulphonation of aromatic sulphonates as a source of sulphur, has been reported (Zürrer

et al., 1987). The capacity of an inoculant to utilise a target compound to satisfy a growth requirement other than carbon, such as the utilisation of NSA as a source of sulphur, may be of benefit in the application of the inoculant for bioaugmentation, i.e. the success of the inoculant may be enhanced by its ability to exploit nutrient source that may not be available to the indigenous population.

The ability of isolates RBN and BAS to utilise NSA as a source of sulphur was investigated. The isolates were grown in a sulphur-free minimal medium with NSA as sole source of carbon, sulphur and energy (section 2.3.2). The data (Figures 4.5 and 4.6) show that RBN utilised NSA as sole source of carbon, energy and sulphur. After 24 h, RBN grew exponentially, reaching maximum turbidity after 48 h (OD_{600nm} = 0.284 ± 0.043). The maximum turbidity was significantly lower than the turbidity at 48 h in MSM plus 500 mg NSA L^{-1} and containing an excess of inorganic sulphate (figure 4.1) (p = 0.037).

Concurrent with the period of exponential growth (24 – 48 h), there was a fall in the concentration of NSA to 51.8% (± 8.6%) of the 0 h concentration. There was a further fall of 13% (± 1.3%) ($p > 0.05$) between 48 h and 96 h. The reduction in NSA concentration after 96 h was significantly greater than that measured when RBN was grown in MSM plus NSA ($p = 0.04$) (section 4.2). In contrast, isolate BAS was incapable of growth utilising NSA as a sole source of carbon and sulphur (Figure 4.6).

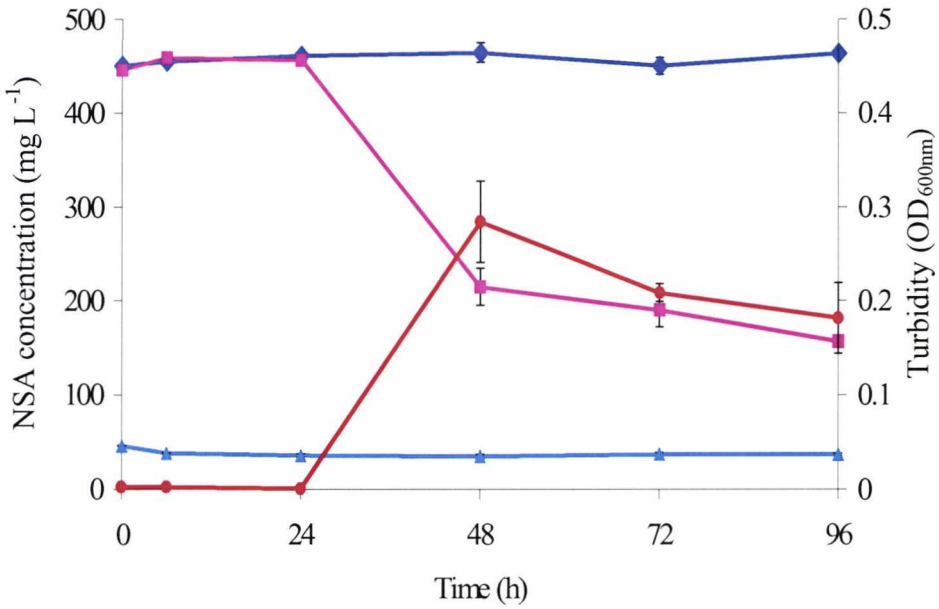

Figure 4.5. Utilisation of NSA and growth profile of isolate RBN in a sulphur-free minimal medium plus 500 mg NSA L^{-1} as sole source of carbon, energy and sulphur. ■ - RBN NSA (mg L^{-1}), ● - RBN turbidity, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

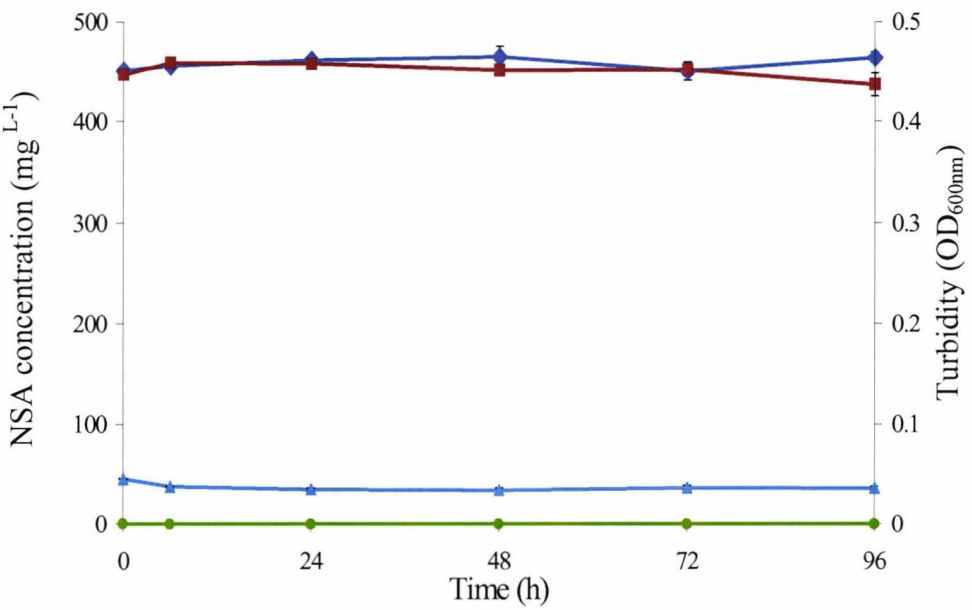

Figure 4.6. Utilisation of NSA and growth profile of isolate BAS in a sulphur-free minimal medium plus 500 mg NSA L^{-1} as sole source of carbon, energy and sulphur (S.E.M shown); ■ - BAS NSA (mg L^{-1}), ● - BAS turbidity, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

The sulphonate moiety of arylsulphonates contributes significantly to the recalcitrance of these compounds (Leidner *et* al., 1980). The utilisation of arylsulphonates as a source of sulphur, but not carbon has been, reported. The oxygenolytic cleavage of the sulphonate group liberates the carbon moiety and increases its biodegradability (Zürrer *et al.*,1987). Therefore, isolates utilising the sulphonate liberated from NSA as a source of sulphur, but not as a source of carbon, may render the carbon moiety more susceptible to subsequent degradation by other microorganisms. This may be of benefit in enhancing NSA degradation in the treatment of wastewaters where other preferentially utilised carbon sources may be present.

The ability of the two isolates to utilise NSA as sole source of sulphur was investigated (section 2.5). The utilisation of NSA and growth of isolates RBN and BAS in the presence and absence of an inorganic sulphur source ($MgSO_4.7H_20$, 0.2 g L^{-1}), and with glucose as the predominant carbon source (2.0 g L^{-1}), are shown in Figures 4.7 and 4.8. The growth profiles of isolate RBN in the unsupplemented and sulphate supplemented sulphur-free media were similar. A small increase in turbidity after 6 h was followed by exponential growth to 24 h. After 24 h, the turbidity of the sulphate-supplemented medium (0.550 ± 0.015) was significantly higher than the unsupplemented medium (0.387 ± 0.003) (p = 0.001) and did not change significantly thereafter. The turbidity in the unsupplemented medium rose significantly from 24 h to the end of the experiment (OD_{600nm} at 96 h = 0.457 ± 00.9) (p = 0.027). The specific growth rate was higher in the supplemented medium (μ = 0.161) than in the unsupplemented medium (μ = 0.136).

After 24 h, similar reductions were measured in the mean concentrations of NSA in both the supplemented medium (61.6 % ± 6.6) and unsupplemented medium (60.7 % ± 3.2). There was no significant change in the concentration of NSA in either media from 24 h until the end of the experiment ($p > 0.05$).

The turbidity of the sulphate-supplemented medium after 96 h was significantly higher (p = 0.001) than in MSM plus 500 mg L^{-1} NSA (section 4.2). There was no significant difference between the unsupplemented medium and MSM plus 500 mg L^{-1} NSA ($p < 0.05$). Maximum turbidity in the unsupplemented sulphur-free medium was not significantly different to that when NSA was supplied as sole source of carbon, energy

and sulphur (Figure 4.5) ($p > 0.05$).There was no significant difference in the reduction of NSA concentration in either the sulphate supplemented or unsupplemented medium and in MSM plus 500 mg L^{-1} NSA, after 96 h.

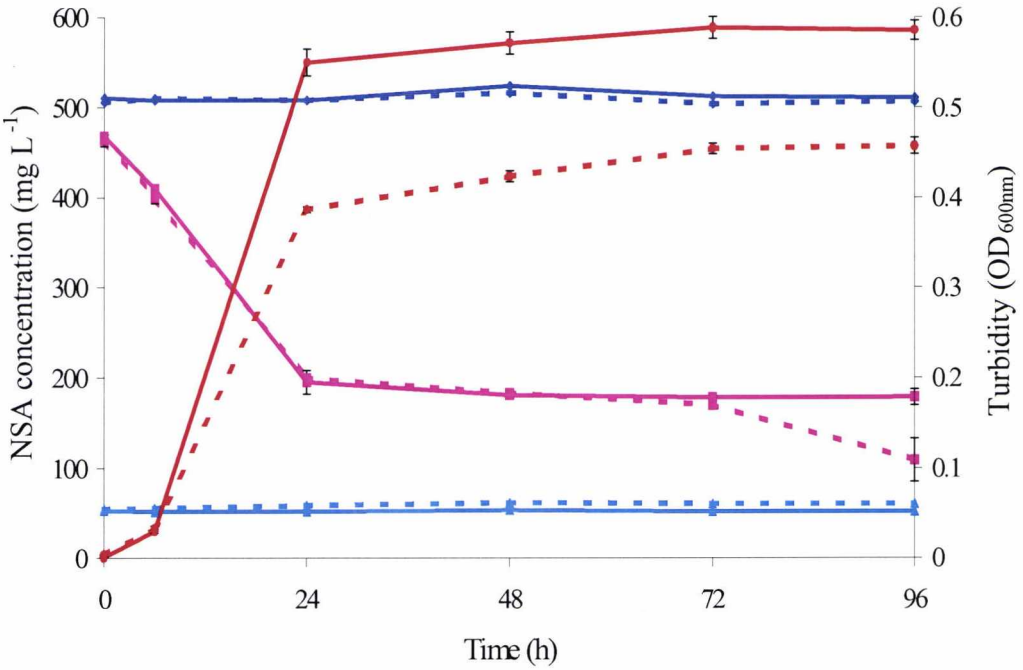

Figure 4.7. Utilisation of NSA and growth of isolate RBN in a sulphur-free medium with and without addition of inorganic sulphate (0.2 g L^{-1} $MgSO_4.7H_2O$). ■ - RBN NSA (mg L^{-1}), ● - RBN turbidity, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control; broken line indicates sulphur-free medium; unbroken line indicates sulphate-supplemented medium. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

The growth profiles for isolate BAS in unsupplemented and sulphur-supplemented media were similar to those of isolate RBN. A lag of approximately 6 h was followed by exponential growth from 6 to 24 h. After 24 h, the OD_{600nm} of the supplemented medium (0.464 ± 0.009) was significantly higher than that of the unsupplemented medium (0.359 ± 0.018) (p = 0.017). As with RBN, the specific growth rate was higher in the supplemented medium (μ = 0.243) than in the unsupplemented medium (μ = 0.222). Significant increases in the turbidity of both the sulphate-supplemented medium (p =

0.0001) and unsupplemented medium (p = 0.005) at 96 h were found when compared with MSM plus 500 mg L^{-1} NSA (section 4.2).

There was no significant difference in the utilisation of NSA by isolate BAS in either medium. The NSA concentration in both media fell rapidly between 0 h and 24 h, to 40.6% (± 1.6), and 39.4% (± 1.2) in the sulphate supplemented sulphur-free medium and unsupplemented sulphur-free medium, respectively. The NSA concentration was further reduced in both the supplemented and unsupplemented media after 96 h (to 30.5% ± 1.5, p = 0.010 and 29.8% ± 1.9, p = 0.008, respectively).

The glucose used in the sulphur-free medium contained a small amount of sulphur (0.005% as sulphate and sulphite, MSDS, Sigma-Aldrich Co. Ltd, England). The influence of inorganic sulphur sources on the growth of the isolates was investigated by inoculating both isolates into the sulphur-free medium plus glucose (2.0 g L^{-1}), as sole source of carbon (Figure 4.9).

The growth profile of isolate RBN in sulphur-free medium plus glucose was similar to that in the sulphur-free medium, with or without additional sulphate (Figure 4.7). A lag of approximately 6 h was followed by exponential growth to 24 h and a small but significant increase from 24 h to 96 h (p = 0.046). The growth profile of isolate BAS in sulphur-free medium plus glucose was similar to those in sulphur-free medium with or without additional sulphate (Figure 4.8), with a lag of approximately 6 h followed by exponential growth to 48 h.

The growth of isolates RBN and BAS in the sulphur-free medium plus glucose was not significantly different from that in the unsupplemented sulphur-free medium (96 h $p >$ 0.05). In contrast, the growth of both isolates in the sulphur-free medium plus glucose was significantly less than in the sulphate supplemented medium (RBN 96 h p = 0.007, BAS 96 h p = 0.002).

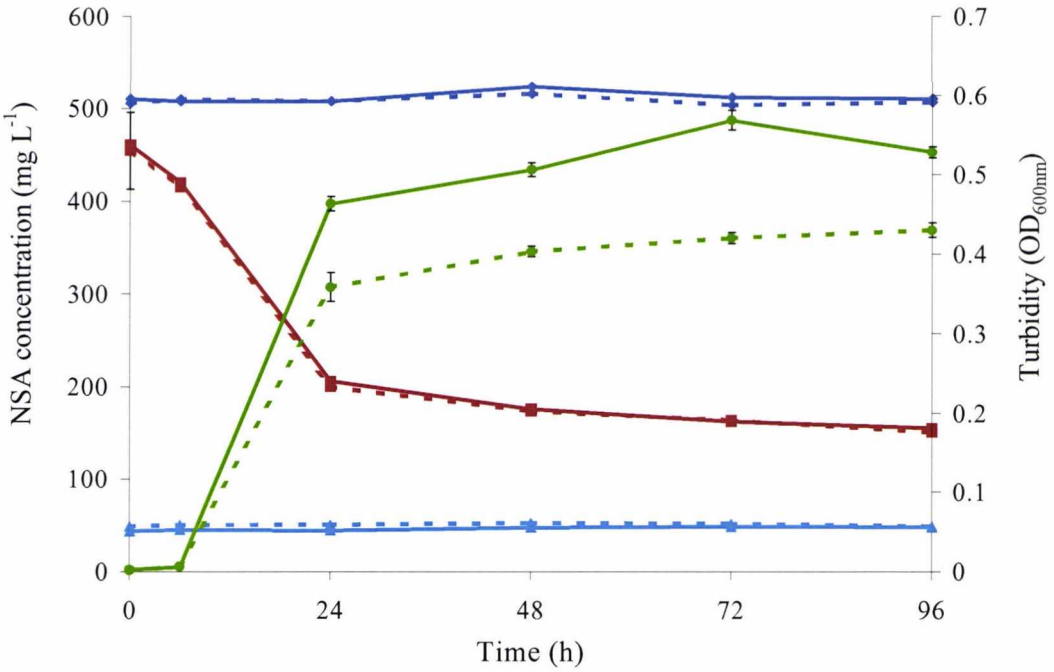

Figure 4.8. Utilisation of NSA and growth profile of isolate BAS in a sulphur-free medium with and without addition of inorganic sulphate (0.2 g L^{-1} $MgSO_4.7H_2O$). ■ - BAS NSA (mg L^{-1}), ● - BAS turbidity, ♦ - uninoculated NSA control (mg L^{-1}), ▲ - uninoculated turbidity control; broken line indicates sulphur-free medium; unbroken line denotes sulphate-supplemented medium. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

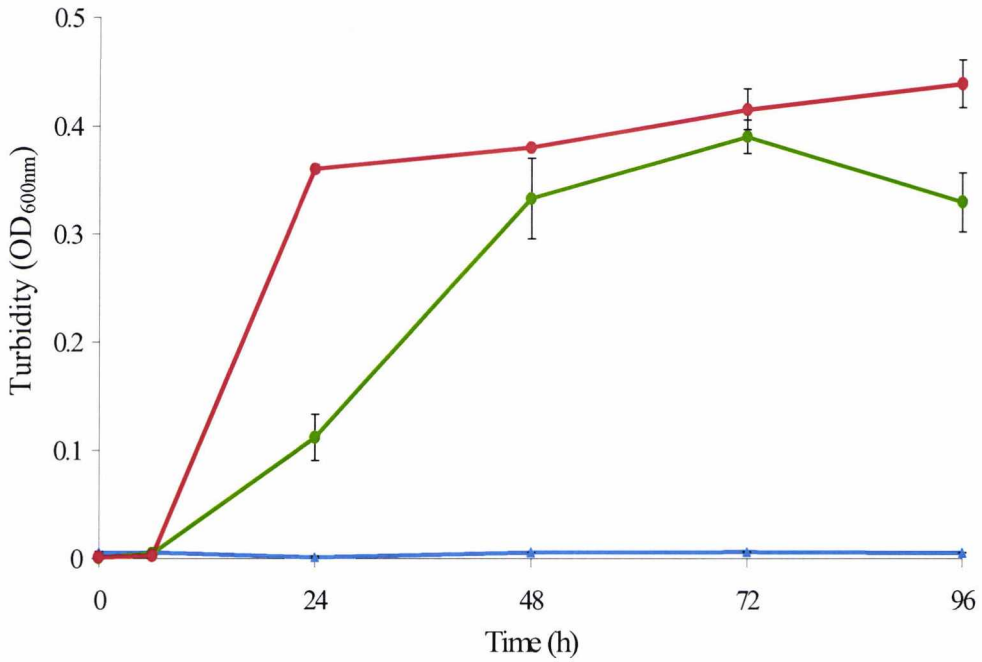

Figure 4.9. Growth profiles of isolates RBN and BAS in sulphur-free medium plus glucose, 2.0 g L^{-1}. • - RBN, • - BAS ; ▲ - control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

4.5. Growth kinetics of isolate RBN with naphthalene sulphonate in continuous culture

Biotreatment plants for the remediation of effluents are dynamic environments with a continuous influx and efflux of pollutant-containing effluent. The kinetics of degradation of an inoculant are, therefore, of great importance. In order to be effective, the inoculant must be able to degrade the target compound in the period of time that it is retained within the biotreatment system. The degradation of NSA by isolate RBN in continuous culture in a chemostat was investigated (section 2.12.3). The chemostat was inoculated with 10 ml of a 48 h growth culture of RBN and run as a batch reactor for 48 h to allow growth of the biomass. After 48 h, the chemostat was run as a continuous culture reactor at a range of dilution rates (Figure 4.10).

During batch culture, a lag period of approximately 36 h was followed by rapid growth and a concurrent fall in NSA concentration between 36 and 48 h. Both the turbidity (48 h OD_{600nm} = 0.672) and fall in NSA concentration (to 0.52% of 0h concentration) were

greater than when RBN was grown in batch in shake flasks (48 h OD_{600nm} = 0.449, fall in NSA to 37.1%) (section 4.2). The specific growth rate (μ) of isolate RBN in the chemostat was 0.192 h^{-1}, higher than both the μmax observed and calculated (0.184 h^{-1} and 0.187 h^{-1}, respectively) in the batch enrichments (section 4.3). A biomass yield of 0.245 g g^{-1}, was estimated from the turbidity by extrapolation of the plot of biomass dry weight (BDW) vs. OD_{600nm} (section 2.4.4).

Following batch growth, the reactor was operated in continuous culture mode at dilution rates (*D*) of 0.02 – 0.16 d^{-1} (HRT 6 - 48 h, organic loading rate of 10.42 – 83.33 mg NSA h^{-1}) with a feed of MSM plus 500 mg NSA L^{-1}. Turbidity (OD_{600nm}) and biomass dry weight (section 2.4.4) and NSA concentration in the effluent (section 2.2.1.4) were measured. The BDW (mg L^{-1}) of isolate RBN increased with dilution rate. However, the removal of NSA was not significantly affected by the changes in *D* or BDW, except after a change in *D* from 0.02 d^{-1} to 0.16 d^{-1} when NSA removal fell to 79.88%. This was followed after 19 h, by a rise in BDW and almost complete removal of NSA.

The steady-state growth kinetics for RBN in continuous culture were calculated (Table 4.3). The theoretical maximum yield Y_E (BDW g L^{-1} when grown on 500 mg NSA L^{-1}, assuming no maintenance) and the maintenance coefficient *m*, were determined by least squares regression analysis of the steady state kinetics (Bainotti & Nishio, 2000). The data are presented as a Tempest plot (Figure 4.11). Y_E was determined as 0.259 g BDW g^{-1} NSA (59.523 g BDW NSA mol^{-1}) and *m* was determined as 0.024 mg NSA g^{-1} BDW (0.105 mmol NSA g^{-1} BDW). Y_E and *m* were then used to calculate Y_S, the theoretical maximum yield (BDW) accounting for the maintenance requirements of the biomass, using the equation (Pirt, 1975):

$$\frac{1}{Y_S} = \frac{m}{\mu} + \frac{1}{Y_E}$$

Y_S (0.250 g BDW g NSA) was in good agreement with the observed yield of isolate RBN when grown in the chemostat in batch mode with 500 mg NSA L^{-1} (Y = 0.245 g BDW g^{-1} NSA) i.e. the predicted maximum yield and observed maximum yield were comparable, indicating that the calculated steady state kinetic values were accurate.

The mass of carbon from NSA converted to biomass was calculated as 0.236 biomass g carbon g^{-1} (23.6%), as shown below, on the assumption that carbon accounts for approximately 50% of the cell biomass (Luria, 1960):

Y = 0.245 g NSA g^{-1}

Yc = 0.245 x 0.5 = 0.123 g

C g NSA g^{-1} = 0.521 g

C converted to biomass = 0.123 / 0.521 = 0.236 biomass g carbon g^{-1}

NSA removal rates (NSA mg BDW mg^{-1} h^{-1}) for each organic loading rate were determined and found to have a linear relationship with the organic loading rate, indicating that the kinetics of removal were first order (Figure 4.12). Degradation kinetics were determined according to Michaelis-Menten kinetics from least square regression analysis of the reciprocal of both sets of data. A Lineweaver-Burk plot of the data is shown in Figure 4.13. The V_{max}, the maximum NSA removal rate (NSA mg BDW mg^{-1} h^{-1}), and K_m, the organic loading rate at which the NSA removal rate equals 0.5 V_{max}, for isolate RBN were calculated as 1.28 mg NSA mg^{-1} BDW h^{-1} and 121.27 mg h^{-1}, respectively.

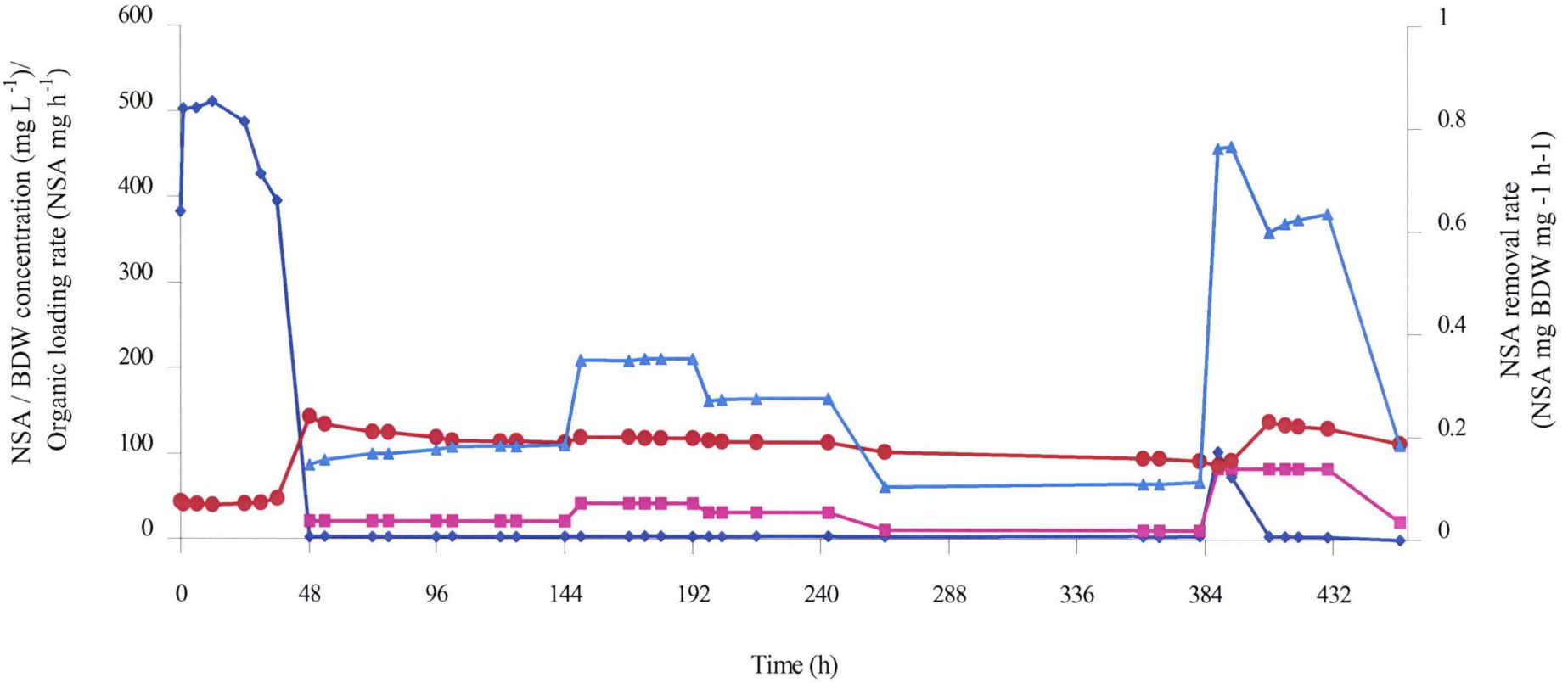

Figure 4.10. Growth and utilisation of NSA by isolate RBN in batch culture (0 – 48 h) and continuous culture (48 – 457 h) at a range of organic loading rates. ♦ - NSA concentration, ● - RBN BDW, ■ - organic loading rate, ▲ - NSA removal rate.

D	n	X	Se	ΔSe	Y_E	q_E
0.02	4	0.096	0.016	2.157	44.547	0.449
0.04	9	0.123	0.012	2.161	56.692	0.738
0.06	4	0.114	0.014	2.160	52.840	1.136
0.08	5	0.118	0.014	2.160	54.848	1.458
0.16	6	0.119	0.139	2.034	58.0792	2.783

Table 4.3. Steady-state values for isolate RBN grown in continuous culture at a range of dilution rates using NSA at an initial concentration of 500 mg L^{-1} (2.173 mmol L^{-1}). All data are the mean of n samples at each dilution rate. D = dilution rate (h^{-1}); X = BDW g L^{-1}; Se = effluent concentration of NSA (mmol L^{-1}); ΔSe = NSA catabolised (mmol L^{-1}); Y_E = true growth yield on NSA catabolised (BDW g NSA mol^{-1} catabolised) where $Y_E = X/\Delta Se$; q_E = specific NSA catabolising rate (NSA mmol BDW g^{-1} h^{-1}) where $q_E = D/Y_E$ (Bainotti & Nishio, 2000).

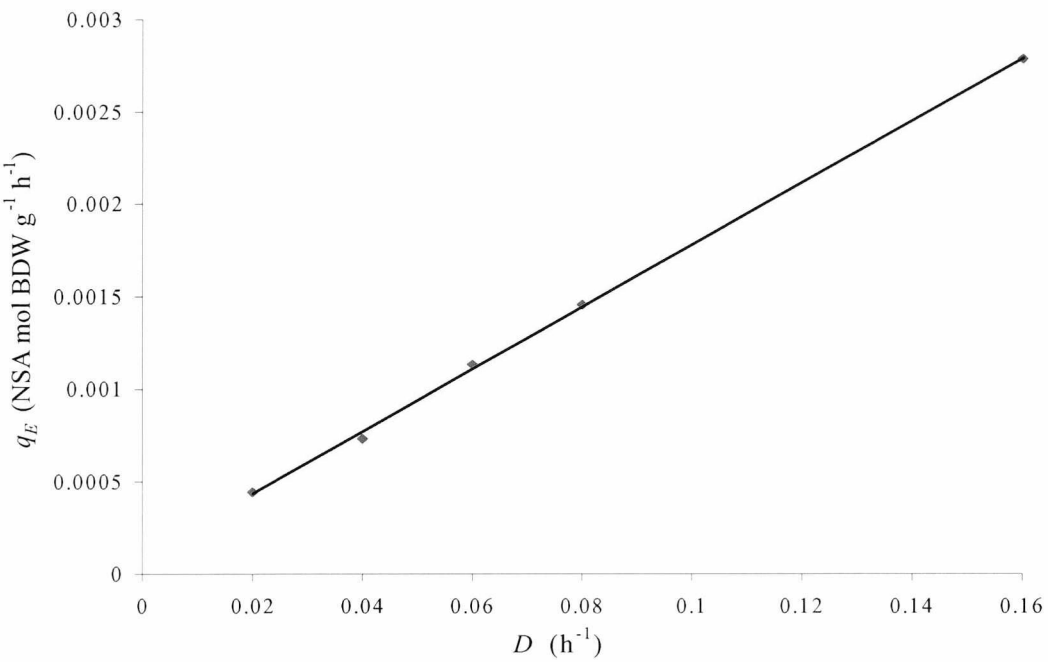

Figure 4.11. Tempest plot for specific rate of NSA consumption as a function of dilution rate in chemostat culture of isolate RBN. Y_E = 59.523 g BDW mol NSA^{-1}, m = 0.105 mmol NSA g BDW^{-1}, r^2 = 0.999.

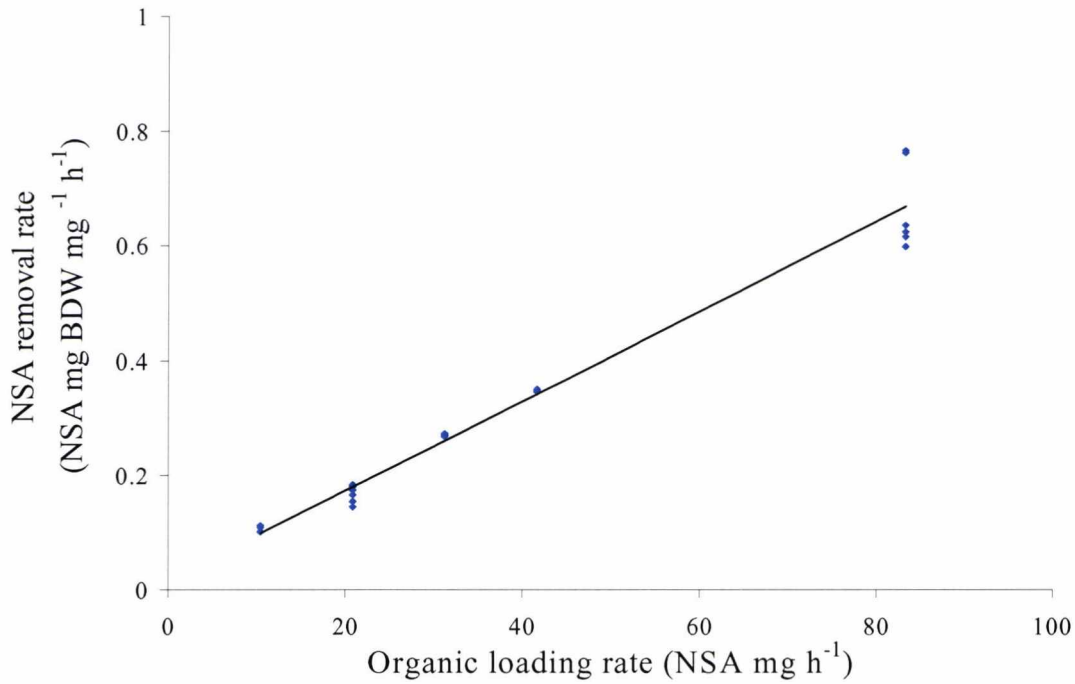

Figure 4.12. NSA removal rate (NSA mg BDW mg^{-1} h^{-1}) versus the organic loading rate (NSA mg h^{-1}) Best fit line is shown, $r^2 = 0.971$

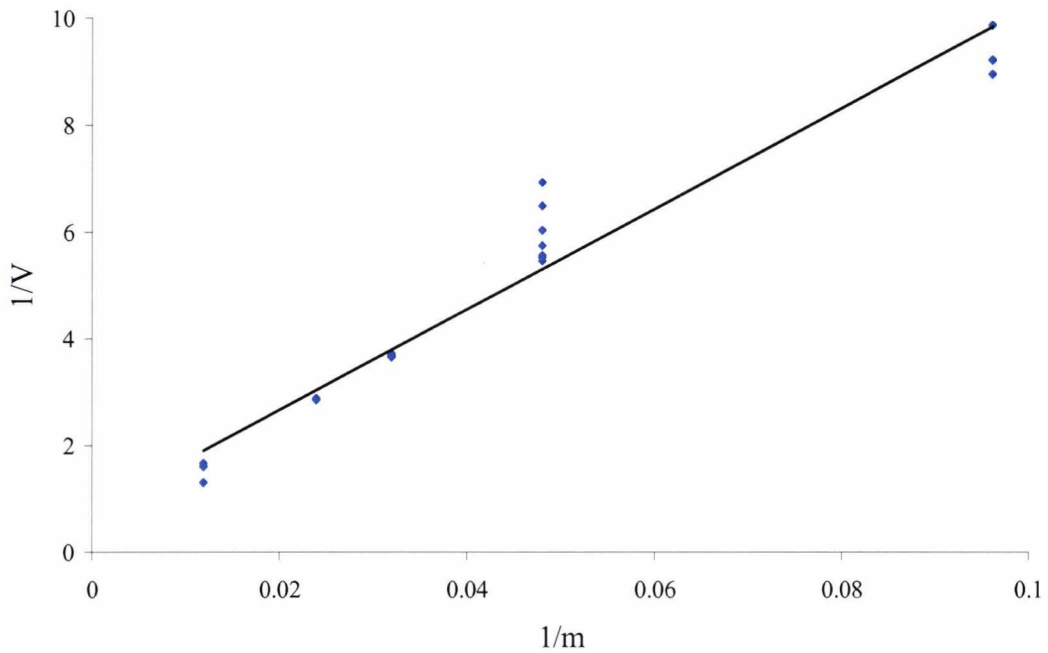

Figure 4.13. Lineweaver-Burk plot of NSA removal rate (V) of isolate RBN at various organic loading rates (m). Best fit line is shown; V_{max} = 1.28 mg BDW mg^{-1} h^{-1}, K_m = 121.27 mg h^{-1}, $r^2 = 0.952$

Discussion

Isolation of a number of bacterial strains capable of degrading NSA has been reported (Brilon *et al.*, 1981; Nortemann *et al.*, 1986; Ohe *et al.*, 1990; Balashov & Boronin, 1996). In this study, two unidentified isolates (designated RBN and BAS), capable of utilising NSA as a sole source of carbon and energy, were isolated by batch enrichment culture inoculated with acclimated sludges from biological wastewater treatment plants. Enrichment culture creates a unique environment, which permits the selection of a desired microorganism or group of microorganisms. This is achieved by: stimulating the growth of the desired microorganism(s) using selective pressures, so that they dominate or form a large part of the microbial community or; facilitating the growth of the desired organism in a manner that allows it to be distinguished from the other micro-organisms present (Aaronson, 1989). Here, a positive selection pressure was applied for the selection of NSA-degrading species from the sludges, by supplying NSA as the sole source of carbon and energy in the enrichment medium.

RBN and BAS were both capable of utilising NSA and removed a total of 62.9% and 65.4% of the NSA, respectively, after 48 h in batch growth culture (figures 4.1 and 4.2). The growth of the isolates was concurrent with the removal of NSA indicating that the isolates were utilising NSA as a source of carbon and energy. The catabolic pathway for NSA is analogous to the naphthalene pathway (Figure 1.4), with degradation proceeding via gentisate or catechol (upper pathway) to the citrate cycle, via pyruvate (lower pathway). Accumulation of gentisate and catechol was observed by HPLC when NSA-degrading isolates were grown with excess NSA (10mM), indicating incomplete metabolism (Brilon *et al.*, 1981b). In this study, no metabolites were detected during the measurement of NSA concentration indicating that both isolates mineralised NSA.

However, growth of both isolates and removal of NSA ceased after 48 hours. The cessation of growth and NSA removal may have been due to fall in pH (from pH 7.0 to pH 4 ± 0.09). As previously described, the degradation pathway of NSA is reported analogous to that of naphthalene (Nörtemann *et al.*, 1986). The initial step of the pathway is catalysed by a dioxygenase (putatively naphthalenesulphonic acid dioxygenase), which adds both atoms of a dioxygen to the ring structure, giving rise to a dihydrodiol intermediate (Figure 1.6). Sulphite is spontaneously eliminated from the intermediate

which then undergoes rearomatisation to 1,2-dihydroxynaphthalene (Kuhm *et al.*, 1991, Carredano *et al.*, 2000). Liberation of sulphite from the degradation of NSA into the medium, may have led to the fall in pH and subsequent inhibition of growth. A similar fall in pH was observed following dioxygenolytic elimination of sulphite during mineralisation of 2-sulphobenzoate by *Pseudomonas* sp. RW611 (Hansen *et al.*, 1992). This hypothesis is supported by the increased growth and NSA removal measured when RBN was cultured in batch in the pH-controlled conditions of a chemostat (section 4.5).

The growth kinetics of the isolates were calculated at a range of NSA concentrations. Isolate RBN grew faster and had a higher μmax and yield than BAS. However, at 500 mg L^{-1} NSA, the reduction of NSA (mg L^{-1}) by isolate BAS was significantly greater. The K_s value of both isolates was 14 mg L^{-1}. K_s represents the affinity of the isolate for the substrate, with lower values indicating a higher affinity. K_s values will vary according to substrate and microbial species; a K_s value for glucose of 0.0071 mg L^{-1} was reported for a *Flavobacterium* sp. (van der Kooij & Hijnen, 1981) compared to a value for glucose of 29 mg L^{-1} reported for a second *Flavobacterium* sp. (Ishida *et al.*, 1982). A single microorganism may also have one K_s at low substrate concentrations and another at high substrate concentrations.

Bacteria with high K_s values are characteristic of nutrient rich environments (Alexander, 1994a). Both isolates came from biological treatment plants receiving effluents containing relatively high concentrations of NSA and NSA-based compounds. These environments can be considered as nutrient-rich and hence both isolates display high K_s values. Enrichment in batch culture often yields only one species or strain per enrichment and, as a result of the high substrate concentrations used (typically many times greater than K_s), this is most likely to be the strain or species with the highest specific maximum growth rate (μmax) (Harder & Dijkhuizen, 1982). The concentration of NSA used in the enrichment medium was in excess of K_s of both isolates and as such, RBN and BAS can be assumed to have been the most rapid NSA-utilising organisms in the two sludges that were enriched.

K_s values are an important determinant for the growth of a bacteria at a given substrate concentration (S). If S is in excess of K_s, then growth will be logarithmic. If S falls below

K_s, then the growth rate will progressively slow as the substrate concentration falls (Alexander, 1994a). This may be of particular importance in the bioaugmentation of biotreatment plants, where a dilution rate greater than the growth rate of the inoculant may result in loss of the organism from the system. In this study, concentrations of NSA between 25.29 mg L^{-1} (± 0.06) and 61.78 mg L^{-1} (± 0.67) were measured in two industrial effluents. These values are in excess of the K_s values of both isolate RBN and BAS, suggesting both isolates would be suitable for the bioremediation of effluents of this type.

The growth kinetics for both isolates were calculated from the observed μ values at a range of NSA concentrations (10 – 1000 mg L^{-1}). Lineweaver-Burk plots indicated that inhibition of growth occurred between 500 and 1000 mg L^{-1}. The inhibition constant of NSA could not be determined without examining the effect of higher concentrations but may have been due to a toxic effect of NSA on the isolates. Acute toxicity of NSA to bacteria has been reported to occur at concentrations in excess of 100 mg L^{-1} (Greim *et al.*, 1994).

The effect of the observed inhibition on the growth kinetics (section 4.3) was investigated by comparing the calculated μ values from the data for 10 – 500 mg L^{-1} NSA and 10-1000 mg L^{-1} with the observed μ values (table 4.2). Good agreement was found between values from 10 – 500 mg L^{-1} for both isolates, but not from the values from 10 – 1000 mg L^{-1}. Monod kinetics assumes a linear relationship between 1/μ and 1/S, and as the effect of inhibition could not be quantified, the data showing inhibition was excluded from the calculation of the growth kinetics. However, further investigation of the effect of higher concentrations of NSA on the growth of the isolates is required. The influence of inhibition on the calculated μ values was markedly less for isolate BAS, suggesting that the growth of BAS was less affected by the inhibitory influence than isolate RBN.

The utilisation of xenobiotic compounds by bacteria, as sources of nutrients other than carbon has been reported. *Comamonas* sp. strain JS765 utilised nitrobenzene as sole source of carbon, nitrogen and energy with liberation of nitrite catalysed by nitrobenzene dioxygenase (Lessner *et al.*, 2002). A *Pseudomonas putida* strain that utilised trimethyl-1,2-dihydroxypropyl ammonium as sole source of carbon, nitrogen and energy has also been isolated (Kaech & Egli, 2001). Additionally, simple sulphonates, such as cysteic

acid and taurine, have been shown to be utilised as sole source of carbon, sulphur and energy (Nortemann *et al.*, 1986; Wittich *et al.*, 1988; King & Quinn, 1997). The ability of the isolates to utilise NSA either as sole source of sulphur or sole source of carbon, energy and sulphur was investigated (section 4.4). The data is summarised in Table 4.4.

The data indicated that isolate RBN could utilise NSA as a source of sulphur but not as efficiently as inorganic sulphate (Figure 4.5). RBN may have utilised the sulphite liberated during the proposed initial step of NSA degradation. Typically, sulphonate assimilation occurs via formation of sulphate, although assimilation of sulphonate-sulphur without the involvement of sulphate, but with the involvement of sulphite, has been established (Uria-Nickelsen *et al.*, 1993).The ability of an isolate to utilise NSA as a source of both carbon and sulphur, may increase the success of that isolate when used in bioaugmentation strategies, due its ability to utilise a nutrient source that may be unavailable to competing microorganisms, or allowing for the engineering of conditions which favour the selected isolate or promote the degradation of NSA. Isolate BAS was unable to grow in sulphate-free medium plus 500 mg L^{-1} NSA and therefore unable to utilise NSA as a source of carbon and sulphur (Figure 4.6).

The assimilation of sulphur by bacteria is governed by the assimilatory sulphate reduction (ASR) pathway. Desulphonation by aerobes, when the sulphonate is acting as a source of carbon, is generally regulated by induction whereas, when acting as a source of sulphur, desulphonation is globally regulated (Kertesz *et al.*, 1994). Desulphonation of arylsulphonates used as a source of carbon involves liberation of the sulphonate moiety as sulphite (Johnston *et al.*, 1975), whereas desulphonation as a source of sulphur involves liberation of the carbon moiety (Zürrer *et al.*, 1987).

Under sulphate-starvation conditions, many bacteria produce sulphate starvation induced (SSI) proteins (Kertesz *et al.*, 1993). Thirteen proteins, expressed during sulphate-starvation, were isolated from *Pseudomonas aeruginosa* PAO1 and included binding proteins with affinity for sulphate and sulphonates, and enzymes involved in sulphonate metabolism (Quadroni *et al.*, 1999). Expression of these proteins facilitates scavenging of sulphur from non-sulphate sources and is regulated by the *ssuADCB* operon, encoding for alkane sulphonate utilisation, with the exception of taurine, which is regulated by the *tauABCD* operon (Eichhorn *et al.*, 2000). The *ssu* operon was also found to facilitate the

utilisation of arylsulphonates as a source of sulphur, but only when expressed in conjunction with the *asf* operon (Vermeij *et al.*, 1999).

Table 4.4. Comparison of the maximum growth (turbidity) and NSA removal by isolates RBN and BAS when utilising NSA as either; sole source of carbon (A), sole source of carbon, energy and sulphur (B), or sole source of sulphur (C). Medium D was the positive growth control for the use of NSA as sole source of sulphur (plus sulphate) and medium E was a sulphur-free medium plus glucose (section 2.5).

Medium	Maximum turbidity (OD_{600nm})		Maximum NSA removal (% reduction from 0 h)	
	RBN	BAS	RBN	BAS
A	0.449	0.348	62.9	65.4
B	0.248	0.000	64.8	0.0
C	0.457	0.430	78.5	70.2
D	0.585	0.528	65.0	69.5
E	0.439	0.390	N/A.	N/A.

Expression of the SSI proteins encoded by the *ssu* operon and those encoded by the *asf* operon, thus facilitating the desulphonation of NSA by the *ssu*-encoded monooxygenase, would explain the ability of isolate RBN to utilise NSA as sole source of carbon, energy and sulphur. Monooxygenation of *p*-toluene sulphonate by *Pseudomonas (Comamonas) testosteroni* gave rise to the corresponding phenol, *p*-cresol (Locher *et al.*, 1989). Liberation of the sulphonate moiety from naphthalene sulphonates by monooxygenation to naphthol has been observed in the green algae, *Scenedesmus obliquus*, under sulphate-limited conditions (Kneifel *et al.*, 1997).

It is conceivable that RBN was able, by expression of the *ssu* operon, to utilise the sulphonate moiety of NSA as a source of sulphur and then degrade the monooxygenase product, naphthol, as a source of carbon. However, dioxygenolytic elimination of sulphite has been reported by *Pseudomonas* sp. RW611, utilising 2-sulphobenzoate as sole source of carbon, energy, and sulphur. The liberated sulphite was slowly oxidised to sulphate, a process that was dependent on the presence of the bacterial cells (Hansen *et al.*, 1992). As

such, it is also plausible that RBN utilised NSA as sole source of sulphur through the same catabolic pathway as for its utilisation as sole source of carbon, i.e. dioxygenation of NSA with spontaneous elimination and subsequent assimilation of sulphite.

The utilisation by bacteria of sulphonates, as sources of sulphur but not carbon, has been reported, including utilisation of *p*-toluene sulphonate and benzene sulphonate by three *Rhodococcus* sp. (Uria-Nickelsen *et al.*, 1993, Chien *et al.*, 1999). Utilisation of aliphatic sulphonates, by isolates from freshwater sediment and soil, as a source of sulphur was reported to be more widespread than their use as source of carbon (King & Quinn, 1997). Four of the isolates were also found to be able to utilise naphthalene-1-sulphonate and *p*-toluene sulphonate as a source of sulphur but not carbon. Six pseudomonad strains, able to utilise methane sulphonate, 1-dodecane sulphonate and *p*-toluene sulphonates as a source of carbon and energy, produced comparable cell yields when utilising the organosulphonates as a source of sulphur in the presence of another carbon source. These strains were also found to be able to utilise the organosulphonates as a source of sulphur after the ability to utilise them as a source of carbon had been lost. This suggests a separate enzymatic pathway for the utilisation of organosulphonates as either a source of sulphur or a source of carbon (Seitz *et al.*, 1993), i.e. a SSI pathway.

The utilisation of NSA as sole source of sulphur for both isolates was investigated in a sulphate-free medium with glucose as an alternative source of carbon (section 2.5). Growth of both isolates was observed in the sulphate-free medium and the sulphate-supplemented positive growth controls (figures 4.7 and 4.8). Growth in the sulphur-free medium suggested that isolates RBN and BAS were able to utilise NSA as a source of sulphur in the presence of an alternative carbon source. There was no significant difference between the NSA removal in either medium by either isolate, or when compared with the NSA removal in MSM plus 500 mg L^{-1} NSA, indicating that the extent of NSA degradation was not influenced by the availability of inorganic sulphate.

However, the glucose used in these experiments contained 0.005% sulphur, as sulphate and sulphite (MSDS, Sigma-Aldrich Co. Ltd, England) which the isolates may have been utilising. Sulphur forms approximately 1% of the total dry mass of the microbial cell (Hines *et al.*, 1997). The medium used contained 2 g L^{-1} of glucose, equivalent to 0.1 mg L^{-1} or 0.3 μM of sulphur in the medium. Contaminating sulphur concentrations of less

than 2 μM were found to give non-turbid growth controls during the investigation of anaerobic desulphonation of arylsulphonates, i.e. the sulphur contamination was insufficient to support growth of the inoculum (Denger *et al.*, 1996). The yield (BDW) values of RBN and BAS in MSM plus 500 mg NSA L^{-1} were 102 mg of RBN and 72 mg BAS, respectively (Table 4.1) These are equivalent to a sulphur requirement at 1% of total BDW of 1.02 mg and 0.72 mg, respectively. The BDW values of the isolates were higher in the presence of glucose, as indicated by the turbidities in the medium. On this basis, the sulphur contamination in the glucose should not have supported the growth of the isolates (Figure 4.7 & 4.8). However, the contamination may have been adequate for the initiation of growth and enzyme synthesis, leading to NSA degradation and subsequent liberation of sulphite for utilisation by the isolates.

The influence of low levels of sulphate and sulphite contamination of the glucose was investigated. Both isolates grew in sulphur-free media plus glucose (Figure 4.9). Additionally, there was no significant difference between the growth of the isolates in the sulphate-free media amended with glucose and NSA and the sulphate-free medium amended with glucose only. The comparable growth of the isolates in the sulphate-free media containing glucose and NSA, and the sulphate-free medium containing glucose only, suggests that the isolates were utilising the sulphate present in the glucose, and not utilising NSA as a source of sulphur. As such, it was impossible to determine from the available data whether either isolate had utilised NSA as a sole source of sulphur in the presence of an alternative carbon source.

Sulphate has been shown to be preferentially used before sulphonate as a source of sulphur (Seitz *et al.*, 1993, Uria-Nickelsen *et al.*, 1994, Chien *et al.*, 1999). When grown with one each of sulphate, cysteine or thiocyanate (group 1) and one each of 4-toluene sulphonate, 4-nitrocatecholsulphate or ethanesulphonic acid (group 2), *Pseudomonas putida* S-313 was found to utilise group 1 in advance of group 2. Exhaustion of the preferred source was followed by a lag period, before utilisation of the less preferred source, suggesting *de novo* synthesis of the required pathway for utilisation, i.e. synthesis of SSI-proteins (Beil *et al.*, 1996).

It is possible, therefore, that in the unsupplemented sulphur-free medium, the isolates utilised the residual sulphate in the glucose and subsequently utilised NSA as a sulphur

source. However, no lag period, consistent with the synthesis of SSI-proteins, was apparent in the growth of the isolates in the unsupplemented sulphate-free medium. It is also conceivable that the low concentration of sulphate in the glucose resulted in concurrent utilisation of the sulphate and induction of the SSI-proteins, and hence, no lag in growth being apparent. The lack of any significant difference between the growth of the isolates in the unsupplemented sulphur-free medium and the minimal medium plus glucose belie this possibility.

Sulphate contamination contributed 0.075 mg sulphate L^{-1} to the sulphate-free medium used in the investigation of the use of 1-naphthalene sulphonic acid as sole source of sulphur by the green alga, *Scenedesmus obliquus*. However, *Scenedesmus obliquus* continued to utilise 1-naphthalene sulphonic acid as sole source of sulphur in the presence of up to 15 mg sulphate L^{-1} and the desulphonation pathway did not need to be induced (Soeder *et al.*, 1987). Therefore, the isolates RBN and BAS may have utilised both the sulphur contamination and the NSA as sources of sulphur. This raises the possibility that the necessary mechanism for utilisation of sulphonate sulphur were being expressed by the isolates without the need for induction, and therefore, the isolates may have utilised NSA as sole source sulphur without an apparent lag in growth.

The removal rates of NSA in the unsupplemented sulphur-free medium were unchanged compared with those in MSM supplemented with 500 mg L^{-1} NSA. The consistency of the rate of removal of NSA may be indicative of its utilisation as a sulphur source. However, the rate of NSA removal was also unchanged in the supplemented sulphur-free medium, i.e. in the presence of excess sulphate. If sulphate was the preferred source of sulphur, as has been reported (Seitz, *et al.*, 1993, Uria-Nickelsen *et al.*, 1994, Beil *et al.*, 1996, Chien *et al.*, 1999), this would suggest that NSA remained the preferred carbon source and did not serve as a source of sulphur. No new peaks were observed suggesting that NSA was completely degraded. This does not preclude the possibility of utilisation of NSA as a source of carbon and sulphur by RBN, and as a source of sulphur by BAS in the presence of glucose. However, it does support the hypothesis that utilisation of NSA as a source of sulphur was concurrent with its utilisation as a source of carbon and therefore, mediated via the same catabolic pathway as for its use as a source of carbon.

The ability of the isolates to utilise NSA as a source of sulphur was investigated. Such a capacity may be exploited in the utilisation of the isolates for the bioremediation of NSA in the environment. Isolate RBN was found to be able to utilise NSA as sole source of carbon, energy and sulphur, but it was it was not possible to determine whether or not either isolate was able utilise it as a sole source of sulphur. Further work is therefore suggested:

- As will be subsequently discussed, both isolates utilised naphthalene as sole source of carbon and energy (section 5.1). The effect of the sulphur contamination of the glucose could be determined by comparison of the growth of the isolates in a sulphur-free medium with glucose, plus naphthalene or NSA. Comparable levels of growth in the two media would suggest that growth was dependent upon the sulphur in the glucose and that NSA was not utilised as a source of sulphur. However, a lack of growth in the medium containing naphthalene would indicate that NSA was used as a source of sulphur.
- The genes for the catabolism of NSA by the isolates were shown to be plasmid-encoded and to be lost after plasmid-curing (section 5.2). Utilisation of NSA as a source of sulphur by plasmid-cured isolates, by expression of constitutively encoded SSI, would result in a decrease in NSA concentration and potentially, an accumulation of naphthol in the medium.
- Comparison of the SSI proteins isolated from isolates RBN and BAS under sulphur-limited conditions, may provide an insight into the different capacities of isolates to utilise NSA as sole source of sulphur.

The purpose of determining steady state kinetics in continuous culture is to allow for development of microorganism and substrate balances, and predictions of effluent microorganism and substrate concentrations (Metcalf & Eddy, 1991), i.e. to determine the operational parameters within which biological treatment will be effective. The steady state kinetics for RBN in continuous culture were determined (section 4.5). The maintenance coefficient, m, was 0.024 mg NSA g^{-1} BDW, comparable to other reported m values (Strachan *et al.*, 1996; Low & Chase, 1999). Maintenance functions include the turnover of cell materials, maintenance of concentration gradients and cell motility. Increasing the environmental stress on the cell will not only increase the value of m as the cell will have invest greater energy into maintaining intracellular homeostasis, but will

also reduce growth of the biomass. The *m* value of *Sacchromyces cerevisiae* was increased by the addition of 1M NaCl to the culture medium with a resultant fall in growth (Strachan *et al.*, 1996). The growth and NSA removal by RBN in chemostat batch culture was greater than that observed in batch in shake flask culture (section 4.5). This supports the hypothesis that a fall in pH, as a result of liberation of sulphite from NSA, limited growth and NSA removal in shake flask culture, but did not limit either in the pH-controlled environment of the chemostat. Increases in *m* values, due to the fall in pH observed in the shake flask cultures of both isolates, may explain the difference in growth and NSA utilisation by the isolates between the shake flask cultures and the chemostat batch culture of RBN.

When grown in continuous culture, isolate RBN maintained a mean NSA removal of 99.4% (± 0.02) from an influent concentration of 500 mg L^{-1}, at a range of *D* from 0.02 to 0.16 (HRT of 6 – 48 h). The growth of RBN showed a linear relationship to the loading rate of NSA or *D*, indicating that the culture was carbon limited. This was expected as $D = \mu$ and μ_{max} (section 4.3) was not reached or exceeded. The removal rate of NSA also showed a linear relationship to *D* and followed first order kinetics. This would also expected as the maximum loading rate was less than K_m.

First order models may approximate degradation kinetics when S is less than K_m, although approximation using Monod kinetics is thought to be more accurate (Bekins *et al.*, 1997). As such, V_{max} and K_m for the removal of NSA during continuous culture were calculated using Michaelis-Menten kinetics, which are analogous to Monod kinetics (Alexander, 1994a). Both V_{max} and K_m were greater than the maximum NSA removal rates and organic loading rates used, confirming that the growth of the culture was carbon-limited. Therefore, under the conditions described, accumulation of NSA in the effluent from biotreatment plants augmented with isolate RBN would not be expected. However, in order to determine their suitability for use in bioaugmentation of biotreatment plants, their persistence and activity in the presence of an indigenous microbial community and alternative carbon sources, required investigation.

Conclusions

The objective of this work was the isolation of NSA degrading isolates for utilisation in the bioaugmentation of biotreatment plants treating effluents containing NSA. Two NSA-degrading isolates were enriched from acclimated activated sludges, and found to extensively degrade NSA in shake flask growth cultures. Growth and NSA degradation were limited by a fall in the pH of the medium, which was thought to be due to the liberation of sulphite from NSA. However, complete degradation of NSA was achieved under pH-controlled conditions. As such, inhibition of the degradation of NSA due to sulphite liberation would not be expected in the pH-controlled conditions of a biotreatment plant. One of the isolates, RBN, was also found to utilise NSA as sole source of carbon, energy and sulphur, although the mechanism by which this was achieved was not elucidated. Similarly, both isolates appeared to utilise NSA as sole source of sulphur in the presence of an alternative carbon source although further investigations of this utilisation are required. Determination of the growth kinetics indicated that the affinity and removal rates of the isolates for NSA, would be appropriate for the degradation of NSA at the concentrations measured in two industrial effluents. The use of these isolates for the bioaugmentation of biotreatment plants treating NSA-containing effluents will subsequently be investigated.

CHAPTER 5

ELUCIDATION OF THE CATABOLIC PATHWAY FOR UTILISATION OF NSA AND PROVISIONAL IDENTIFICATION OF THE ISOLATES

Chapter 5

ELUCIDATION OF THE CATABOLIC PATHWAY FOR UTILISATION OF NSA AND PROVISIONAL IDENTIFICATION OF THE ISOLATES

5.1. The metabolic pathway for the degradation of the naphthalene sulphonate by isolates RBN and BAS

The degradation of NSA is thought to occur via a catabolic pathway analogous to the naphthalene degradation pathway. The initial dioxygenation of naphthalene is catalysed by naphthalene dioxygenase (Jeffrey *et al.*, 1984). Isolates expressing dioxygenases will transform indole to the blue dye, indigo (Dagher *et al.*, 1997) and this transformation was used to screen isolates RBN and BAS (section 2.9). Both RBN and BAS formed dark blue coloured colonies indicating that these isolates were capable of transforming indole to indigo and therefore, may have been expressing a dioxygenase (Figure 5.1).

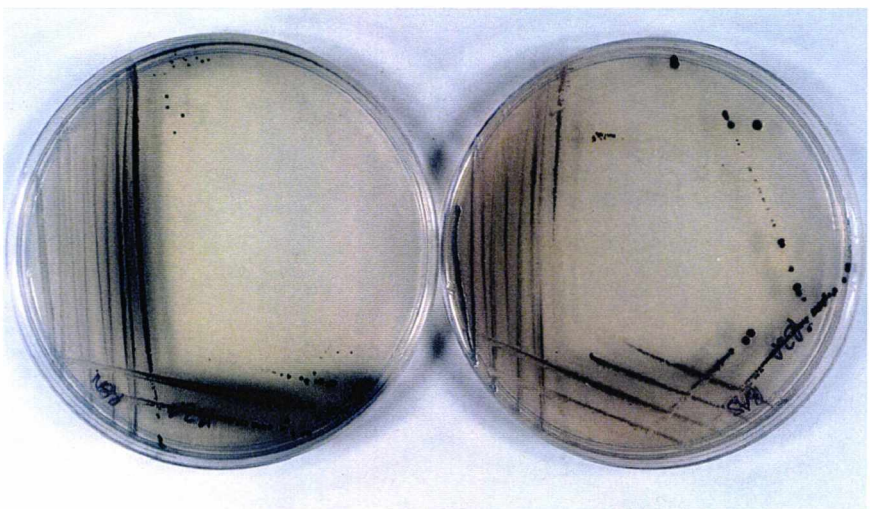

Figure 5.1 – Transformation of indole to indigo by isolates RBN (left) and BAS indicating the activity of a dioxygenase. Isolates grown on TSA at 25°C.

Both isolates were examined in batch culture for their ability to utilise naphthalene (section 2.6) or PSA (section 2.3.2) as sole sources of carbon and energy. Both isolates grew rapidly on naphthalene after an initial lag period of approximately 24 h (Figure 5.2). After 96 h, the turbidity of both isolates was found to be significantly greater (RBN p =

0.006, BAS $p = 0.001$) than that observed during growth in MSM plus 500 mg NSA L^{-1} (section 4.2).

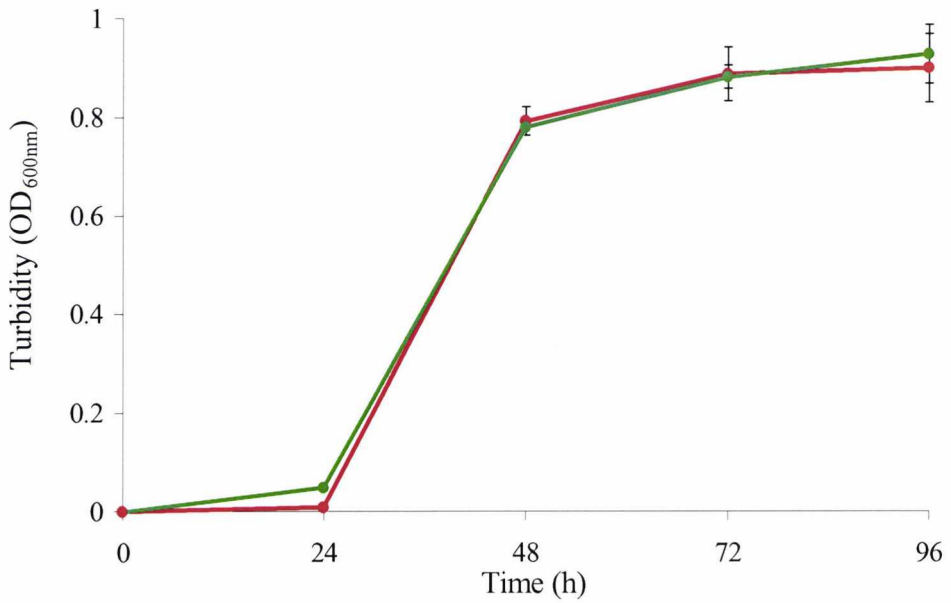

Figure 5.2. Growth profiles of isolates RBN and BAS in MSM plus naphthalene, 500 mg L^{-1}; • - RBN, • - BAS. Vertical bars indicate S.E.M (n = 3); for symbols without bars, the limits of error were within the confines of the symbol.

The BDW of the isolates grown with naphthalene was estimated from the turbidity by extrapolation of the plot of biomass dry weight vs. OD_{600nm} (section 2.4.4). The BDW with 500 mg naphthalene L^{-1}, were 0.179 g L^{-1} (RBN) and 0.183 g L^{-1} (BAS). Assuming that 100% of the naphthalene was utilised (i.e. the minimum cell yield g^{-1} naphthalene), the yields of RBN and BAS were calculated as 0.359 g BDW g^{-1} naphthalene and 0.367 g BDW g^{-1} naphthalene, respectively. These yields are 1.75- and 2.54-fold higher than those measured when the isolates were grown in batch culture with NSA as sole source of carbon and energy (section 4.3).

However, neither isolate was able to utilise PSA as sole source of carbon and energy (figure 5.3). These observations support the hypothesis that the initial step in the catabolism of NSA by these isolates, was carried out by a naphthalene dioxygenase-like enzyme.

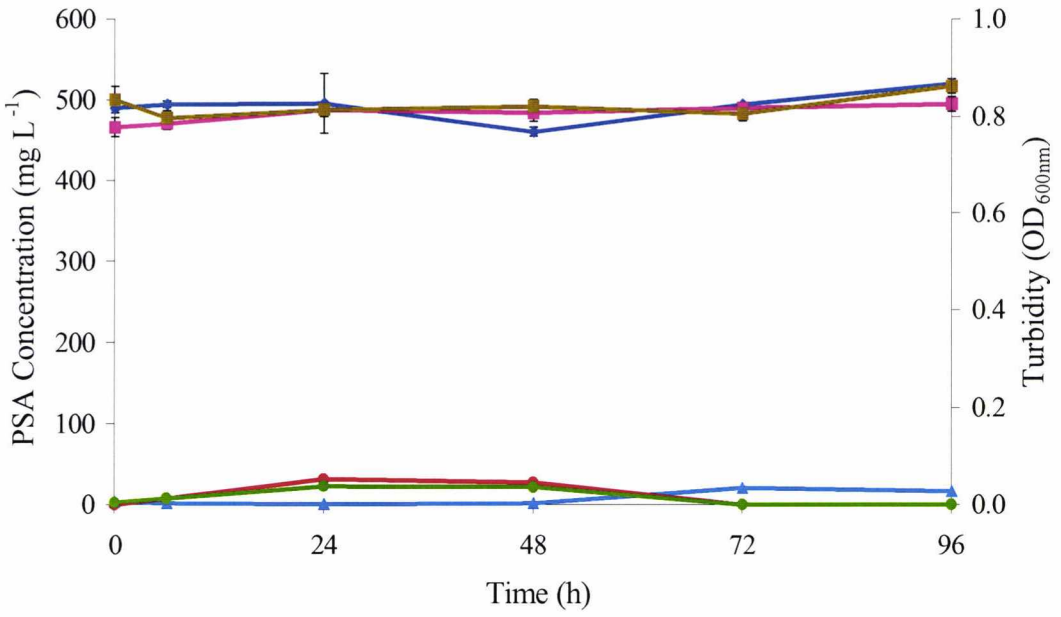

Figure 5.3. Utilisation of PSA and growth profile of isolates RBN and BAS in MSM plus 500 mg PSA L^{-1}; ■ - RBN PSA, ● - RBN turbidity, ■ - BAS PSA, ● - BAS turbidity, ♦ - PSA control, ▲ - turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

In order to confirm the presence of a naphthalene dioxygenase, the DNA of the isolates was probed for regions encoding for the *nah*Ac region of naphthalene dioxygenase. PCR amplification of extracted DNA (section 2.10.1) was carried out using primers designed to distinguish *nah*Ac-type dioxygenases from other characterised dioxygenases (Wilson *et al,* 1999) (sections 2.10.4 and 2.1.4). The amplicons were separated on a 2.0% agarose gel and visualised by UV-excitation (section 2.10.2) as shown in Figure 5.4. Amplicons of approximately 500 base pairs were amplified from the DNA of both isolates and subsequently cloned and sequenced (section 2.10.7, appendix 2.1) The sequence data was aligned using CLUSTAL W, with closely related sequences recovered from Genbank (section 2.10.8). The data are shown as a distance tree (figure 5.5).

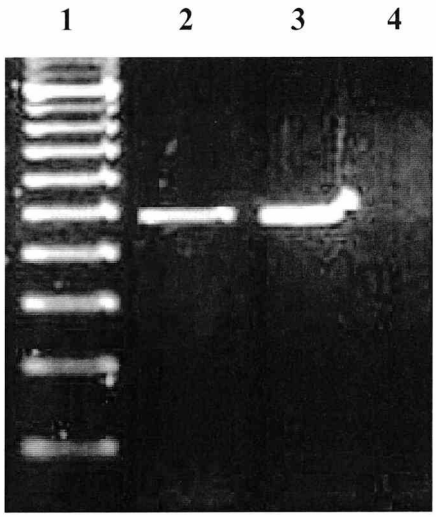

Figure 5.4. Gel electrophoresis of *nah*Ac amplicons. Lanes: 1, molecular ruler (100 base pair increment); 2, *nah*Ac-PCR of isolate RBN DNA; 3, *nah*Ac-PCR of isolate BAS DNA; 4, negative control (PCR-grade water).

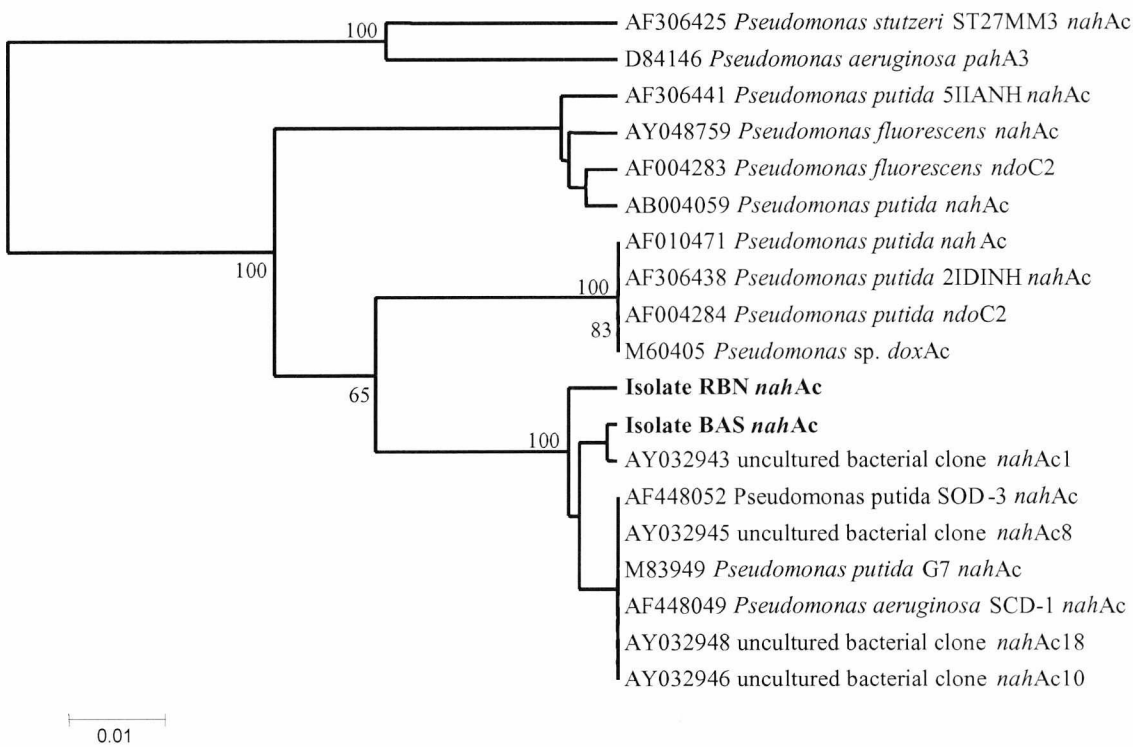

Figure 5.5. Nucleotide distance tree of partial *nah*Ac sequences (471 bp). The scale bar represents the number of changes per sequence position. Numbers on branches represent bootstrap confidence values as the percentage of 100 bootstrap replications. Bootstrap percentages of less than 50% are not shown. Sequences generated in this study are shown in bold

5.2. Location of the catabolic genes for naphthalene sulphonate degradation.

The locations of the NSA-degrading genes in isolates RBN and BAS were determined by plasmid curing in Luria-Bertani (LB) medium (section 2.8.). The cured isolates were inoculated into MSM plus 500 mg NSA L^{-1} and assessed for growth and NSA removal (Figure 5.6), as described previously (section 4.2). Neither isolate was able to utilise NSA as sole source of carbon and energy after serial subculturing in LB medium, and subsequent incubation in MSM plus NSA, at either 25°C or 37°C. The results indicated that NSA catabolism in both isolates was plasmid-encoded and that both isolates had been cured (Feng *et al.*, 1997) .

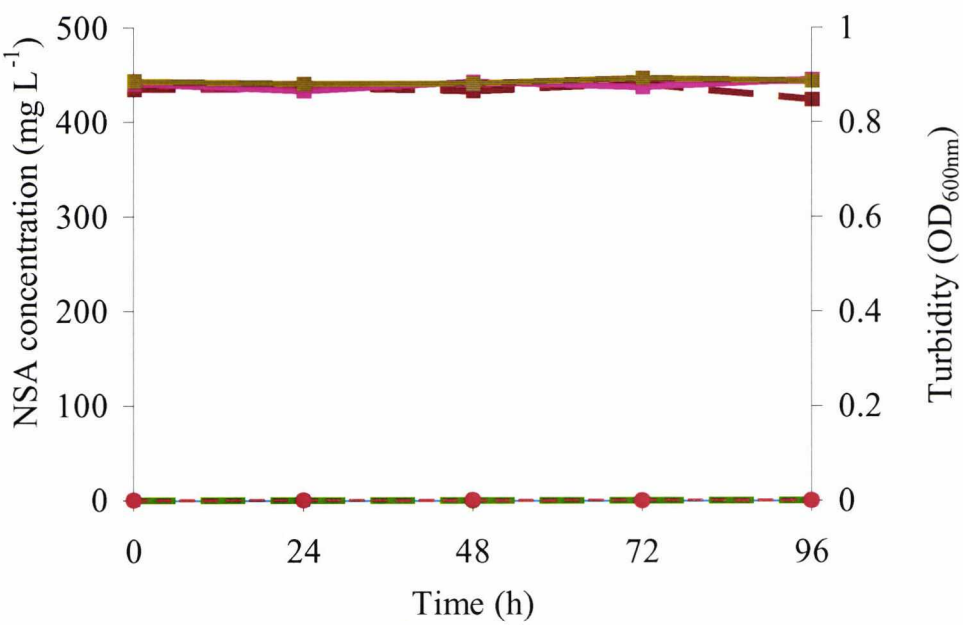

Figure 5.6. Utilisation of NSA and growth profile of plasmid-cured isolates RBN and BAS in MSM plus 500 mg NSA L^{-1}: ■ - RBN NSA (mg L^{-1}), ● - RBN turbidity, ■ - BAS NSA (mg L^{-1}), ● - BAS turbidity; ------- incubation at 25°C; —— incubation at 37°C. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

The DNA of the plasmid cured isolates was extracted and amplified by PCR with *nah*Ac primers as previously described. The amplicons were separated on a 2.0% agarose gel and visualised by UV-excitation (figure 5.7). No *nah*Ac products were amplified from the

DNA of the cured isolates indicating that the genes encoding for this region of naphthalene dioxygenase had been eliminated by curing.

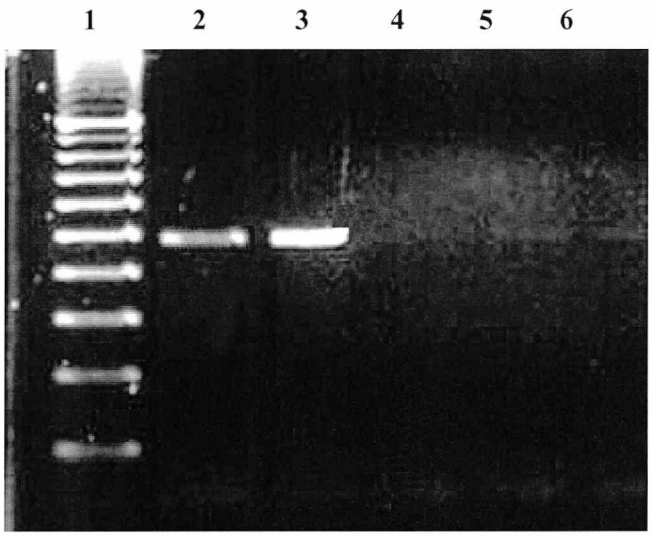

Figure 5.7. Gel electrophoresis of *nah*Ac amplicons from the wild type and plasmid cured isolates. Lanes: 1, molecular ruler (100 base pair increment); 2, *nah*Ac-PCR of isolate RBN DNA; 3, *nah*Ac-PCR of isolate BAS DNA; 4, *nah*Ac-PCR of plasmid cured isolate RBN; 5, *nah*Ac-PCR of plasmid cured isolate BAS; 6, negative control (PCR-grade water).

5.3. Provisional identification of the isolates

The phenotypes of the isolates were examined after streaking on TSA (Figure 5.8). Isolate RBN produced orange, opaque, regular, smooth, convex, round colonies with a diameter of 1 – 3 mm; BAS produced pale yellow, translucent, regular, smooth, convex, round colonies with a diameter of 1 – 3 mm. Gram staining and microscopic examination determined that both isolates were Gram-negative rods and suggested that both were in pure culture.

The purity of the isolates was confirmed by PCR amplification of the 16S rDNA with Com1/Com2 primers (sections 2.10.4 and 2.1.4) and separation of the amplicons by SSCP gel electrophoresis, without prior λ exonuclease digestion (section 2.10.9), as shown in figure 5.9. SSCP gel electrophoresis produced two bands per isolate, indicating the presence of a single strain for each isolate .

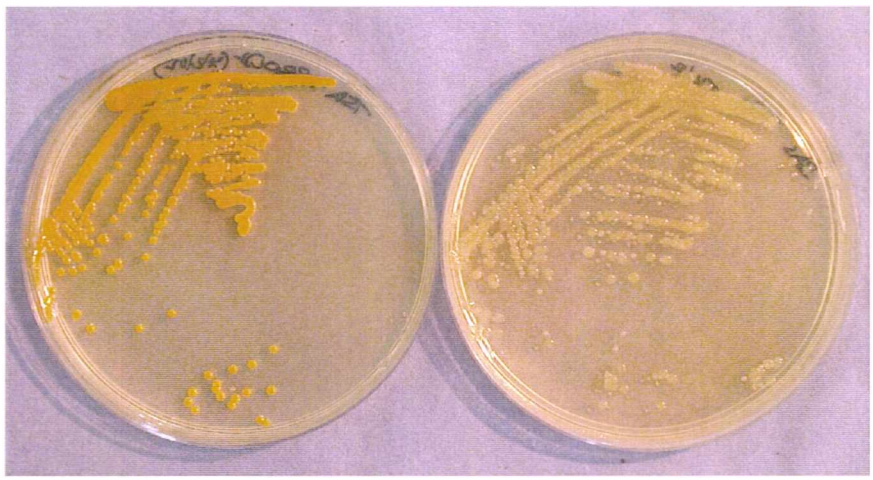

Figure 5.8 – Plate cultures of isolates RBN (left) and BAS on TSA.

The partial 16S rDNA amplicons were cloned and sequenced (section 2.10.7, appendix 2.2). The sequences were analysed and phylogenetic trees constructed for each isolate by sequence alignment with closely related 16S rDNA sequences from Genbank (section 2.10.8). The phylogenetic trees for isolate RBN and BAS are shown in Figures 5.10 and 5.11. Similarity and divergence matrices, comparing the partial 16S rDNA sequences of the isolates with those of the closely related sequences identified by Genbank, were constructed using the Similarity Matrices Program on the Ribosomal Database Project website (Appendices 2.3 & 2.4).

Isolate RBN clustered with the 2 unidentified *Sphingomonas* strains but showed the greatest sequence homology (98.1%) with *Sphingomonas* sp. JS1 (Accession number AJ427917) over the 386 base pair region examined. Isolate BAS clustered with *Comamonas testosteroni* strains and showed a 100% sequence homology with this species over the 375 base pair region examined.

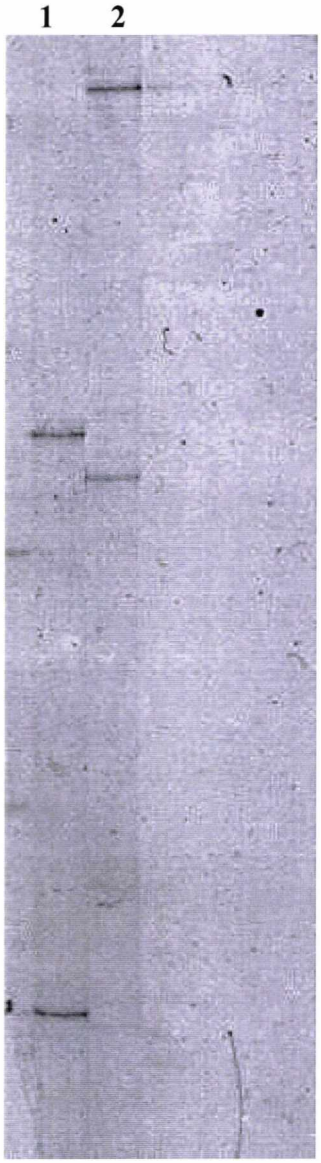

Figure 5.9 – SSCP gel electrophoresis of partial 16S rDNA amplicons for isolates RBN (lane 1) and BAS (lane 2), showing 2 bands per isolate.

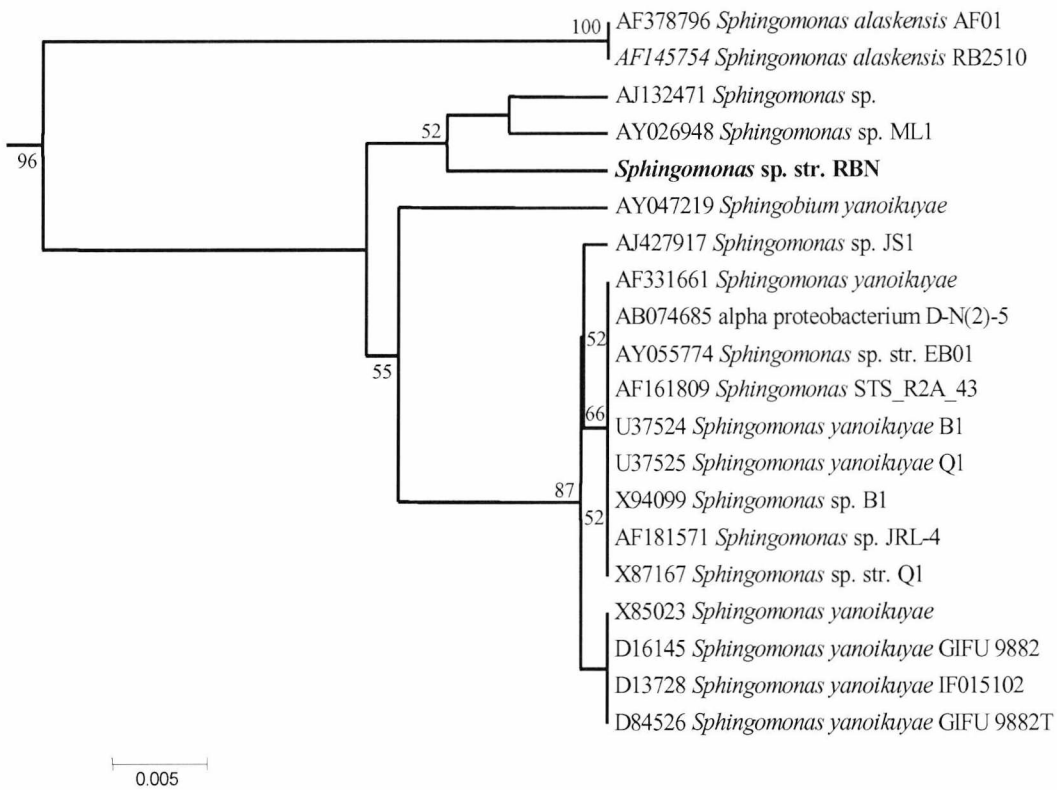

Figure 5.10 – Phylogenetic relationship of partial 16S rDNA sequences of isolate RBN and closely related sequences. A 386 bp alignment was produced using the CLUSTAL W algorithm. The nucleotide distance matrix was calculated using DNADIST from the PHYLIP package. Phylogenetic relationships were inferred by UPGMA distance analysis with Kimura two-parameter genetic distances (DNADIST 2:1 transition to transversion ratio). The scale bar represents the number of changes per sequence position. Numbers at branches represent bootstrap confidence values as the percentage of 100 bootstrap replications. Bootstrap percentages of less than 50% are not shown. The sequence generated for RBN is shown in bold

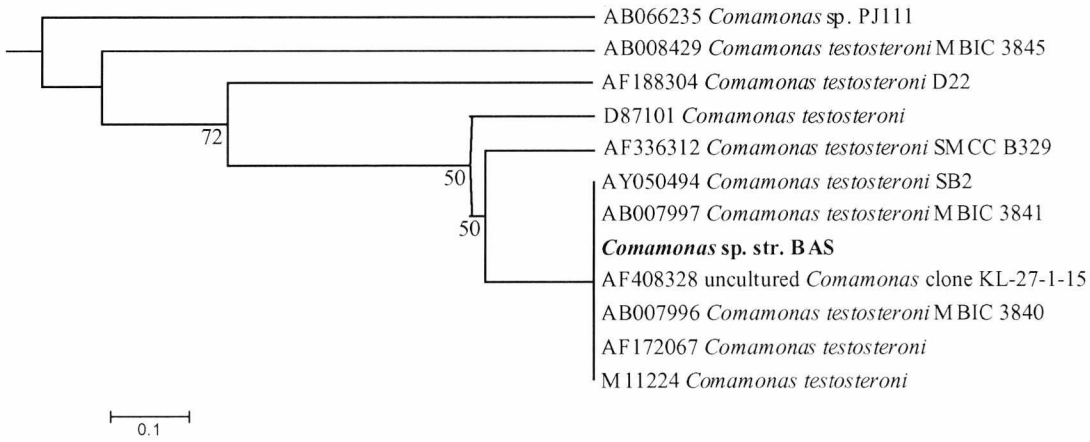

Figure 5.11 – Phylogenetic relationship of partial 16S rDNA sequences of isolate BAS and closely related sequences. A 375 bp alignment was produced using the CLUSTAL W algorithm. The nucleotide distance matrix was calculated using DNADIST from the PHYLIP package. Phylogenetic relationships were inferred by UPGMA distance analysis with Kimura two-parameter genetic distances (DNADIST 2:1 transition to transversion ratio). The scale bar represents the number of changes per sequence position. Numbers at branches represent bootstrap confidence values as the percentage of 100 bootstrap replications. Bootstrap percentages of less than 50% are not shown. The sequence generated for BAS is shown in bold

Discussion

The putative pathway for the degradation of NSA is analogous to the pathway for the degradation of naphthalene. The initial step in the naphthalene pathway is catalysed by naphthalene dioxygenase and involves the incorporation of both atoms of a dioxygen into the 2 position of the naphthalene ring (Kuhm *et al.*, 1991, Carredano *et al.*, 2000). The transformation of indole to indigo is a property of dioxygenase systems that form *cis*-dihydrodiols from aromatic hydrocarbons, including benzene and toluene dioxygenases (Ensley *et al.*, 1983). Naphthalene dioxygenase will also catalyse the transformation of indole to the blue dye indigo (Dagher *et al.*, 1997). Both isolates were found to be expressing a dioxygenase by screening for the ability to transform indole to indigo.

Both isolates utilised naphthalene as sole source of carbon and energy. The BDW of both isolates was found to be considerably higher when grown with naphthalene than when grown with NSA. The carbon content of naphthalene is greater than that of NSA (1.68 : 1 w/w). However, the ratio of yields for the isolates grown on naphthalene, relative to their growth on NSA (*Sphingomonas* sp. strain RBN = 1.75, *Comamonas testosteroni* strain BAS = 2.54), were found to be greater than the carbon ratio of naphthalene and NSA, i.e. the increase in growth was not solely dependent on the increased availability of carbon. This may be indicative of a reduction in the amount of maintenance energy required by the isolates (Low & Chase, 1999) as the degradation of naphthalene did not result in the fall in pH, and the subsequent increase in environmental stress on the isolates, observed during the degradation of NSA (section 4.2).

The degradation of phenol, toluene, *p*-cresol and other monocyclic aromatic compounds may also be initiated by dioxygenases (Ensley *et al.*, 1983, Heinaru *et al.*, 2000). Neither isolate was able to utilise PSA as sole source of carbon and energy indicating a degree of substrate specificity of the dioxygenases expressed by the isolates for NSA and naphthalene. As such, the transformation of indole to indigo, utilisation of naphthalene as a source of carbon, and the inability to utilise PSA as a source of carbon and energy, indicated that both isolates were expressing naphthalene dioxygenase-like enzymes.

The isolates were examined for genes encoding for naphthalene dioxygenases by amplification of their DNA with primers, Ac114f and Ac596r, with specific affinity for a

region of the *nah*Ac gene of naphthalene dioxygenases (Wilson *et al.*, 1999). This region encodes for the large α sub-unit of the terminal Rieske non-heme iron oxygenase component of the naphthalene dioxygenase system, which has been implicated in the determination of the substrate specificity of the enzyme (Parales *et al.*, 1998). Both isolates were found to contain regions of DNA encoding for *nah*Ac and the sizes of the amplified products, approximately 500 base pairs, corresponded to that predicted by Wilson *et al.* (1999) (figure 5.4).

The *nah*Ac amplicons were cloned and sequenced, and the sequences aligned with other *nah*Ac sequences from the NCBI database (figure 5.5). The *nah*Ac sequences of the isolates showed a high degree of homology with other *nah*Ac sequences, indicating the capacity for expression of naphthalene dioxygenases. The very high degree of similarity between all of the *nah*Ac sequences examined, including those of the isolates, is unsurprising. High degrees of similarity between analogous components encoding for the catabolism of naphthalene from different bacterial strains have been reported, indicating a common ancestor for these genes (Denome *et al.*, 1993, Suen & Gibson, 1993, Eaton, 1994, Takizawa *et al.*, 1994). More specifically, the α subunits of all Rieske non-heme iron oxygenases, such as those encoded for by *nah*Ac, have been shown to be related to each other. Four dioxygenase families, naphthalene, toluene/benzene, biphenyl and benzoate/toluene, were identified on the basis of amino acid sequencing of the oxygenase α subunits (Werlen *et al.*, 1996). As such, it would be expected that the dioxygenase responsible for the initial dioxygenation of NSA would be largely homologous with other naphthalene dioxygenases.

However, funnelling of naphthalenesulphonates into the naphthalene degradation pathway is thought to necessitate a unique desulphonating 1,2-dioxygenase, NSA dioxygenase. The NSA-degrading isolate *Pseudomonas* BN6 was found to be incapable of degrading naphthalene, suggesting that the intial dioxygenation of NSA was catalysed by a unique enzyme with a narrow substrate specificity. The NSA-induced BN6 cells did not express 1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase, the second enzyme in the naphthalene degradation pathway. This enzyme is thought to be redundant in the NSA degradation pathway as the product of its activity, 1,2-dihydroxynaphthalene, is formed by elimination of sulphite and spontaneous rearomatisation of the intermediate product of NSA dioxygenase. However, the degradation of NSA by BN6 was competitively

inhibited by naphthalene, indicating that the dioxygenase responsible had an affinity for naphthalene (Kuhm *et al.*, 1991). Similarly, *Pseudomonas (Comamonas) testosteroni* strain T-2 was reported as being able to degrade toluene sulphonate but not toluene (Locher *et al.*, 1989).

Comparison of the *nah*Ac sequences from *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS with those of other naphthalene-degrading strains provided no evidence of expression of a unique NSA 1,2-dioxygenase. However, while dioxygenases show a high degree of homology, small changes in the α subunit have been reported to give rise to remarkable differences in substrate specificities of those dioxygenases (Furukawa *et al.,* 1993, Gibson *et al.,* 1993). The α and β subunits of the biphenyl dioxygenases of strains KF707 and LB400 showed a high degree of homolgy, with only 20 and 1 amino acid differences respectively, yet the enzymes were found to have very different specificities for polychlorinated biphenyl cogeners. As such, a NSA 1,2-dioxygenase could show a high degree of sequence homology with other naphthalene dioxygenases, but be possessed of a different substrate specificity.

In contrast to other isolates reported to express arylsulphonate dioxygenases and which were incapable of catabolising the unsulphonated aromatic analogue (Kuhm *et al.,* 1991, Locher *et al.,* 1989), both *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS were capable of utilising naphthalene as sole source of carbon and energy. Coexistence of homologous catabolic genes in isolates has been reported. *Pseudomonas* sp. strain Cg7 was reported as containing 2 *nah*Ac genes (Herrick *et al.* 1997) as were several naphthalene-degrading isolates from the Mediterranean Sea (Bosch *et al.*, 1999; Ferrero *et al.*, 2002). This gives rise to the possibility that separate enzyme systems were responsible for the catabolism of NSA and naphthalene by the isolates. Any differences between the enzymes involved in the catabolism of NSA and naphthalene could have been elucidated by RT-PCR of the transcribed mRNA during growth of the isolates on either substrate. However, attempts to elucidate the enzyme being actively transcribed by the isolates during the catabolism of NSA, by RT-PCR amplification of the *nah*Ac region, were unsuccessful.

While arylsulphonatases have been found to have specific substrate selectivity, they are also reported to catalyse a wide range of analogous substrates. The naphthalene

dioxygenase of *Pseudomonas putida* G7 was found to capable of oxygenating biphenyl (Barriault & Sylvestre, 1999). A naphthalene sulphonate-assimilating bacteria, *Pseudomonas* sp. TA-2 was able to convert 1- and 2-naphthoate to their corresponding *cis*-dihydrodiols. This conversion was attributed to a dioxygenase with a relaxed regioselectivity (Ohmoto *et al.*, 1996). Similarly, a naphthalene-degrading strain, *Pseudomonas fluorescens* strain TTC1, was found to oxidise 1- and 2-naphthol to dihydroxynaphthalene. This transformation was thought to proceed through the formation of unstable dihydrodiol intermediates, which were then transformed to dihydroxynaphthalene by the spontaneous elimination of water (Bianchi *et al,*. 1997), a transformation mechanism analogous to that proposed for the transformation of NSA. Brilon *et al.* (1981a) observed that, of the isolates enriched from a naphthalene-degrading sewage sludge community, by extended enrichment with concurrently increasing concentrations of NSA and diminishing concentrations of naphthalene, few were capable of growth on both naphthalene and NSA. Two of those isolates were found to preferentially utilise naphthalene before NSA, as a source of carbon and energy, when grown in the presence of both substrates. Utilisation of NSA occurred only after the naphthalene had been exhausted, but there was no evidence of biphasic growth (diauxy). Naphthalene was also found to inhibit the removal of NSA from cell-free extracts, demonstrating that both substrates were hydroxylated by the same enzyme (Brilon *et al.*, 1981b). This suggests that the initial oxidation of NSA may be catalysed by a naphthalene dioxygenase with a relaxed substrate specificity rather than a unique NSA-dioxygenase.

The initial dioxygenation of NSA may be catalysed by either a unique NSA-dioxygenase or a naphthalene dioxygenase with a relaxed substrate specificity and further investigations are required. However, it is clear that both isolates have the facility for the expression of a naphthalene dioxygenase and it is, therefore, likely that both isolates are catabolising NSA via the classic naphthalene degradation pathway, following initial dioxygenation of NSA by a naphthalene dioxygenase.

The location of the genes for the catabolism of NSA was determined by plasmid curing the isolates in LB medium (section 5.2). Plasmids are dispensable as their function is only of benefit to the cell in a limited set of environmental conditions. Plasmid loss will, therefore, occur spontaneously in the absence of the appropriate environmental selection

pressure (Parker, 2000). After curing, both isolates were found to have lost the ability to utilise NSA as sole source of carbon and energy (Figure 5.6), indicating that the genes for NSA catabolism were plasmid encoded. The loss of the catabolic genes was confirmed by amplification of the DNA of the cured isolates with primers for *nah*Ac. Amplification products were absent from both cured isolates (Figure 5.7).

Bacterial plasmids may have a significant role in the development of catabolic pathways for xenobiotic chemicals in the environment. Most catabolic genes are carried by wide host range, conjugative or mobilisable plasmids. The spread of these catabolic genes is due to horizontal transfer coupled with selective pressure from the increasing presence of those compounds in the environment (Van der Meer *et al.,* 1992). Plasmid encoded pathways for the degradation of a number of environmentally significant compounds have been reported including, 2,4-D (Ka *et al.*, 1994), carbofuran (Feng *et al.*, 1997), nitrobenzene (Park & Kim, 2000) and fenitrothion (Hayatsu *et al.*, 2000). The catabolic genes for naphthalene are generally found on self-transmissible plasmids (Sayler *et al.* 1990). *In-situ* transfer of naphthalene catabolic genes, assessed by monitoring of the transfer of *nah*Ac alleles, has been observed in coal tar contaminated sites, where a taxonomically diverse range of hosts displayed identical sequences of *nah*Ac. (Herrick *et al.*, 1997). Restriction fragment length polymorphism of the naphthalene-catabolic plasmids from 12 isolates from the same site, showed a close relationship of the plasmids to each other, and to the pDTG1 plasmid from the naphthalene-degrading strain *Pseudomonas putida* NCIB 9816-4, indicating horizontal transfer of a highly conserved allele between diverse species (Stuart-Keil *et al.*, 1998).

Horizontal transfer may also lead to the evolution of novel degradative pathways for xenobiotic compounds. Horizontal gene transfer and recombination were suggested to have resulted in the capacity of *Ralstonia* sp. strain JS705 to degrade chlorobenzene in contaminated water (Van der Meer *et al.*, 1998). Similarly, transfer of the self-transmissible *clc* element, from 3-chlorobenzoate-degrading *Pseudomonas* sp. strain B13 to toluene-degrading *Pseudomonas putida* F1, and subsequent complementation of the two catabolic pathways, resulted in transconjugants expressing a novel metabolic pathway for the degradation of mono- or 1,4-dichlorobenzene, a substrate that neither parent strain could utilise. This complementation only occurred in the presence of an appropriate selective pressure, i.e. mono- or 1,4-dichlorobenzene. Complementation was

also found to occur when both strains were inoculated into an activated sludge, and was shown to occur with at least one indigenous toluene-degrading strain, after inoculation of the activated sludge with *Pseudomonas* sp. strain B13 only (Ravatn *et al.*, 1998).

Plasmid encoding of the requisite genes for the catabolism of a recalcitrant environmental contaminant may be of benefit in bioremediation. Bioaugmentation of the indigenous microbial population of contaminated sites with xenobiotic-degrading isolates will fail if the introduced organism is unable to incorporate itself into the existing community because of the prevailing environmental conditions (Alexander, 1994b). However, horizontal transfer of catabolic plasmids from inoculated isolates to indigenous bacterial strains has been reported.

Horizontal transfer of a catabolic plasmid from *Pseudomonas putida* HC1 conferred the ability to degrade crude oil hydrocarbons upon marine bacteria. The transconjugant bacteria maintained the plasmid when subjected to a selective pressure, although the plasmid was progressively lost under non-selective conditions (Latha & Lalithakumari, 2001). The plasmid pJP4, which encodes for the partial degradation of 2,4-D, was detected throughout the entire length of a soil column and in a number of indigenous transconjugants, following inoculation with donor *E. coli* D11. These results suggest that plasmid transfer to indigenous species can lead to significant dispersal of genes through the environment (Newby & Pepper, 2002).

This transfer of catabolic genes from inoculated species to indigenous species has also been shown to be of benefit in the bioremediation of recalcitrant organic compounds. Two new chlorobenzoate-degrading strains were isolated from a contaminated aquifer after inoculation with the 3-chlorobenzoate–degrading strain *Pseudomonas* sp strain B13. Prior to inoculation, no chlorobenzoate-degrading strains were isolated from the aquifer. Both strains contained *clc* elements that were identical to that of *Pseudomonas* sp strain B13 indicating that the genes for 3-chlorobenzoate had been transferred from the inoculated strain (Zhou & Tiedje, 1996). Inoculation of 3-chlorobenzoate-degrading *Pseudomonas putida* BN210, and subsequent transfer of the *clc* element into strains indigenous to a membrane biological reactor, led to the isolation of a number of novel chlorobenzoate-degrading strains. These strains were not detected in an uninoculated reactor. Furthermore, two of the novel chlorobenzoate-degrading strains were found to

have higher growth rate on 3-chlorobenzoate than *Pseudomonas putida* BN210. One of these strains also outcompeted *Pseudomonas putida* BN210 when simultaneously inoculated into a membrane reactor fed with 3-chlorobenzoate (Springael *et al.*, 2002).

The use of plasmids for the dissemination of catabolic genes in contaminated environments has been proposed (Rittmann *et al.* 1995). The previously described studies demonstrate that transfer of catabolic genes from inoculated organisms to indigenous organisms, which are already well-adapted to the environment, may ensure that those genes persist and proliferate in the environment, even if the inoculated organism does not. Therefore, plasmid-encoding of xenobiotic genes can be considered as a positive trait in isolates selected for bioremediation purposes. As such, the location on a plasmid of the NSA-degrading genes in both RBN and BAS, may be viewed as desirable characteristic of these organisms and their application in the bioremediation of NSA-contaminated effluents.

Growth on TSA indicated that both isolates were axenic (Figure 5.8). It has long been recognised that batch enrichment culture limits the number of species isolated from the environment. A reduced diversity of PAH-degrading isolates were obtained by enrichment culture (12) compared to a biofilm culture method (36). The diversity of PAH-catabolic genes was also significantly reduced in the enrichment culture, with all the isolates expressing an identical *nah*Ac-like dioxygenase, whereas the isolates from the biofilm culture were found to express *cnd*A-, *nah*Ac- and *phn*Ac-like dioxygenases, reflecting the diversity of isolates in the biofilm culture (Stach & Burns, 2002).

Enrichment in batch culture often yields only one species or strain per enrichment and, as a result of the high substrate concentrations used (typically many times greater than K_S), this is most likely to be the strain or species with the highest specific maximum growth rate (μmax) (Harder & Dijkhuizen, 1982). Enrichment of soil slurry with PCP gave a greatly reduced diversity compared to enrichment with a wood preservative containing several pollutants, including PCP. SSCP analysis revealed that 96% of the clones from the PCP-enriched slurry had the same profile, indicating that only one organism had been enriched, compared with 4 different profiles from the wood preservative-enriched slurry, which accounted for between 14 and 41% of the clones screened (Beaulieu *et al.*, 2000).

Dunbar *et al.* (1997) reported significantly reduced diversity when 2,4-D-degrading organisms were isolated from a 2,4-D-treated soil by enrichment, compared with direct plating of the soil. Only 7 genotypically distinct populations were isolated from 74 enrichments, as determined by rep-PCR, and not more than one isolate was isolated per enrichment. In contrast, 25 distinct rep-PCR populations were obtained by direct plating. Five of the seven isolates obtained from the enrichments were not obtained by direct plating, highlighting the potential for selection pressure in direct plating for organisms that can grow on the medium used.

Isolation of strains that are individually capable of degrading NSA may provide a simpler option for their application for bioaugmentation of biological treatment plants receiving NSA-containing effluents than bioaugmentation with an NSA-degrading consortium. Optimisation of conditions will be more readily achievable for a single species or strain than for a consortium. The ability to utilise a recalcitrant compound as sole source of carbon and energy may provide the strain with a hitherto unexploited nutritional niche in the inoculated environment. A fast-growing strain may also be better able to compete and establish itself within the indigenous microbial population. As such, while the selection pressures of enrichment culture may reduce the number of isolates, those isolates that are selected may possess characteristics desirable in bioaugmentation.

Sphingomonas sp. strain RBN and *Comamonas testosteroni* strain BAS were shown to be axenic by extraction of the DNA from batch cultures, PCR amplification of the 16S rDNA and SSCP separation of the amplicons, without λ exonuclease digestion (section 5.3). SSCP utilises the formation by single stranded DNA of unique conformations, according to their nucleotide sequence and environmental conditions, when denatured prior to electrophoresis. These conformations can then be separated by electrophoresis due to their differing electrophoretic mobilities. The diversity of bacteria in a sample may be assessed by SSCP by examining the number of conformations arising after amplification of a conserved region of the bacterial DNA, such as 16S rDNA. The examination of environmental samples may be simplified by the use of a phosphorylated primer during the amplification of the DNA, which allows for subsequent λ exonuclease digestion of one DNA strand of each amplicon, leaving one strand per strain in the sample (Schwieger & Tebbe, 1998). As phenotypic examination of the isolates indicated that the isolates were axenic, digestion of the amplicon was not deemed to be necessary.

SSCP electrophoresis showed two bands for each isolate, i.e. two denatured single stranded DNA confirmations per isolate, confirming that both isolates were axenic.

The isolates were provisionally identified by sequencing and cloning of partial 16S rDNA sequences, and alignment with closely related 16S rDNA sequences retrieved from Genbank. RBN was grouped with the α-Proteobacteria and found to share the greatest sequence homology with an unidentified *Sphingomonas* isolate. RBN showed a 98.1% sequence homology with *Sphingomonas* sp. JS1 (Accession number AJ427917), differing by 7 base pairs. *Sphingomonas* sp. JS1 was phylogenetically grouped with a number of *Sphingomonas yanoikuyae* strains but RBN was phylogenetically grouped with 2 other unidentified isolates (figure 5.10). As such, RBN can only be identified as a *Sphingomonas* species. BAS was grouped with the β-Proteobacteria and showed a 100% sequence homology with 5 *Comamonas testosteroni* strains. BAS was also phylogenetically grouped with those strains (figure 5.11).

Conclusions

NSA has been detected in, and found to persist after biological treatment of, two industrial effluents (Chapter 3). As such, the bioaugmentation of biological effluent treatment plants with microorganisms capable of degrading NSA, may be of benefit in the treatment of effluents containing NSA. Two NSA-degrading strains, *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS, were isolated and shown to be capable of utilising NSA as a source of carbon and energy (Chapter 4). Here, the initial step of the catabolic pathway for the degradation of NSA by the isolates has been elucidated. The location of the catabolic genes on plasmids in both isolates has also been demonstrated. As both isolates have the capability to degrade NSA and the potential, by plasmid-mediated horizontal transfer, to disseminate those catabolic genes, their use for the bioaugmentation of biological treatment systems treating NSA-containing effluents will be investigated.

Chapter 6

BIOAUGMENTATION OF BIOLOGICAL TREATMENT SYSTEMS WITH NAPHTHALENE SULPHONATE-DEGRADING ISOLATES

Chapter 6

BIOAUGMENTATION OF BIOLOGICAL TREATMENT SYSTEMS WITH NAPHTHALENE SULPHONATE-DEGRADING ISOLATES

6.1. Degradation of NSA in a medium containing multiple carbon sources by *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS.

Bioaugmentation of biotreatment plants, populated with a diversity of adapted microorganisms, may fail for a number of reasons. One reason may be that, due to catabolite repression, isolates shown to utilise the target xenobiotic compounds as a sole source of carbon in the laboratory, may preferentially utilise other carbon sources when inoculated into the contaminated environment, leaving the target compound undegraded (Goldstein *et al.*, 1985). Deutz *et al.* (1994) reported repression of toluene utilisation by *Pseudomonas putida* in the presence of succinate and glucose. The expression of styrene monooxygenase, the first enzyme in the styrene degradation pathway, was also repressed in the presence of succinate (O'Leary *et al.*, 2002). The degradation of NSA (at 500 mg L^{-1} and 50 mg L^{-1}) by *Sphingomonas* sp. RBN and *Comamonas testosteroni* sp. BAS, in a medium containing a number of readily-degradable carbon sources (CM), including glucose and propionic acid, was assessed (section 2.7).

In CM containing 500 mg NSA L^{-1} (equivalent to approximately 52% of the total carbon), *Sphingomonas* sp. strain RBN reduced the NSA concentration to 43.7% (± 2.8) of the initial concentration after 24 h (Figure 6.1). The NSA concentration was further reduced to 34.1% (± 10.5) of the initial concentration after 96 h. The turbidity in the medium rose to a maximum OD_{600nm} of 0.7 (± 0.002) after 24 h. This increase was concurrent with the fall in NSA concentration. The growth profile was comparable to that observed when RBN was grown with NSA as sole source of carbon and energy (section 4.2). After 96 h, the maximum reduction in NSA concentration was not significantly different to that observed with NSA as sole source of carbon and energy ($p > 0.05$), but the maximum turbidity was significantly greater ($p = 0.00002$).

Comamonas testosteroni strain BAS reduced the concentration of NSA to 48.4% (± 9.8%) of the initial concentration after 24 h, and to 37.4% (± 9.7%) after 96 h. During this period, the turbidity (OD_{600nm}) increased of 0.467 (± 0.005) after 24 h, and reached a maximum OD_{600nm} of 0.511 (± 0.001) after 72 h. As for RBN, the maximum NSA removal was not significantly different ($p > 0.05$) to that measured when NSA was supplied as sole source of carbon and energy but the maximum turbidity was significantly greater ($p = 0.0004$) (section 4.2).

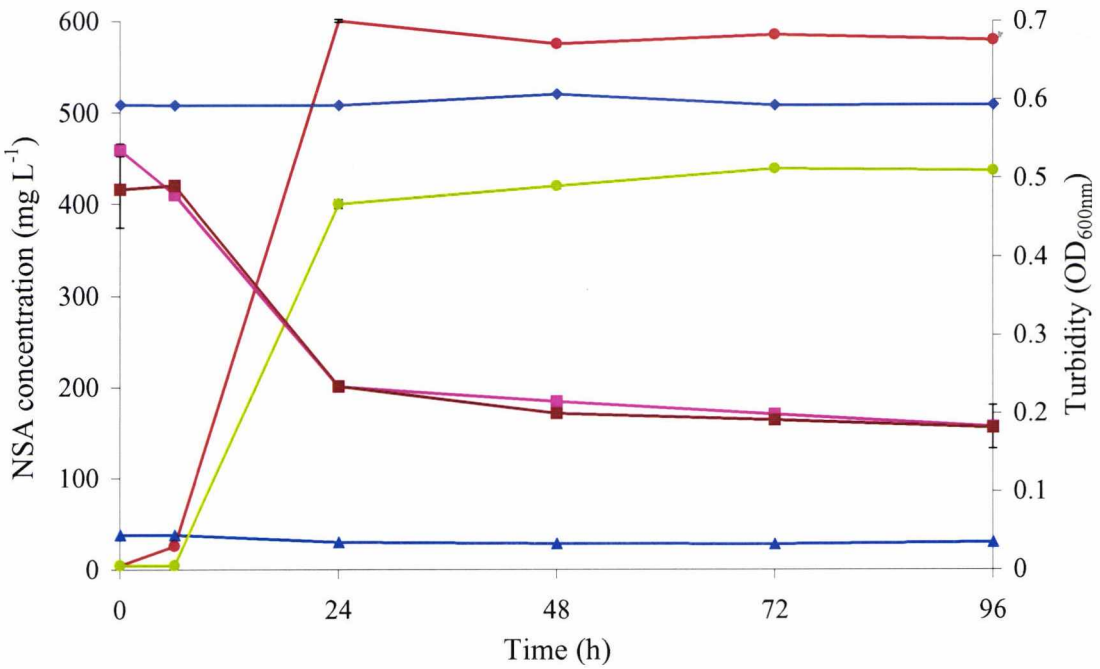

Figure 6.1. Utilisation of NSA and growth of *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS in CM plus 500 mg NSA L^{-1}: ■ - RBN NSA, ● - RBN turbidity, ■ - BAS NSA, ● - BAS turbidity, ♦ - uninoculated NSA control, ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

As previously discussed, enrichments are typically undertaken at substrate concentrations that are many times greater than the K_s of the isolate(s) eventually isolated However, the concentration of the target compound in a wastewater may be relatively low. In order for bioaugmentation to succeed, the inoculated microorganism(s) must be capable of degrading the target compound over a range of a concentrations. Consequently, the

degradation of 50 mg NSA L^{-1} in CM by the isolates, was assessed (Figure 6.2). This was equivalent to the concentration of NSA measured in a biologically treated tannery effluent (section 3.2) and, therefore, represented a relevant concentration.

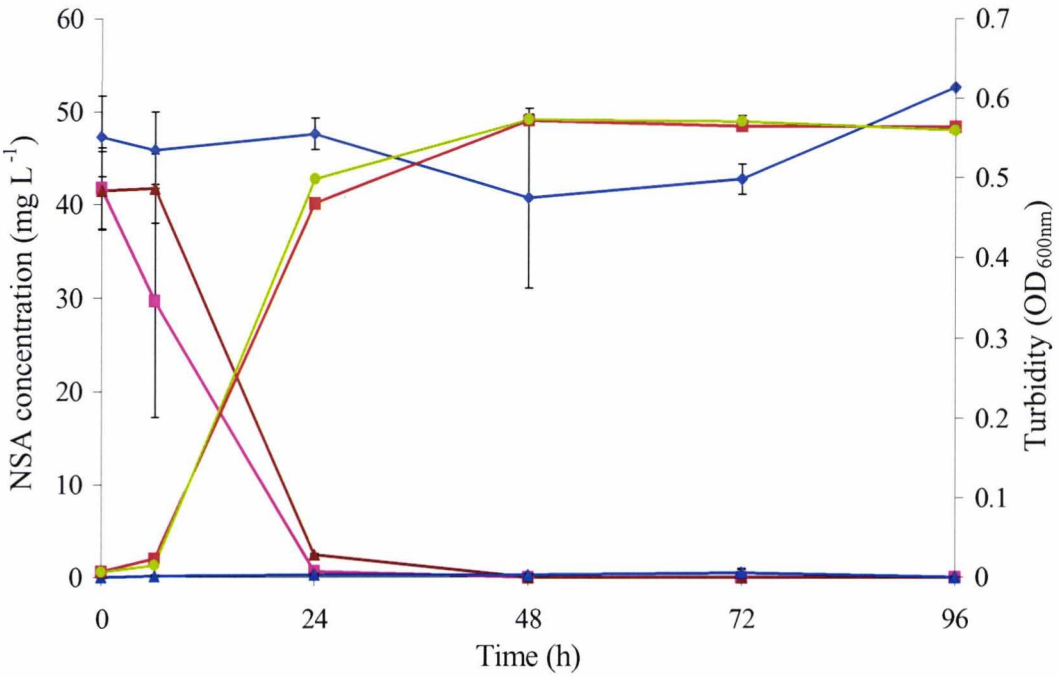

Figure 6.2. Utilisation of NSA and growth profile of isolates RBN and BAS in CM plus 50 mg NSA L^{-1}; ■ - RBN NSA, ● - RBN turbidity, ■ - BAS NSA, ● - BAS turbidity, ♦ - uninoculated NSA control, ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

The concentration of NSA in CM medium inoculated with *Sphingomonas* sp. strain RBN was reduced to 1.6% (± 0.5%) of the initial concentration after 24 h, and below detectable levels (< 1 mg L^{-1}) after 48h. A concurrent increase in turbidity to a maximum OD_{600nm} of 0.572 (± 0.004) after 48 h was measured. The maximum turbidity (after 48 h) was significantly greater (p = 0.0004) than that measured when RBN was grown in MSM plus NSA as sole source of carbon and energy (after 24 h) (section 4.2), but significantly lower (p = 0.0002) than that measured when RBN was grown in CM plus 500 mg NSA L^{-1}. The lower level of growth (turbidity) in CM at 50 mg NSA L^{-1} compared to that

measured in CM plus 500 mg NSA L^{-1}, indicated that NSA continued to be used as a source of carbon in the presence of alternative carbon sources, albeit that the total growth was greater in the presence of those carbon sources.

The concentration of NSA in the CM medium inoculated with *Comamonas testosteroni* strain BAS was reduced to 5.9% (± 1.6%) of the initial concentration after 24 h, and to below detectable levels after 48h. A concurrent increase in turbidity to a maximum OD_{600nm} of 0.574 (± 0.006) after 48 h was measured. As for *Sphingomonas* sp. strain RBN, the maximum growth (OD_{600nm}) was significantly greater (p = 0.0003) than that measured when BAS was grown in MSM with NSA as sole source of carbon and energy. However, the growth of BAS in CM plus 50 mg NSA L^{-1} was also significantly greater (p = 0.004) than that measured in CM plus 500 mg NSA L^{-1}.

Neither isolate showed a growth profile that would indicate diauxic growth (MacGregor *et al.*, 1991), nor was a lag phase apparent before the utilisation of NSA in either medium. This suggests that the utilisation of NSA was concurrent with utilisation of the other carbon sources, and thus, was not hindered by preferential degradation of more readily degradable carbon sources.

6.2. Bioaugmentation with *Sphingomonas* sp. strain RBN of a suspended floc reactor receiving a multi-component feed containing NSA

Both bacterial isolates degraded NSA in the presence of other, readily degradable carbon sources (section 6.1). However, in order for an isolate used for bioaugmentation of a biotreatment plant to succeed, it must be capable of survival, growth and degradative activity in the presence of a diversity of established microorganisms. The use of one of the isolates, *Sphingomonas* sp. strain RBN, for the bioaugmentation of a suspended floc reactor (section 2.12.1) was investigated. RBN was chosen for bioaugmentation because the previously reported data had shown that it grew more rapidly and to a greater BDW than *Comamonas testosteroni* strain BAS.

A suspended floc reactor was inoculated with sludge 4 (section 2.1.1) to give a MLSS in the aeration tank of 175 mg L^{-1}. The reactor was fed continuously with CM medium and operated with a hydraulic retention time of 16 h. The reactor feed and effluent were sampled for COD (section 2.2.1.1), and the aeration tank liquor was sampled for MLSS (section 2.12.1). The reactor was allowed to equilibrate for 17 d, at which point the COD removal rate was approximately stable. After 17 d, the reactor feed was supplemented with 50 mg L^{-1} of NSA, PSA and compound #21, a PSA-based syntan (section 2.1.3). This provided a mixture of biogenic and recalcitrant organic compounds, as would be expected in an industrial effluent. Thereafter, the effluent was sampled for NSA, PSA and compound #21 and analysed by HPLC (section 2.2.1.4). The reactor was equilibrated for a further 10 days and inoculated on day 27, with 1 l of a 48 h enrichment of RBN grown in MSM plus 500 mg NSA L^{-1}. This addition was calculated as 21 mg BDW L^{-1} in the reactor, by extrapolation of the plot of biomass dry weight (BDW) vs. OD_{600nm} (section 2.4.4). The feed, effluent and aeration tank liquor were sampled at hourly intervals for 6 hours, and thereafter at 24 h intervals. The experiment was terminated on day 29 due to failure of the feed pump. The COD removal and NSA effluent concentrations are shown in Figure 6.3. The COD, NSA concentration and MLSS were used to calculate U, the specific utilisation rate (mg COD mg^{-1} BDW h^{-1}), and q_E, the specific substrate utilisation rate (mg NSA mg^{-1} BDW h^{-1}) (section 4.5). The data are summarised in Table 6.1.

A mean COD removal of 85.9% (± 2.3%) and a mean U of 0.179 mg COD mg^{-1} BDW h^{-1} (± 0.01 mg COD mg^{-1} BDW h^{-1}) were measured in the 17 d period after start-up of the reactor. The mean COD removal fell to 66.9% (± 1.7%) after the addition of NSA, PSA and compound #21 to the reactor feed on day 17. However, mean U rose to 0.193 mg (± 0.02 mg) COD mg^{-1} BDW h^{-1}. The increase in U was due to a fall in mean MLSS, suggesting that the addition of the recalcitrant organic compounds had stressed the biomass leading to an increase in m, and a fall in the ratio of biomass production to COD removal (Low & Chase, 1999). During this period, the mean NSA levels in the effluent rose to a concentration of 49.37 (± 0.47) mg NSA L^{-1}. Six days after the addition of NSA, PSA and compound #21, the mean COD removal had increased to 77.5% and the mean NSA concentration in the effluent had fallen to 32.66 mg L^{-1} (q_E = 0.008 mg NSA

mg^{-1} BDW h^{-1}), indicating that the biomass had adapted to facilitate limited degradation of NSA. The mean *U* rose to 0.250 mg COD mg^{-1} BDW h^{-1}, again due to a fall in mean MLSS. Due to coelution of other materials, it was not possible to quantify the concentrations of PSA and compound #21 in the reactor effluent.

After inoculation of the reactor with *Sphingomonas* sp. strain RBN, the mean COD removal fell to 74.4% (± 3.2%) and the mean *U* fell to 0.247 mg COD mg^{-1} BDW h^{-1} (± 0.01 mg COD mg^{-1} BDW h^{-1}), due to the increase in MLSS subsequent to the addition of RBN. The effluent NSA concentration fell during the 6 h after inoculation and was reduced to 15.26 mg L^{-1} (qE = 0.017 mg NSA mg^{-1} BDW h^{-1}) after 24 h, indicating that addition of RBN to the reactor had enhanced the removal of NSA.

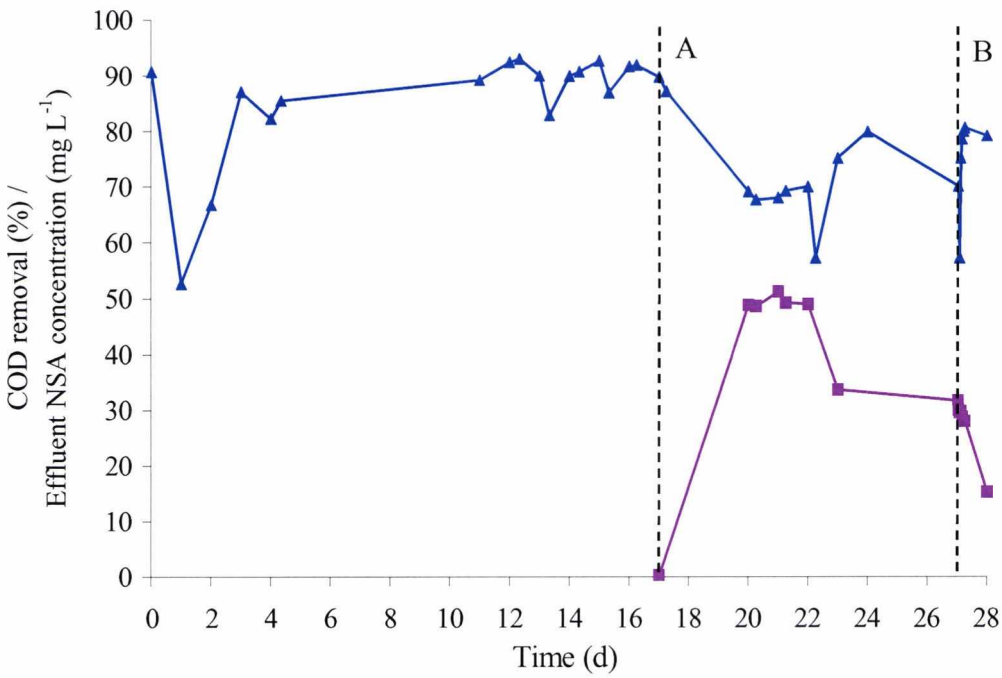

Figure 6.3. COD removal and effluent NSA concentration of a suspended floc reactor fed with CM after addition of NSA on day 17 (A) and inoculation with *Sphingomonas* sp. strain RBN on day 27 (B); ▲ - COD removal, ■ - effluent NSA concentration.

Gross COD removal and *U* values may not give a true representation of the efficiency of effluent treatment in the reactor. In particular, *U* values only describe the relationship between the degraded COD and biomass (MLSS, mg L^{-1}) and do not account for the residual COD in the effluent from the reactor. As such, the apparent COD removal rate

(mg COD mg $MLSS^{-1}$ h^{-1}) may increase as either a result of a change in the biomass to a microbial population with reduced numbers but with a greater efficiency for the degradation of the biodegradable COD, or an increase in *m* and a fall in the ratio of biomass production to COD removal (Low & Chase, 1999). However, while the rate of relative COD removal may rise, the total COD removal may fall, as observed after the addition of the recalcitrant compounds to the reactor feed (Table 6.1).

Table 6.1. Mean values for COD removal, mixed liquor suspended solids (MLSS), the specific utilisation ratio (*U*), effluent NSA concentration and (specific substrate utilisation rate (q_E) for a suspended floc reactor fed with CM after addition of NSA on day 17 (A) and inoculation with *Sphingomonas* sp. strain RBN on day 28 (B). Sample numbers are shown in parentheses. Values given for effluent NSA concentration and q_E after (B) are for 1 - 6 h and 24 h after inoculation. NA = not applicable.

Reactor event	MLSS (mg L^{-1})	COD removal (%)	*U* (mg COD mg BDW^{-1} h^{-1})	Effluent NSA (mg L^{-1})	q_E (mg NSA mg BDW^{-1} h^{-1})
Naïve biomass	168 (12)	85.98 (19)	0.179 (12)	NA	
Addition of NSA (A)	152 (6)	66.87 (6)	0.193 (6)	49.37 (5)	0.0002
Adaptation of biomass	135 (2)	77.52 (2)	0.250 (2)	32.66 (2)	0.008
Addition of RBN (B)	125 (7)	74.39 (7)	0.247 (7)	29.20 (6) 15.26 (1)	0.010 0.017

The efficiency of the biological treatment of a complex effluent can be described by determining *k*, the maximum organic removal rate. The *k* values for the reactor were determined using the equation (Eckenfelder & Musterman, 1995):

$$k = Se(So\text{-}Se)/XvT$$

where *So* = COD of the influent (mg L^{-1})

Se = COD of the effluent (mg L^{-1})

Xv = MLSS in the aeration tank (mg L^{-1})

T = HRT (d^{-1})

(Section 2.12.1)

The change in k values for the different reactor conditions were then calculated by linear regression analysis of k versus Se (Eckenfelder & Musterman, 1995). The values are shown in Table 6.2. The addition of the recalcitrant organic compounds to the reactor feed led to fall in k, i.e. a fall in the maximum organic removal rate. Bioaugmentation of the biomass with *Sphingomonas* sp. strain RBN increased k, indicating that inoculation with a metabolically-competent microorganism had increased the biological treatment efficiency of the reactor biomass. This was concurrent with a reduction in the NSA concentration measured in the reactor effluent, suggesting that increase in k was, in part, due to the degradation of NSA by RBN. Although the effluent concentrations of PSA and compound #21 could not be determined, the incomplete recovery of k after inoculation of the reactor, and the extensive reduction in NSA, suggests that these compounds were largely undegraded. As previously described (section 5.1), RBN was unable to degrade PSA and, therefore, the persistence of these compounds in the effluent, after bioaugmentation of the biomass with RBN, would be expected. This does not exclude the possibility that the existing biomass may have been capable of degradation of these compounds, albeit that the data suggests that this would have been limited.

Table 6.2. Maximum organic removal rate, k, for a suspended floc reactor fed with CM, after addition of NSA, PSA and compound #21, and inoculation with *Sphingomonas* sp. strain RBN.

Reactor condition	k (d^{-1})
Naïve biomass plus CM	39.41
Naïve biomass plus CM plus recalcitrant organic compounds	15.62
RBN-augmented biomass plus CM plus recalcitrant organic compounds	20.30

6.3. Labelling of the isolates with green fluorescent protein (*gfp)*

It is possible to conclude empirically, from observed improvements in the total organic removal rate (k) or specific substrate (q_E) removal, that the inoculation of a biomass with an isolate has improved the biological treatment of an effluent containing recalcitrant organic compounds. However, it is impossible to draw any conclusions from these data on the survival and growth of the inoculated isolate.

In order to study their persistence when inoculated into a complex, multispecies biomass, the isolates were chromosomally marked with green fluorescent protein (*gfp)* by plate-mating with *Escherichia coli* strain S17/1λpir (section 2.11.1). Strain S17/1λpir harboured the plasmid vector pUT-miniTn5*gfp*Km, encoding for *gfp* and kanamycin resistance. After mating, transconjugants were selected by plating on selective agar, and subsequent inoculation of growing colonies into MSM plus 500 mg NSA L^{-1} and 50 mg kanamycin L^{-1}.

Transconjugants of *Sphingomonas* sp strain RBN (RBN*gfp*) were successfully isolated. The labelling of RBN with *gfp* was confirmed by epifluorescent microscopy (section 2.11.2) and PCR amplification of the total DNA with *gfp* primers (section 2.10.4). The putative transconjugant, RBN*gfp*, fluoresced weakly when viewed by epifluorescent microscopy with blue light illumination. *Gfp* amplicons were produced by PCR of the DNA of RBN*gfp* and *Escherichia coli* strain S17/1λpir, with *gfp* primers. No *gfp*-PCR products were amplified from the wild type RBN (Figure 6.4). The growth and degradation characteristics of RBN*gfp* were determined in MSM plus 500 mg NSA L^{-1} and 50 mg kanamycin L^{-1} (section 2.3.2). The maximum growth and NSA removal were not significantly different ($p < 0.05$) to that of the wild type RBN strain (Figure 6.5). Neither the wild type RBN nor *Escherichia coli* strain S17/1λpir were able to grow in MSM plus 500 mg NSA L^{-1} and 50 mg kanamycin L^{-1}. All attempts to isolate a *gfp*-transconjugant of strain BAS proved unsuccessful.

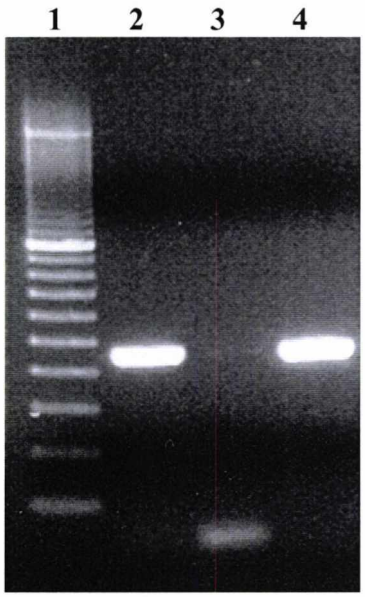

Figure 6.4. Gel electrophoresis of *gfp* amplicons from *Escherichia coli* strain S17/1λpir (pUTminiTn5*gfp*Km) and putative transconjugant RBN*gfp*. Lanes: 1, molecular ruler (100 base pair increment); 2, *gfp*-PCR of strain RBN*gfp* DNA; 3, *gfp*-PCR of wild-type strain RBN DNA; 4, *gfp*-PCR of *Escherichia coli* strain S17/1λpir.

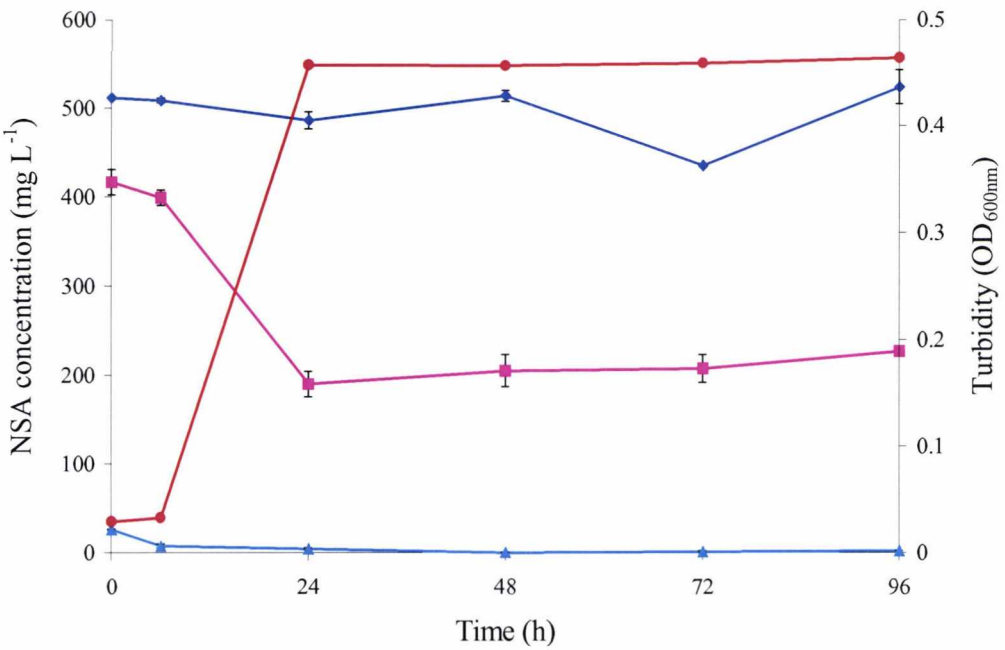

Figure 6.5. Utilisation of NSA and growth profile of transconjugant RBN*gfp* in MSM plus 500 mg NSA L^{-1} and 50 mg L^{-1} kanamycin: ■ - RBN NSA, ● - RBN turbidity, ♦ - uninoculated NSA control, ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

6.4. Bioaugmentation of a bacterial community receiving a multi-component feed containing NSA, with *Sphingomonas* sp. strain RBN*gfp*

The kinetics of NSA degradation in a suspended floc reactor were enhanced after inoculation with *Sphingomonas* sp. strain RBN (section 6.2). However, it was not possible to assess the survival of RBN after inoculation. The survival of RBN was assessed after inoculation of suspended floc reactors with RBN*gfp* (section 2.12.5).

Matched chemostat reactors, containing 1 l of CM supplemented with 45.9 mg NSA L^{-1} (10% of the total carbon in the feed) were inoculated with 15 ml of sludge 3 (section 2.1.1), equivalent to 5 x 10^4 CFU ml^{-1} in the reactors, and operated as batch reactors for 48 h. After 48 h, the reactors were switched to continuous culture, and fed with CM supplemented with 45.9 mg NSA L^{-1} at a dilution rate of 0.04 (HRT = 24 h). Both reactors were fed from a single source. After 28 d of operation, the reactors were inoculated with 10 ml of a 48 h culture of RBN*gfp* (reactor 1), or RBN*gfp*C, a strain of RBN*gfp* rendered incapable of degrading NSA by plasmid-curing (reactor 2). Eight days after inoculation, both reactors were shock-loaded by the addition of 250 mg NSA L^{-1}. During the experiment, the reactors were sampled for NSA quantification (section 2.2.1.4), and the reactor MLSS sampled for plate counts on TSA and TSA plus 50 mg kanamycin L^{-1} (section 2.12.5), and total DNA extraction (section 2.10.1). The DNA was amplified by PCR with *gfp* primers (section 2.10.4) and the amplicons quantified spectrophotometrically (section 2.10.3). The data for NSA removal, CFU counts on TSA plus kanamycin (CFUKm) and *gfp* quantification, are shown in Figures 6.6, 6.7 and 6.8.

The NSA concentration in reactor 2 fell to a mean of 10.98 (± 2.13) mg NSA L^{-1} after 72 h, whereas the concentration in reactor 1 remained unchanged. After 6 d of operation, the NSA concentrations in both reactors were reduced to below detectable levels, indicating that the biomass had adapted to degrade NSA. There was no significant difference between reactors of either the mean total CFU or the mean kanamycin-resistant CFU (CFUKm) ($p < 0.05$, to 28 d). Kanamycin-resistant CFU accounted for 4.2% (± 1.5) and 2.3% (± 0.9) of the total CFU in reactor 1 and reactor 2, respectively, with both reactors showing a gradual increase in CFUKm numbers to 28 d.

Inoculation of the reactors with either RBN*gfp* (reactor 1) or RBN*gfp*C (reactor 2), led to a peak in the numbers of CFUKm on 28 d. The mean CFUKm measured immediately after inoculation were not significantly different in either reactor ($p < 0.05$). In contrast, the concentration of *gfp*-PCR product from reactor 1 (1698.11 ng μl^{-1}) was greater than from reactor 2 (923.92 ng μl^{-1}). In both reactors, CFUKm numbers returned to preinoculation levels 24 h after inoculation. The CFUKm numbers were not significantly different, when compared between reactors ($p < 0.05$, CFUKm as % of total CFU), or pre- and post inoculation (4.3% ± 1.1% and 2.7% ± 0.8%, reactor 1 and reactor 2, respectively) to day 36. However, the concentration of *gfp*-PCR product from reactor 1 rose to 2891.22 ng μl^{-1} on day 29 before dropping to 1044.02 ng μl^{-1} on day 31. The concentration of *gfp*-PCR product from reactor 2 remained level during this period (to 36 d) and was consistently lower than from reactor 1. As such, both isolates were persistent in the reactors but were present at less than 4.3% (reactor 1) and 2.8% (reactor 2) of the total population.

Twenty-four hours after shock loading of the reactors with NSA, there was a significant increase in CFUKm numbers ($p = 0.005$) from 4.8% (± 0.5%) to 10.8% (± 1.1%) of the total CFU, and a two-fold increase in the concentration of *gfp*-PCR products, from 1564.81 ng μl^{-1} to 3378.09 ng μl^{-1}, in reactor 1. The concentration of NSA in reactor 1 was reduced from a peak of 51.37 (± 1.92) mg NSA L^{-1} to below detectable levels (< 1 mg L^{-1}) after 24 h. The CFUKm numbers and concentration of *gfp*-PCR products returned to pre-shock loading levels after 96 h and 72 h, respectively. In contrast, the CFUKm numbers in reactor 2 did not change significantly ($p < 0.05$) and were significantly lower than those in reactor 1 ($p = 0.016$) after shock loading. The concentration of *gfp*-PCR products from reactor 2 also did not change. NSA was still detectable in reactor 2 24 h after shock loading (4.90 mg NSA L^{-1} ± 0.01 mg L^{-1}) and was reduced to below detectable levels after 72 h, from a peak of 163.04 mg NSA L^{-1} (± 4.09 mg NSA L^{-1}). After 41 d, the reactors were stopped.

The introduction of an isolate or consortium may have an impact on the structure of the inoculated microbial community. The impact of bioaugmentation on the bacterial communities in the reactors was assessed using SSCP gel electrophoresis (section 2.10.9)

of the amplification products of partial 16S rDNA-PCR of the reactor DNA with Com1/Com2 primers (section 2.1.4). The results are shown in Figure 6.9. The communities were dissimilar and dynamic in structure. Both reactors displayed changes in banding profiles and the intensity of individual bands, indicating variation in the diversity and numbers of individuals of species over the course of the experiment. Inoculation with RBN*gfp* and RBN*gfp*C did not produce any additional bands in the profiles of either reactor. However, as indicated in Figure 6.9, the profile of reactor 1 revealed decreases in the intensity of two bands subsequent to inoculation of the reactor with RBN*gfp*, suggesting an impact of bioaugmentation on the community structure in the reactor. There were no similar impacts apparent after bioaugmentation of reactor 2 with RBN*gfp*C. There was no evidence of impact on the bacterial community of either reactor as a result of shock loading.

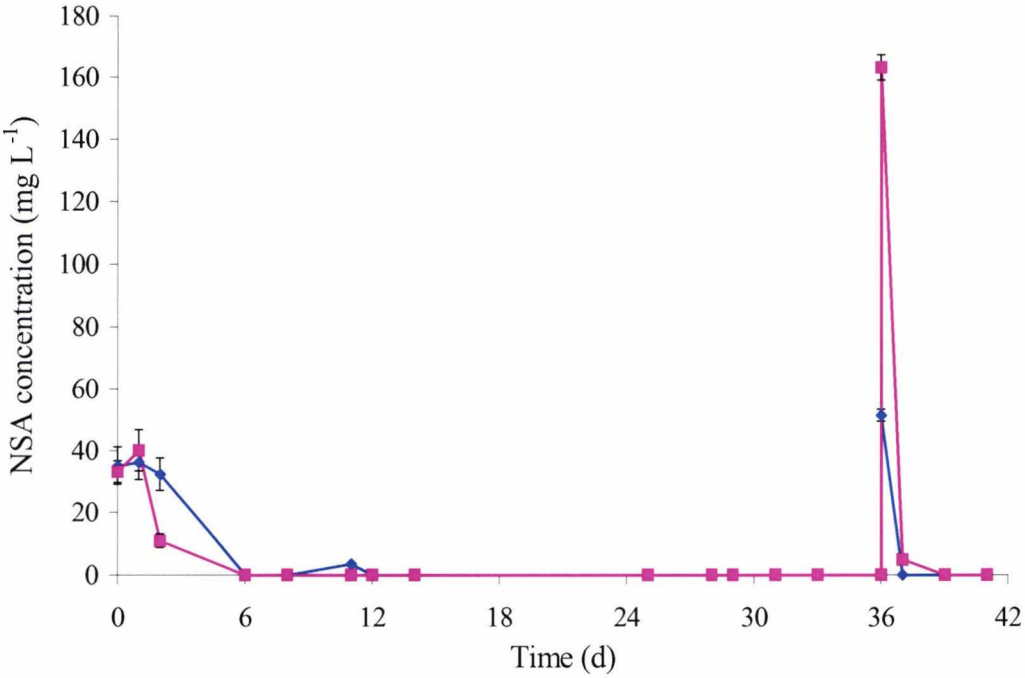

Figure 6.6. NSA concentrations in chemostat reactors inoculated on day 28 with *Sphingomonas* sp. strain RBN*gfp* (♦, reactor 1) and *Sphingomonas* sp. strain RBN*gfp*C (■, reactor 2) and shock-loaded with 250 mg NSA on day 36.

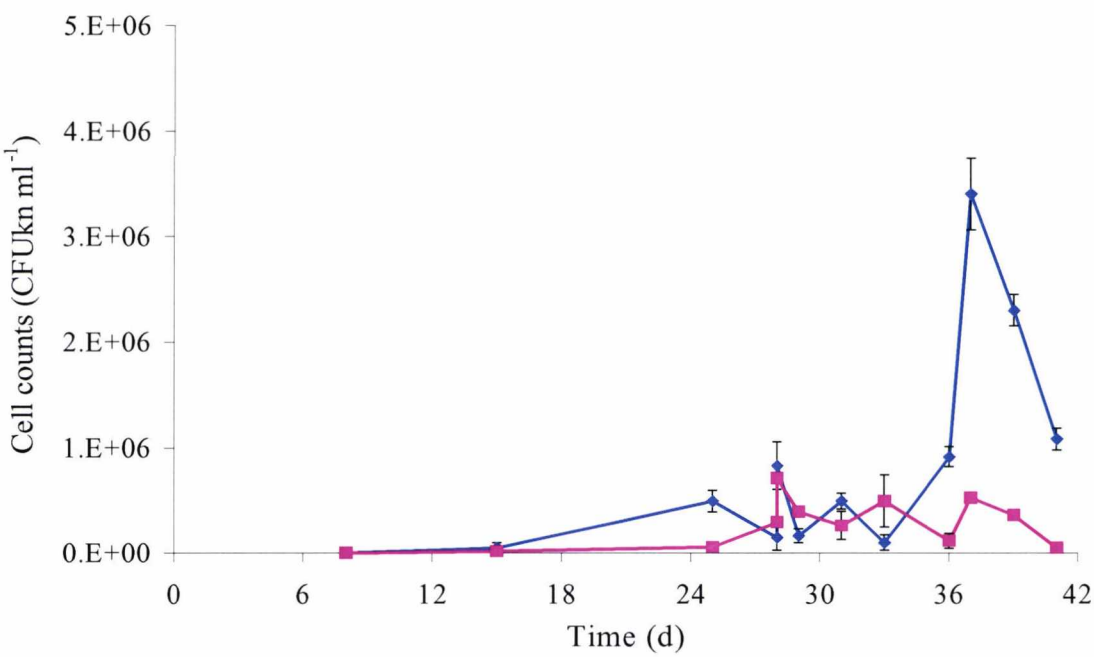

Figure 6.7. Kanamycin-resistant CFU numbers in the biomass from chemostat reactors inoculated on day 28 with *Sphingomonas* sp. strain RBN*gfp* (♦, reactor 1) and *Sphingomonas* sp. strain RBN*gfp*C (■, reactor 2) and shock-loaded with 250 mg NSA on day 36.

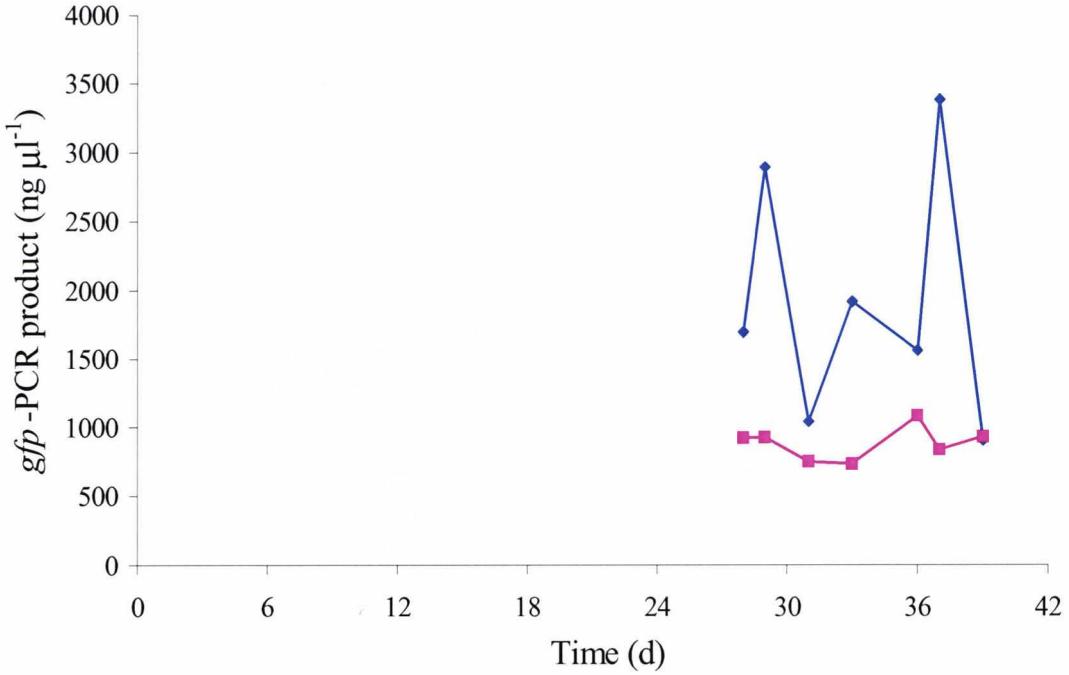

Figure 6.8. Concentration of *gfp*-PCR products amplified from the total DNA extracted from the biomass of chemostat reactors inoculated on day 28 with *Sphingomonas* sp. strain RBN*gfp* (♦, reactor 1) and *Sphingomonas* sp. strain RBN*gfp*C (■, reactor 2) and shock-loaded with 250 mg NSA on day 36.

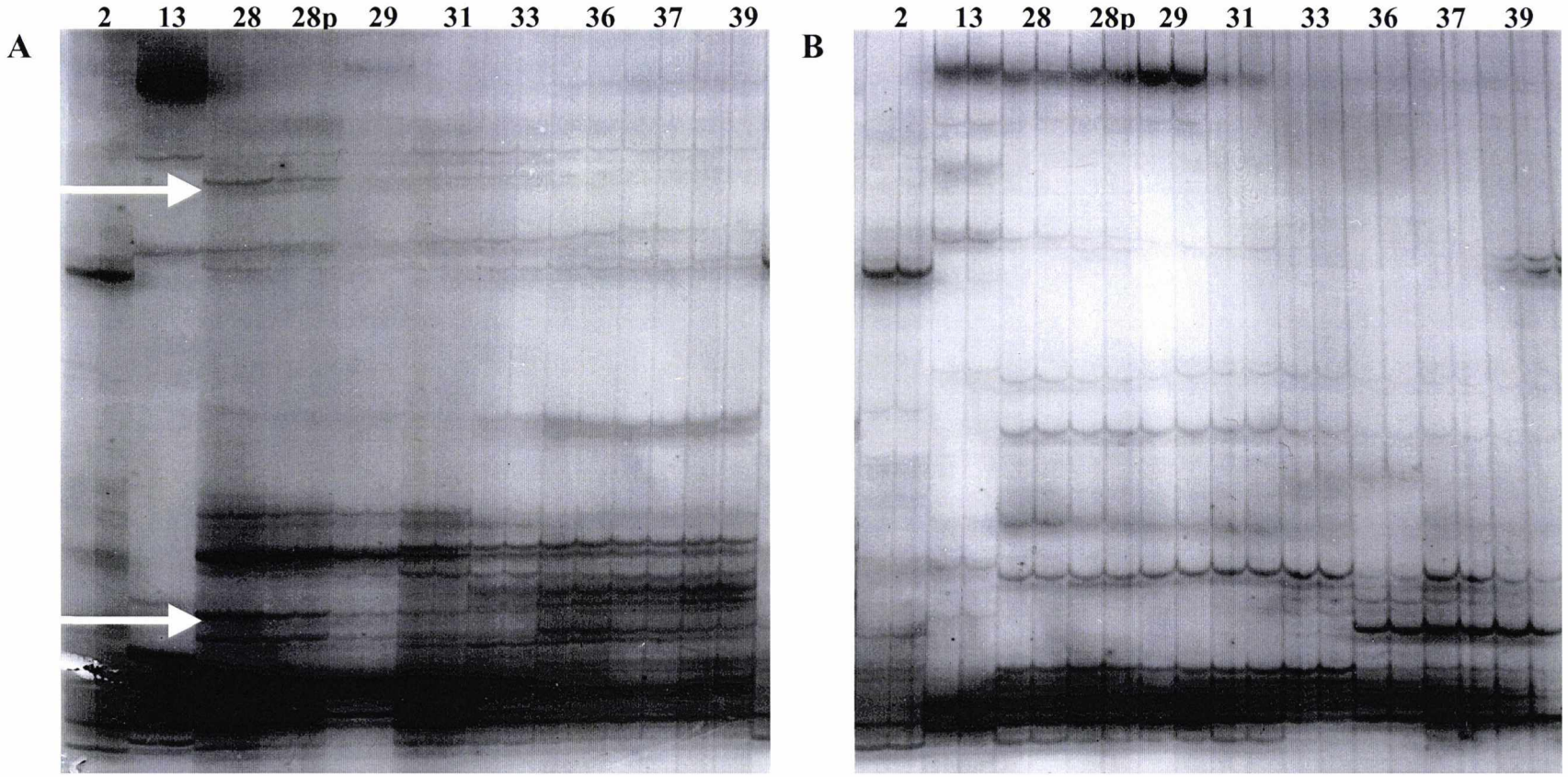

Figure 6.9. SSCP gel electrophoresis of partial 16S rDNA amplicons from reactor 1 (A) and reactor 2 (B) before and after inoculation with *Sphingomonas* sp. strain RBN*gfp* (A) or *Sphingomonas* sp. strain RBN*gfp*C (B). Samples taken on the indicated day numbers (28p sampled immediately after inoculation). Each sample was run in duplicate. Regions displaying a possible impact of bioaugmentation in reactor 1 are indicated by arrows.

Discussion

Bioaugmentation (of biotreatment plants) is the accelerated removal of undesired compounds by the use of indigenous or allochthanous wild-type or genetically-modified microorganisms (Boon *et al.* 2000). Inoculation of activated sludges with metabolically competent isolates to facilitate or enhance the degradation of recalcitrant pollutants is frequently reported (Selvaratnam *et al.*, 1995; Watanabe *et al.*, 1996; Yu & Mohn, 2001; Cavalca *et al.*, 2002). However, the degradation of a target compound by an isolate shown in the laboratory, may not occur when that isolate is inoculated into an environment containing an established microbial community and numerous other carbon sources. Addition of a PCB-degrading *Arthrobacter* sp. strain B1B did not enhance degradation of PCB in a contaminated soil (Gilbert & Crowley, 1998). Similarly, a 2,4-D-degrading *Pseudomonas* sp. strain did not mineralise 2,4-D when inoculated into contaminated sewage (Goldstein *et al.*, 1985).

Failure of an inoculated isolate, shown to be competent in the laboratory, to degrade a target compound may be due to catabolite repression. Catabolite repression is the regulatory mechanism by which the expression of genes required for the utilisation of secondary carbon sources is suppressed in the presence of a preferred substrate. In the presence of a range of carbon sources, bacteria are able to preferentially utilise the source that allows for the highest growth rate and may only synthesise the enzymes required to utilise that source (Stülke & Hillen, 1999). As such, it may be that when exposed to the target compound at environmental concentrations, the catabolic pathways for the degradation of the target compound are repressed in favour of more abundant or more readily degradable carbon sources.

Catabolite repression is widespread among bacteria. The synthesis and secretion of the enzymes for degradation of natural polymers, such as starch and cellulose, by saccharolytic *Clostridia* sp., were repressed in the presence of the rapidly metabolised monomeric component, such as glucose (Mitchell *et al.*, 1995). Repression of the catabolic pathways for the degradation of xenobiotics has also been reported. Deutz *et al.* (1994) reported repression of toluene utilisation by *Pseudomonas putida* in the presence

of succinate and glucose. The expression of styrene monooxygenase, the first enzyme in the styrene degradation pathway, was also repressed in the presence of succinate (O'Leary *et al.*, 2002). The degradation of NSA and BSA by *Pseudomonas maltophila* BSA6, was almost entirely repressed by the presence of substrates which allowed for rapid growth, such as acetate (Lee & Clark, 1993).

The effect of other carbon sources on the degradation of NSA by *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS was investigated in batch culture using CM plus NSA. The removal of NSA (500 mg L^{-1}) by the isolates in CM (Figure 6.1), was not significantly different to that measured when NSA was supplied as sole source of carbon and energy (section 4.2). Complete degradation of NSA was measured when NSA was present at 50 mg L^{-1} (Figure 6.2). The growth of the isolates at both concentrations of NSA was also similar to that observed when NSA was supplied as sole source of carbon and energy. The utilisation of NSA suggested neither isolate was subject to catabolite repression, due to the presence of alternative carbon sources, at the concentrations of NSA examined.

Diauxy is a phenomenon of catabolite repression in batch culture, during which the growth profile shows a number of distinct growth phases separated by a lag(s). This profile is due to sequential expression of catabolic pathways, regulated by catabolite repression mechanisms and favouring the preferred carbon source(s) (MacGregor *et al.*, 1991). Complete catabolite repression of the utilisation of phenol by *Ralstonia eutropha* 335, resulting in diauxic growth, was observed during growth on a mixture of phenol and benzoate (Ampe *et al.*, 1998). There was no evidence of polyphasic growth by either isolate at either concentration of NSA (Figures 6.1 & 6.2), indicating that NSA utilisation by the isolates was not subject catabolite repression, under the described conditions. The continued utilisation of the target compound by an inoculated microorganism in the presence of other carbon sources, is clearly essential to the success of bioaugmentation.

In the presence of a mixture of carbon sources, under carbon-limited conditions, bacteria will utilise a number of carbon sources simultaneously (mixed substrate growth).

Bacteria that utilise a wide range of carbon sources simultaneously may grow more rapidly than those with a narrow substrate spectrum (Lendenmann & Egli, 1998). When grown with lactate, pyruvate or a mix of the two, the mixed substrate maximum specific growth rate of *Escherichia coli* K12 was greater than either of the single substrate maximum specific growth rates (Narang *et al.*, 1997). Mixed substrate growth may increase the degradation rate of a target compound. Ellis *et al.* (1998) observed an increase in phenol degradation in a biomass fed a mixture of carbon sources, compared to when phenol was supplied as sole source of carbon and energy.

The maximum growth of both isolates in CM plus NSA was significantly greater at both 500 mg NSA L^{-1} and 50 mg NSA L^{-1} than that measured when NSA was supplied as sole source of carbon and energy (section 4.2). However, the utilisation of NSA at 500 mg L^{-1} was not significantly different to that previously measured (section 4.2), indicating that the increased growth was due to utilisation of the other carbon sources present. Similarly, the carbon content of 50 mg NSA L^{-1} would have been insufficient to support the growth observed in CM plus NSA, if it had been the source of carbon exclusively utilised by the isolates. Similar patterns of growth and NSA utilisation were measured when the isolates were grown in MSM plus 500 mg NSA L^{-1} supplemented with glucose (2 g L^{-1}) as the most abundant carbon source (section 4.4). Clearly, both isolates were utilising the alternative carbon sources in the media, and the absence of diauxy in the growth profiles indicated that this was concurrent with the utilisation of NSA.

The continued utilisation of NSA in the presence of alternative carbon sources by the isolates may have been due to the preinoculation growth conditions, i.e. culture of the isolates in MSM plus NSA. Preinoculation induction of catabolic pathways has been shown to reduce or negate the repressive effect of other carbon sources to which the inoculant is subsequently exposed. Narang *et al.* (1997) found that the growth rate of *Escherichia coli* K12 on mixtures of sugars was determined by the sugar used in the preinoculation media. Glucose repression of glucitol metabolism in *Clostridium pasteurianum* was reduced if the cells were precultured with glucitol (Roohi & Mitchell, 1987). Preinoculation culturing of the isolates in MSM plus NSA may have predisposed the cells to NSA utilisation, even when NSA was not present as the predominant carbon

source. The preinoculation culture conditions of isolates may therefore, have a significant effect on the success of bioaugmentation with those isolates.

As previously discussed, bioaugmentation may fail in its primary purpose, if the inoculated organism preferentially utilises more readily degradable carbon sources (Goldstein *et al.*, 1985). Mixed substrate growth, which may facilitate faster growth than single substrate growth and increase the survival potential of the isolate is, therefore, a desirable characteristic. The results of this study demonstrate that, in contrast to some reported NSA-degrading isolates (Lee & Clark, 1993), both isolates continued to degrade NSA, as both the predominant carbon source and at a concentration relevant to industrial effluent (section 3.2), in the presence of alternative carbon sources. The degradation of NSA was also concomitant with the utilisation of the alternative carbon sources (i.e. mixed substrate growth). Therefore, both *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS may be deemed to be metabolically competent for bioaugmentation of wastewaters containing NSA and other carbon sources.

The success or failure of a bioaugmentation depends on two conditions: the survival of the isolate and the demonstration of its degradative activity (Stephenson & Stephenson, 1992). The degradation kinetics of a suspended floc reactor, fed CM plus three recalcitrant compounds, NSA, PSA and compound #21 (section 2.1.3) and following bioaugmentation with *Sphingomonas* sp. strain RBN, was assessed (section 6.2). The data are summarised in Table 6.1 and 6.2. The addition of all three compounds to the reactor lead to a fall in k, from 39.41 d^{-1} to 15.62 d^{-1}. The removal rates for NSA (q_E) were initially low, although 6 d after addition of the three compounds, an increase in q_E, from 0.0002 mg NSA mg BDW^{-1} h^{-1} to 0.008 mg NSA mg BDW^{-1} h^{-1}, indicated that the indigenous biomass had adapted to allow for limited degradation of NSA. Bioaugmentation of the reactor with RBN resulted in a 112.5% increase in q_E and a 29.9% increase in k, in the 24 h subsequent to inoculation. Due to coelution of other materials, it was not possible to quantify the concentrations of PSA and compound #21 in the reactor effluent. The extent to which the improvements in treatment were maintained could not be investigated beyond 24 h due to failure of the feed pump.

There are numerous reports of the successful bioaugmentation of biological treatment systems receiving feeds containing recalcitrant organic compounds. Inoculation of reactors fed peptone and phenol, with two phenol-degrading organisms shortened the start-up period for phenol removal (Watanabe *et al.*, 1996). Increasing rates of *p*-toluene sulphonate (pTS) removal were measured with increasing inoculum size when a pTS-degrading isolate, *Comamonas testosteroni* T-2, was added to domestic activated sludge (Bokhamy *et al.*, 1997). Inoculation of membrane separation reactors (MBR) with the 3-chlorobenzoate (3CBA)-degrading *Pseudomonas putida* BN210 increased the resistance of the MBR to shock-loading with 3CBA (Ghyoot *et al.*, 2000). Bioaugmentation has also been shown to improve the total organic removal kinetics. Inoculation of a conventional activated sludge reactor (CASR) treating fish market waste with phototrophic bacteria (PTB), increased k compared to an uninoculated reactor (Huang *et al.*, 1999). Similarly, inoculation of a CASR treating anaerobically pre-treated piggery waste with a *Rhodobacter* sp., increased k and reduced the fraction of recalcitrant organic materials in the effluent, compared to an uninoculated CASR (Huang *et al*, 2001).

In this study, the effect of bioaugmentation of a suspended floc reactor on the specific substrate and total organic removal kinetics, was determined. The data show that inoculation with *Sphingomonas* sp. strain RBN improved the treatment efficiency (k) of the reactor and that this improvement was due to an increase in q_E. Therefore, targeting a specific recalcitrant organic pollutant, NSA, by inoculation of the reactor with a NSA degrading isolate, was shown to be a successful strategy for improving the treatability of the feed. However, inoculation of the reactor with RBN did not return k to the value calculated prior to the addition of the recalcitrant compounds to the reactor feed. The relatively reduced value of k after inoculation may have been due to the presence of PSA and compound #21 in the effluent from the reactor. While it was not possible to measure the concentrations of these compounds in the effluent, the reduced levels of COD removal (%) and reduced value of k, concurrent with the increased removal of NSA, before and after inoculation of the reactor, suggest that these compounds were not degraded to any significant extent.

The improvements in total organic removal rate (k) and specific substrate removal rate (q_E) indicated that the use of *Sphingomonas* sp. strain RBN for the bioaugmentation of a suspended floc reactor had been successful. However, the survival and growth of RBN, subsequent to inoculation, could not be determined. A number of strategies have been applied for determining the fate of microorganisms inoculated into contaminated environments (section 1.10). Examples of these include: i) enumeration of two 3-CBA-degrading isolates, *Pseudomonas putida* BN210 and *Ralstonia eutrophia*-like AE2250, in activated sludge by plating on selective agar (Ghyoot *et al.*, 2000); ii) quantitative-PCR with strain-specific *gyr*B primers of two phenol-degrading isolates, *Pseudomonas putida* BH and *Comamonas* sp. strain EH6, in activated sludge (Watanabe *et al.*, 1998); and iii) *gfp*- and *lux*-marking to determine the survival and activity, respectively, of a 4-chlorophenol-(4-CP)-degrading isolate, *Arthrobacter chlorophenolicus* A6L, after inoculation into 4-CP-contaminated soil (Elväng *et al.*, 2001).

In this study, attempts were made to chromosomally tag *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS with *gfp*. As previously described (section 1.10), *gfp* is a useful marker for monitoring microbial populations. RBN was chromosomally marked with *gfp* by plate-mating with *Escherichia coli* S17/1λpir(pUTminiTn*gfp*Km) (section 2.11). The plasmid vector, pUTminiTn*gfp*Km, carries a gene fusion of the *gfp* variant P11 to a strong, constitutive, broad host-range promoter, P*psb*A, and the *nptII* gene, encoding for kanamycin resistance. A transconjugant of RBN, RBN*gfp*, was successfully isolated. The presence of *gfp* in the transconjugant was confirmed by epifluorescent microscopy and *gfp*-PCR (figure 6.4). The transconjugant showed the same growth profile and NSA degradation as the original strain, in MSM plus 500 mg NSA L^{-1} (figure 6.3), indicating that RBN had been successfully marked with *gfp*.

The *gfp* marked strain was used to evaluate the bioaugmentation of matched suspended floc reactors receiving CM plus 45.9 mg NSA L^{-1} (section 6.4). The concentration of NSA in both reactors was reduced to below detectable levels 6 d after seeding with sludge 3 (Figure 6.6), indicating that the biomass had adapted for the degradation of NSA. NSA has been detected before and after biological treatment, in industrial effluents

discharged to municipal treatment works (Reemtsma & Jekel, 1994; Castillo *et al.*, 2001) and it is conceivable that the inoculum sludge 3 may have had previous exposure to NSA. Ghyoot *et al.* (2000) reported adaptation of the indigenous biomass for the degradation of 3- and 4-CBA, 6 – 9 d after start-up of semi-continuous activated sludge reactors. Magbanua *et al.* (1998) reported the degradation of a number of synthetic organic compounds, including 4-chlorophenol, 4-nitrophenol and *m*-toluate, by an activated sludge from a municipal wastewater treatment plant fed a mixture of biogenic and synthetic organic compounds. As shown here, the facility for the degradation of a given xenobiotic compound, such as NSA, may already exist in activated sludges from municipal wastewater treatment plants.

After 28 d of operation, the reactors were inoculated with RBN*gfp* (reactor 1) and RBN*gfp*C, the plasmid-cured, non-NSA-degrading strain (reactor 2). Inoculation of reactor 2 with RBN*gfp*C served as a control, with both reactors receiving the same shock load of inoculated microorganisms but with reactor 2 receiving no increase in metabolic capacity for the degradation of NSA. The inoculation of RBN*gfp*C also allowed for the separate investigation of the survival of the isolate after inoculation, in the absence of the ability to utilise NSA as source of carbon and energy.

The number of CFUKm in both reactors peaked at similar levels immediately after inoculation and then fell back to preinoculation levels ($p < 0.05$) of between 4.3% and 2.7% (reactor 1 and reactor, respectively) 24 h thereafter (Figure 6.7). In contrast to the CFUKm, the measured *gfp* levels in reactor 1 rose sharply in the 24 h subsequent to inoculation (Figure 6.8), suggesting a rapid increase in the population of RBN*gfp*, which was not reflected in the CFUKm counts. However, the persistence of *gfp* is independent of the metabolic status of the cell (Unge *et al.*, 1999) and hence, the measured *gfp* levels may have been affected by the presence of dead, metabolically inactive or non-culturable cells. It is conceivable that RBN*gfp* grew rapidly immediately after inoculation but that 24 h after inoculation, the culturable population had declined, resulting in the observed disparity between measured *gfp* levels and CFUKm. This may also explain the apparent stability of *gfp* levels, compared to the fall in CFUKm, measured in reactor 2. It is also possible that the difference in *gfp* levels between reactor 1 and 2 during the 24 h

subsequent to inoculation, indicated that RBN*gfp* grew more rapidly than RBN*gfp*C, suggesting that the ability to degrade NSA conferred a competitive advantage on RBN*gfp*. Further investigation of the population dynamics in the reactors during the 24 h period after inoculation would be required to confirm this.

Watanabe *et al.* (1998) reported a biphasic fall in the population of phenol-degrading *Pseudomonas putida* BH after inoculation into a phenol-digesting activated sludge, with a fast decline phase, where the population fell by several orders of magnitude, followed by a slow decline phase. In contrast, the population of 3-CA-degrading *Comamonas testosteroni* I2*gfp* inoculated into an activated sludge incapable of degrading 3-CA remained at the inoculated value or above in the three weeks subsequent to inoculation (Boon *et al.*, 2000). This contrast suggests that the population size of an inoculated isolate may be determined, in part, by the presence of indigenous organisms capable of degrading the target compound. Therefore, the population size of RBN*gfp* may have been limited by the presence of other NSA-degrading strains in the biomass. However, the proportion of competent biomass capable of degrading synthetic organic compounds, in an activated sludge fed a mixture of biogenic and synthetic compounds, was estimated as between 3.1% and 6.4% (Magbanua *et al.*, 1998). As such, the population size of RBN*gfp* may have been related to its ability to utilise NSA. These factors would not however, explain the persistence RBN*gfp*C.

The survival of an isolate may not be solely dependent on or determined by its ability to degrade a target compound (section 1.9). In contrast to a number of other isolates, AS2, a 3-CBA degrading activated sludge isolate, was found to establish a stable population after inoculation into an activated sludge, which was attributed to its ability to form flocs (McClure *et al.*, 1991). *Sphingomonas paucimobilis* 551 (pS10–45), a floc-forming isolate genetically modified to degrade phenol, maintained population levels of 3 – 4 times greater than that of the similarly modified non-floc-forming *Escherichia coli* HB101 (pS10-45), after inoculation into an activated sludge. Again, the greater population numbers were attributed to the ability of *Sphingomonas paucimobilis* 551 (pS10-45) to form flocs (Soda *et al.*, 1999).

In this study, both *Sphingomonas* sp strain RBN*gfp* and *Sphingomonas* sp strain RBN*gfp*C were found to persist after inoculation into a NSA-degrading activated sludge. The survival of RBN*gfp*C suggests that the incorporation of these isolates was not related to their ability to exploit a 'specialist' niche, the degradation of NSA, but to another characteristic of the original isolate, *Sphingomonas* sp. strain RBN. A number of xenobiotic-degrading sphingomonad strains have been isolated from activated sludges, including strains degrading substituted naphthalenesulphonates (Stolz, 1999), dehydroabietic acid (Muttray & Mohn, 1999) and PCP (Tiirola *et al*, 2002). Sphingomonads are also reported as being abundant in activated sludges, accounting for between 5 – 10% of the total cells (Neef *et al.*, 1999). Therefore, it is perhaps unsurprising that the *gfp* modified strains of RBN, a xenobiotic-degrading *Sphingomonas* sp. isolated from activated sludge, persisted after inoculation into reactor biomass.

Shock loading of the reactors with NSA resulted in a significant increase in both CFUKm (Figure 6.7) and *gfp* levels (Figure 6.8) in reactor 1, indicating a proliferation of RBN*gfp* in response to the shock load. Both *gfp* and CFUKm returned to pre-shock loading levels after 48 h and 96 h, respectively. A similar increase was not observed in reactor 2. The concentration of NSA in reactor 1 was reduced to below detectable levels after 24 h but was still detectable in reactor 2 after 24 h, falling below detectable levels after 72 h (Figure 6.6). Inoculation with a metabolically competent isolate, RBN*gfp*, was shown to increase the resistance of a suspended floc reactor to a shock load of NSA. This was demonstrated by the rapid increase in the size of RBN*gfp* population in response to the shock load, and the enhanced rate of removal of NSA, compared to that measured in a reactor inoculated with RBN*gfp*C.

Proliferation of metabolically competent isolates in response to shock loads has been previously reported. Shock loading of a phenol-digesting sludge inoculated with phenol-degrading *Pseudomonas putida* BH, lead to a 10-fold increase in the numbers of strain BH. The population rapidly declined to pre-shock load numbers after the shock loading was stopped (Watanabe *et al.*, 1998). Inoculation of activated sludges with metabolically competent organisms has also been shown to increase resistance to shock loading.

Inoculation with 3CBA-degrading *Pseudomonas putida* BN210 conferred a greater degree of resistance to 3-CBA shock loads on a MBR than on a similarly inoculated CAS reactor. This greater resistance was attributed to the retention of BN210 by the MBR, and hence, the increased metabolic capacity for the degradation of 3-CBA (Ghyoot *et al.*, 2000).

The impact of bioaugmentation and shock loading on the bacterial communities in the reactors was assessed by SSCP gel electrophoresis of the 16S rDNA amplicons of PCR amplification of the DNA extracted from the biomass in each reactor over the course of the experiment (Figure 6.9). Although seeded with the same inoculum, the communities in the reactors were found to be dissimilar and dynamic. The banding patterns in both reactors changed with time, indicating shifts in the structure of the communities. The evolution of dissimilar and dynamic community structures, in identical reactors seeded with a common inoculum, have been reported elsewhere (Boon *et al.*, 2000; Miskin *et al.*, 2001).

Inoculation did not appear to have any distinct impact on the community structure of either reactor. It was not possible to distinguish the inoculated *Sphingomonas* sp strain RBN strains in either reactor. PCR-SSCP represented 69% of the total diversity indicated by a clone library, of the 16S rDNA of microbial community of from an anaerobic digester, indicating a minimum cell number threshold for the detection of strains in a community (Delbès *et al.*, 2000). The detection limit for individual populations by SSCP is approximately 1.5% of the total population of a bacterial community (Lee *et al.*, 1996). The CFUKm of both reactors indicated that the populations of RBN*gfp* could account for between 2.3% (reactor 2) and 4.2% (reactor 1) of the total CFU. The concurrrent increase in measured *gfp* levels and CFUKm in reactor 1 (to 10.8% of total CFU) after shockloading would suggest that the increase in CFUKm was due to an increase in the numbers of RBN*gfp*. Assuming a detection limit for an individual population of 1.5%, it was expected that the change in the RBN*gfp* population would have been detectable by SSCP. However, this was not the case and no banding pattern associated with either the inoculation or shockloading of the reactor was apparent. Ghyoot *et al.* (2000) and Boon *et al.* (2000) also failed to distinguish inoculated strains

in activated sludge, using DGGE of 16S rDNA amplicons, even immediately subsequent to inoculation.

A fall in intensity of 2 bands amplified from reactor A was noted 24 h after inoculation with RBN*gfp* and it is possible that the change in intensity was indicative of a change in the community structure as a result of inoculation with RBN*gfp*. This period is coincidental with a peak in *gfp* product observed 24 h after inoculation and may indicate a temporary repression of one or more of the strains indigenous to the reactor biomass. However, as inoculation could not be distinguished by the appearance of new bands in the banding pattern on the gel, it was not possible to compare the intensity of the inoculum bands with the impacted bands. Both impacted bands showed increased intensity in the sampling periods thereafter. There was no apparent impact of inoculation on reactor 2. Shock loading also had no visible impact on the bacterial communities in either reactor.

Conclusions

As previously stated, the success or failure of bioaugmentation depends on two conditions: the survival of the isolate and the expression of its degradative activity (Stephenson & Stephenson, 1992). Here, *Sphingomonas* sp. strain and *Comamonas testosteroni* strain BAS were shown to utilise NSA in the presence of other carbon sources. As such, both isolates would be expected to utilise NSA, at the concentrations evaluated, after inoculation into wastewaters. *Sphingomonas* sp. strain RBN was shown to persist in activated sludge regardless of its metabolic capacity for the degradation of the target compound. Inoculation with RBN was also shown to improve both the steady state kinetics of NSA degradation (section 6.2), and NSA shock load resistance (section 6.4), of activated sludges. The bioaugmentation of these sludges with RBN was, therefore, successful.

CHAPTER 7

BIOLOGICAL AND PHYSICOCHEMICAL DEGRADATION OF POLYMERIC NAPHTHALENE SULPHONATE-FORMALDEHYDE CONDENSATES

Chapter 7

BIOLOGICAL AND PHYSICOCHEMICAL DEGRADATION OF POLYMERIC NAPHTHALENE SULPHONATE-FORMALDEHYDE CONDENSATES

7.1. Degradation of a naphthalene sulphonate-formaldehyde condensate-based syntan (compound #22) by *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS.

Bioaugmentation of suspended floc reactors with NSA-degrading isolates was shown to enhance the degradation of NSA (section 6.2) and improve resistance to NSA shock loads (section 6.4). Therefore, inoculation with appropriate isolates may be of benefit in the biological treatment of NSA-contaminated effluents. However, NSA may be present in effluents as a contaminant arising from the production and use of formaldehyde condensate polymers of NSA (SNFC). Significant concentrations of SNFC were measured in two industrial effluents (section 3.2). Consequently, the degradation of a SNFC-based syntan, compound #22, by RBN and BAS was investigated (section 2.3.2).

The growth of *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS in MSM plus 500 mg #22 L^{-1} was similar (Figure 7.1). Maximum turbidities of 0.058 (± 0.001) and 0.049 (± 0.001) were measured after 24 h, in the medium inoculated with RBN and BAS, respectively. These turbidities were comparable to those measured when both isolates were grown with 50 mg NSA L^{-1} as sole source of carbon and energy (section 4.3). Compound #22 was shown to contain NSA (56.82 ± 0.49 mg NSA L^{-1}) (section 3.1). The increase in turbidity was concurrent with the disappearance of NSA from the inoculated media indicating that the NSA in compound #22 was utilised by the isolates as a source of carbon and energy. However, no significant change in the concentration of compound #22 was measured in the medium after inoculation with either isolate ($p > 0.05$, 0 h vs. 96 h) suggesting that neither isolate was able to utilise compound #22 as sole source of carbon and energy.

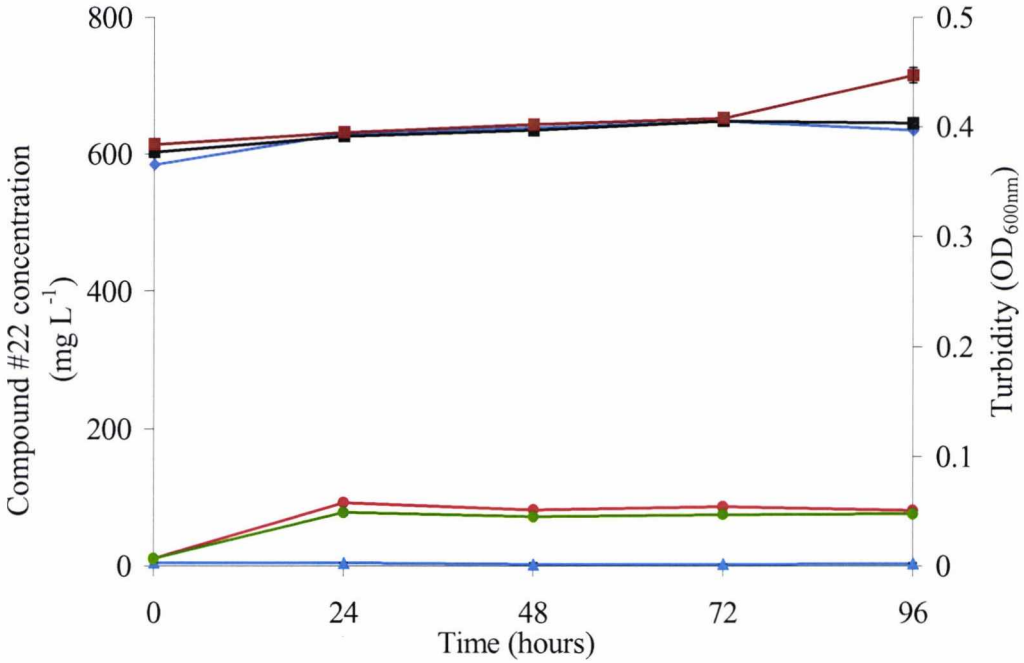

Figure 7.1. Utilisation of compound #22 and growth profile of isolates RBN and BAS in CM plus 500 mg #22 L^{-1}: ■ - RBN #22, ● - RBN turbidity, ■ - BAS #22, ● - BAS turbidity, ♦ - uninoculated #22 control, ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

7.2. Utilisation of compound #22 by *Sphingomonas* sp. strain RBN as sole source of carbon, energy and sulphur

Sphingomonas sp. strain RBN was shown previously (section 4.4) to utilise NSA as sole source of carbon, sulphur and energy. The catabolic pathways involved in utilisation of organosulphonates as sole source of carbon, energy and sulphur may differ from those involved in their utilisation as sole source of carbon (Seitz *et al.*, 1993). Therefore, the induction of different catabolic pathways by sulphate limitation, on the utilisation of compound #22 as a source of carbon, energy and sulphur was investigated (section 2.5).

The growth profile of RBN in sulphur-free minimal medium plus 500 mg #22 L^{-1} (Figure 7.2) was not significantly different ($p > 0.05$) to its growth profile in MSM plus 500 mg #22 L^{-1} (section 7.1). A maximum turbidity (OD_{600nm}) of 0.053 (± 0.001) was measured after 24 h. There was no significant change in the concentration of #22 in either the

inoculated or uninoculated media over the course of the experiment ($p > 0.05$, 0 h to 96 h). The small increase in turbidity measured suggests that, as previously observed (section 7.1), RBN was unable to utilise compound #22, as a growth substrate, but was utilising the free NSA present in compound #22. It should be noted that the composition of compound #22 is largely unknown and, while the growth of the isolates suggests that NSA was the only readily degradable source of carbon present, it was not known if compound #22 contained other utilisable sources of sulphur, such as inorganic sulphate.

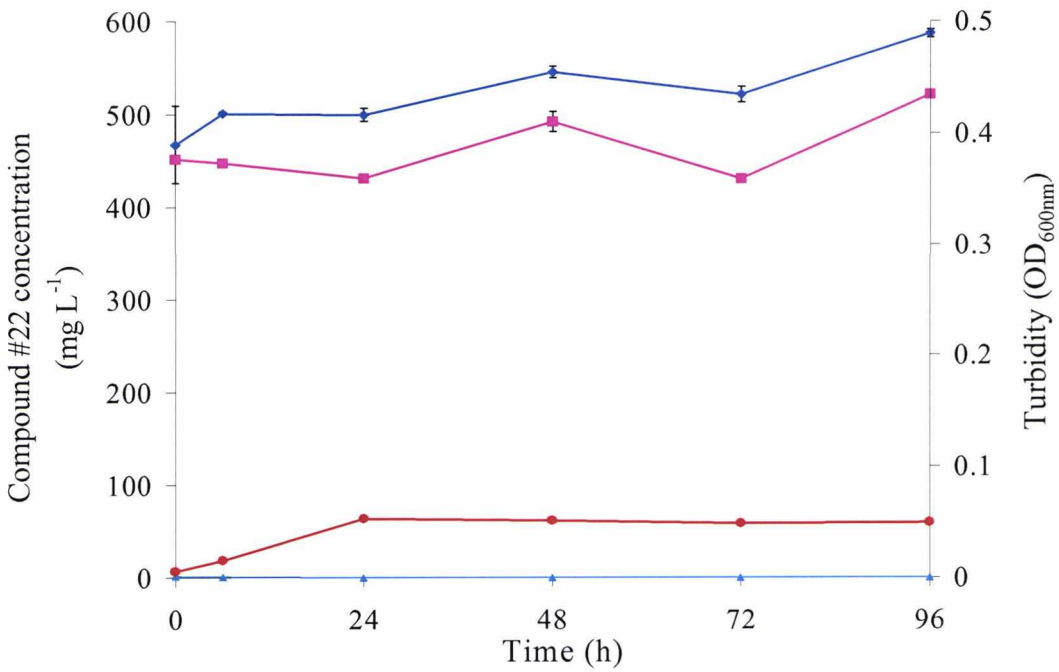

Figure 7.2. Utilisation of compound #22 and growth profile of isolate RBN in sulphur-free medium plus 500 mg #22 L^{-1}; ■ - RBN #22, ● - RBN turbidity, ♦ - uninoculated #22 control, ▲ - uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

7.3. Inhibition of growth and cometabolic degradation of compound #22 by *Sphingomonas* sp. strain RBN

A number of factors may have prevented the utilisation of compound #22 including toxicity of the compound to the isolates. It is also conceivable that compound #22 could not be utilised as sole source of carbon and energy but may have been cometabolically

degraded in the presence of other carbon sources. These possibilities were investigated (section 2.12.4).

RBN was inoculated into a chemostat reactor containing MSM plus 500 mg #22 L^{-1} and sampled for HPLC analysis of compound #22 and turbidity (Figure7.3). The turbidity of the medium rose to OD_{600nm} 0.072 before reaching a plateau after 24 h. There was no significant change in the concentration of compound #22 during this time. The COD of the medium fell from 678.5 mg L^{-1} (± 0.5) to 575 mg L^{-1} (± 3.5) after 48 h. This fall was equivalent to the COD of the free NSA contamination of compound #22 (section 3.3) indicating that RBN was utilising the NSA as a source of carbon and energy. After 72 h, NSA was added to the reactor to a final concentration of 500 mg L^{-1}. Twenty-four hours after the addition of NSA (96 h), the turbidity in the reactor had increased to a maximum OD_{600nm} of 0.678. This increase was concurrent with a reduction of NSA concentration in the reactor to below detectable levels (< 1 mg L^{-1}). The increase in COD, due to the addition of NSA, was also reversed and COD levels were reduced to that measured prior to the addition of NSA. The maximum turbidity and NSA removal were comparable to that measured when RBN was grown in batch in a chemostat reactor with NSA as sole source of carbon and energy (maximum OD_{600nm} = 0.672, NSA removal = 99.48 %) (section 4.5).

The reactor was operated for a further 203 h after addition of the NSA, but the concentration of compound #22 and COD in the reactor remained unchanged. During this period, the turbidity of the medium fell to OD_{600nm} 0.453, indicating that the biomass concentration was falling (specific decay rate, b = 0.001 h^{-1}). The results show that compound #22 did not inhibit the growth or utilisation of NSA by RBN. However, as previously observed (sections 7.1 and 7.2), RBN was not only unable to utilise compound #22 as a sole source of carbon and energy, but was also unable to degrade it cometabolically in the presence of NSA.

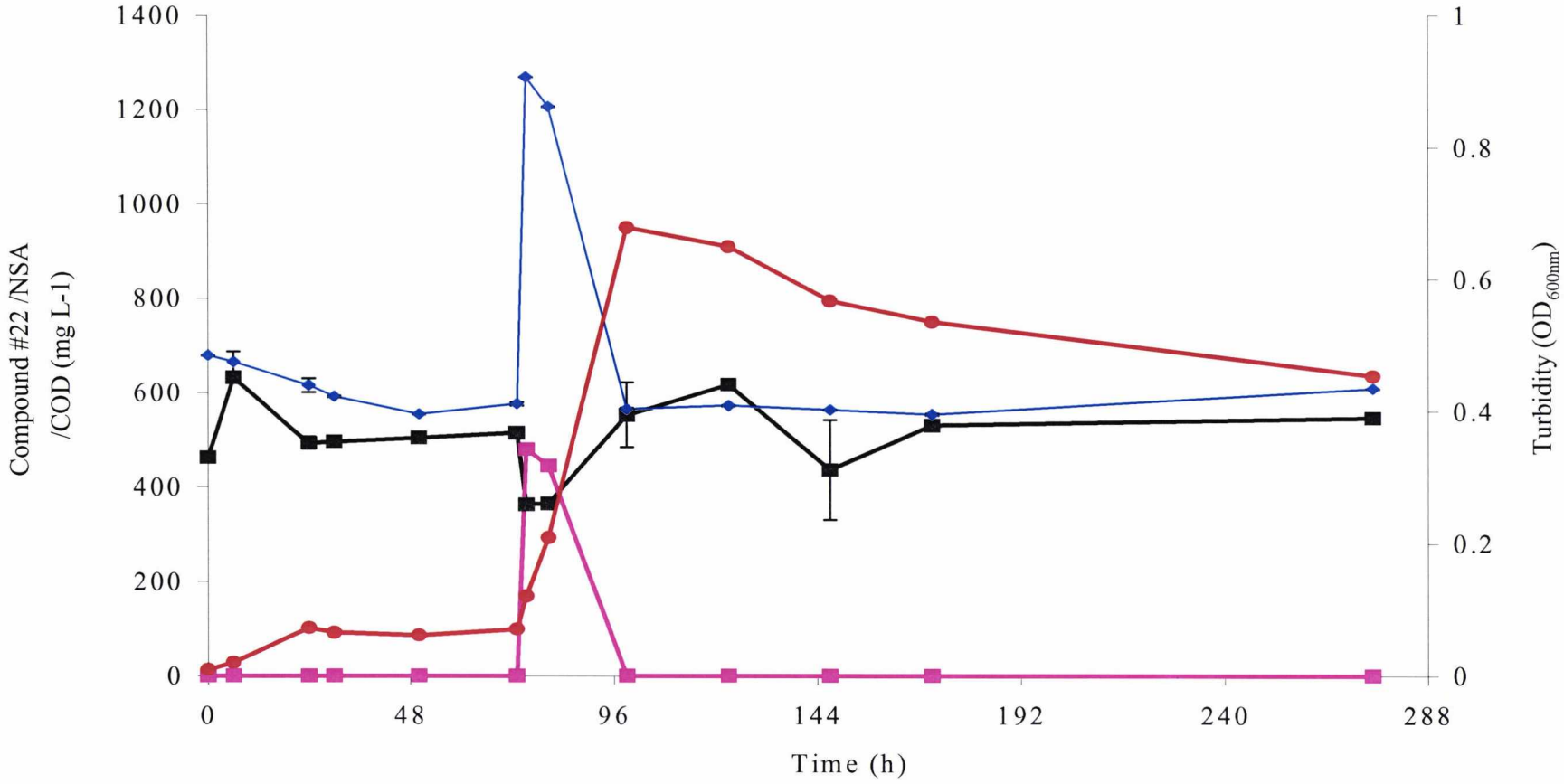

Figure 7.3. Assessment of the effect of NSA (500 mg L^{-1}) on the growth and degradation of compound #22 by RBN in batch culture in a chemostat reactor: ■ - #22 concentration, ■ - NSA concentration, ♦ - COD, ● - turbidity. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

7.4. Physicochemical degradation of compound #22 by combined treatment with UV-light and Fentons reagent

The use of physicochemical techniques, particularly chemical oxidation, for the destruction of recalcitrant organic materials in effluents is widely reported. Treatment with ozone was found to decrease the COD and TOC concentrations of substituted naphthalene sulphonic acids in solution (Rivera-Utrilla *et al.*, 2002; Shiyun *et al.*, 2002). Similarly, the COD of an industrial wastewater contaminated with naphthenic acid was found to be significantly reduced by a combined treatment with UV-light (UV) and Fentons reagent (hydrogen peroxide (H_2O_2) and an iron catalyst) (Chen *et al.*, 1997). Therefore, the oxidative degradation of compound #22, by a combined treatment with UV and Fentons reagent, was investigated.

The presence of residual H_2O_2 in the samples taken from the reaction solutions would have led to the continued degradation of #22 after sampling and consequently, invalidated subsequent measurements of compound #22 (Lin *et al.*, 1999). The Fenton reaction was halted by the addition of phosphate buffer (0.05 M final concentration), increasing the pH to 7.0 and treating the samples with catalase to degrade any residual H_2O_2. The stability of catalase-treated samples was confirmed by measurement of the concentration of compound #22 in samples after treatment with concentrations of H_2O_2 and $FeCl_3$ required for 100% of ThOD, and with or without subsequent catalase treatment. The reaction solutions were sampled before and after addition of H_2O_2 and $FeCl_3$ (0 h), and after storage in the dark at 4°C for 24 h, replicating the conditions that HPLC samples were kept under prior to analysis. The samples were then analysed by HPLC analysis (section 2.2.1.4) (Figure 7.4). Addition of H_2O_2 and $FeCl_3$ resulted in degradation of compound #22 to below the limit of detection (< 10 mg L^{-1}) in the uncatalased samples, indicating that oxidation of compound #22 had occurred. However, there was no significant reduction ($p > 0.05$) in the concentration of compound #22 in the samples treated with catalase, indicating that the residual H_2O_2 had been removed.

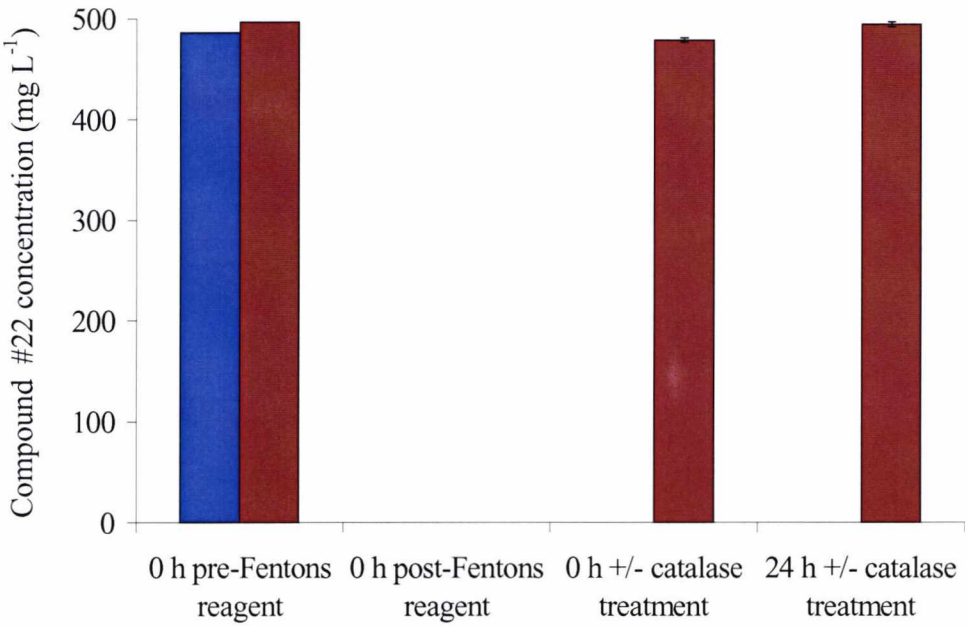

Figure 7.4. Stability of compound #22 samples before and after treatment with Fentons reagent, and with or without subsequent catalase treatment; ■ - uncatalased samples, ■ - catalase-treated samples. Treatments without columns were below the limit of quantification. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

Photooxidation of compound #22 was carried out in 500 mg L^{-1} solutions treated with 100%, 50%, 25% and 10% of the H_2O_2 and $FeCl_3$ (Fentons reagent) required to achieve the theoretical oxygen demand (ThOD) for complete oxidation of the carbon content of compound #22 (section 2.13.1). Photochemical oxidation of *p*-chlorophenol was achieved by a combined treatment of UV and H_2O_2 in the absence of a catalyst (Ghaly *et al.*, 2001). As such, the degradation of compound #22 by H_2O_2 was also investigated in the absence of $FeCl_3$. The solutions were sampled for COD and HPLC analysis (section 2.2.1.1 and 2.2.1.4) immediately before and after addition of the Fentons reagent, and after 30, 60, 90, 120 and 240 min of UV treatment. All samples were catalase-treated prior to analysis. Photo-Fenton treatment with the catalyst also led to the development of colour in the test solutions. The depth of colour increased with increasing strength (%) of treatment. The colour development after 120 min of UV-treatment at different treatment strengths are shown in Figure 7.5.

Figure 7.5. Colour development in solutions of compound #22 after 120 min of photo-Fenton treatment showing increasing colour with increasing strength of treatment. Treatment (from left to right): 0%, 10%, 25% and 50% of ThOD.

Combined UV and H_2O_2 at 100% of ThOD, in the absence of $FeCl_3$, was not effective for the degradation of compound #22. Addition of Fentons reagent increased the COD from 523.67 mg L^{-1} to 932 mg L^{-1} (measured without catalase treatment) but thereafter, neither the concentration of compound #22 nor the COD of the reaction solution were significantly reduced, after treatment with UV for 240 min ($p > 0.05$) (Figures 7.6 and 7.7). Conversely, treatment at 100% of ThOD with $FeCl_3$ significantly reduced the COD after 30 min UV-treatment ($p < 0.0001$), with a maximum reduction to 3.4% (± 0.6%) of the initial COD after 120 min. The concentration of compound #22 was also reduced to below detectable levels after 30 min. Treatment at 50%, 25% and 10% of ThOD significantly reduced COD ($p < 0.0001$, $p < 0.0001$ and $p = 0.004$, respectively) to 48.8% (± 3.4%) and 73.6% (± 1.1%) after 120 min and 86.8% (± 1.7%) after 240 min, of the initial COD concentration, respectively. Treatment at 10% and 25% of ThOD reduced the concentration of compound #22 to 20.8% (± 2.3%) after 120 min and 0.8% (± 0.2%) after 90 min, respectively. Treatment with 50% of ThOD reduced the concentration of compound #22 by 99.5% (± 1.6%) after 30 min and to below detectable levels after 60 min. The data are summarised in table 7.1.

The data indicate that compound #22 was degraded by combined treatment with UV and Fentons reagent. Combined treatment also significantly reduced the concentration of compound #22 in the reaction solution. However, as indicated by the residual COD in the reaction solution, the partial oxidation of compound #22, at 10% 25% and 50% of ThOD, led to the production of unidentified intermediate compounds. These compounds were not detectable by UV-HPLC. A linear relationship was found between the concentration of oxidising agent, H_2O_2, and the reduction of COD in the reaction solution indicating that the reactions were proceeding according to the calculated stoichiometry (section 2.13.1) (Figure 7.8).

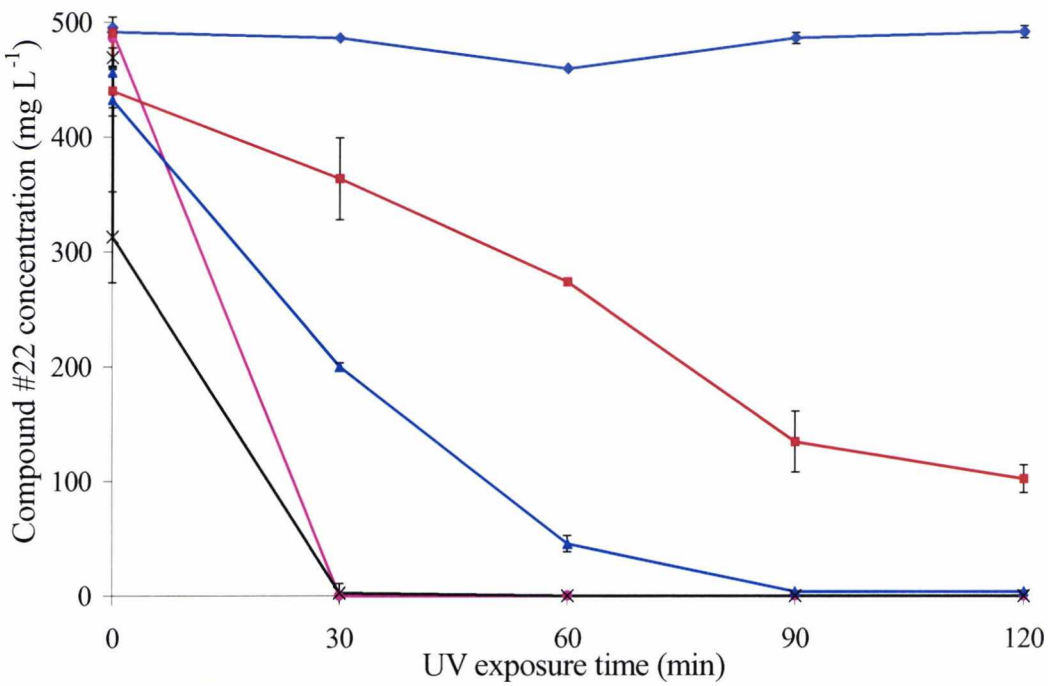

Figure 7.6. Degradation of compound #22 by combined treatment with UV and Fentons reagent: effect of varying concentrations of Fenton reagent. ♦ - Fentons reagent required for 100% of ThOD in the absence of catalyst; ● - Fentons reagent required for 100% of ThOD; x - Fentons reagent required for 50% of ThOD; ▲- Fentons reagent required for 25% of ThOD; ■ - Fentons reagent required for 10% of ThOD. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

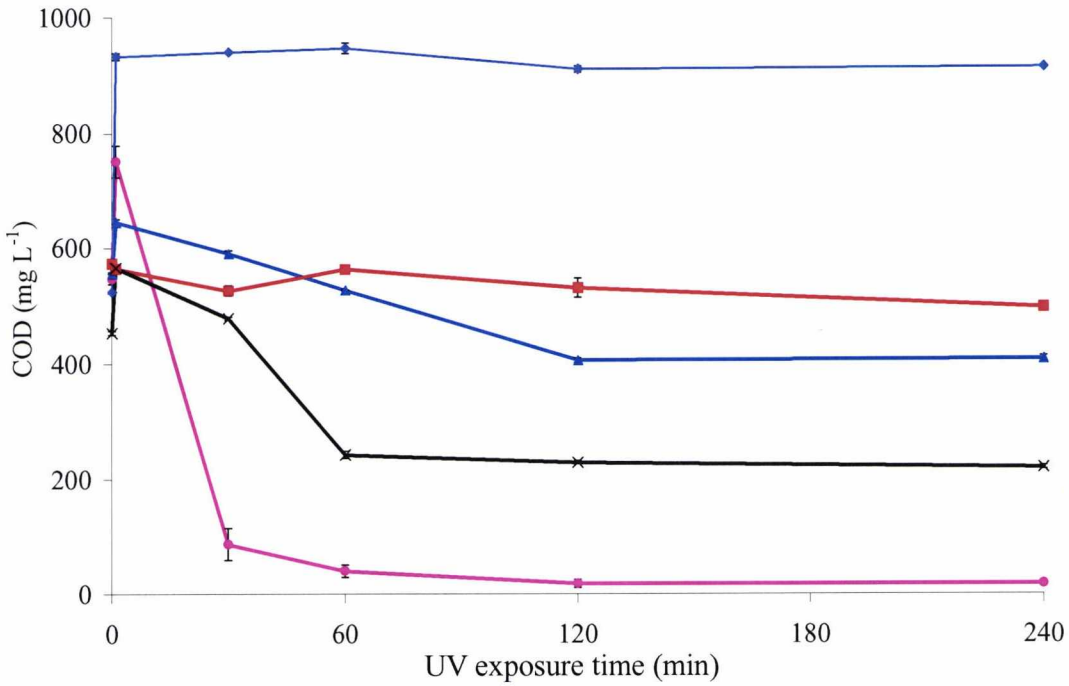

Figure 7.7. COD removal by combined treatment with UV and Fentons reagent: effect of varying concentrations of Fenton reagent. ♦ - Fentons reagent required for 100% of ThOD in the absence of catalyst; ● - Fentons reagent required for 100% of ThOD; x - Fentons reagent required for 50% of ThOD; ▲- Fentons reagent required for 25% of ThOD; ■ - Fentons reagent required for 10% of ThOD. Vertical bars indicate S.E.M (n=4); for symbols without bars, the limits of error were within the confines of the symbol.

Table 7.1 – Reduction (%) in concentration and COD of compound #22 after photo-Fenton treatment. Fenton reagents given as percentage of concentration required to achieve the theoretical oxygen demand (ThOD) for complete oxidation of the carbon content of compound #22

UV-Fenton treatment (% of ThOD)	Compound #22 (% of initial concentration)	COD (% of initial concentration)
100% - no catalyst	99.2 (± 1.0)	98.1 (± 0.8)
10%	79.2 (± 2.3)	86.8 (± 1.7)
25%	0.8 (± 0.2)	73.6 (± 1.1)
50%	0	48.8 (± 3.4)
100%	0	3.4 (± 0.6)

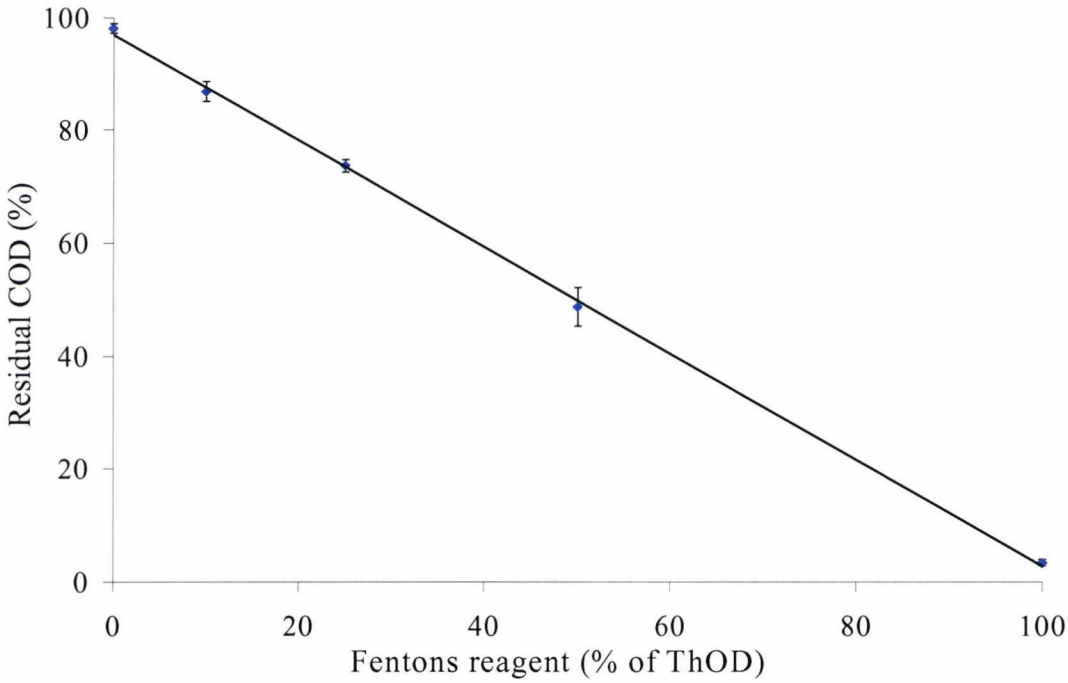

Figure 7.8. Relationship between residual COD and Fentons reagent concentration (r^2 = 0.999). Vertical bars indicate S.E.M (n=4).

7.5. Influence of physicochemical treatment on the biodegradation of compound #22 by *Sphingomonas* sp. strain RBN

Pretreatment by photocatalytic oxidation (Bolduc & Anderson, 1997) or ozonation (Shiyun *et al.*, 2002) has been shown to increase the biodegradability of recalcitrant organic compounds. More specifically, photo-Fenton pretreatment was found to render of *p*-nitrotoluene-ortho-sulphonic acid susceptible to subsequent biological treatment (Pulgarin *et al.*, 1999). The previously reported data (section 7.3) have shown that limited photochemical oxidation could be used to transform compound #22. The use of photochemical oxidation, by combined treatment with UV and Fenton reagent at 0%, 10%, 25% and 50% of ThOD, as a pretreatment to biological treatment of compound #22 with *Sphingomonas* sp. strain RBN, was investigated (section 2.13.2).

Solutions of compound #22 were photo-Fenton treated and subsequently inoculated with *Sphingomonas* sp. strain RBN. The solutions were incubated for 192 h and sampled for turbidity HPLC, and COD. Turbidity was measured after 24 h in all of the inoculated media except that treated with 50% of ThOD (Figure 7.9 a). The mean turbidities of the

media showed significant treatment-dependent variation (ANOVA, F = 109.5, $p < 0.0001$). The turbidity measured in the medium treated with Fentons reagent and UV to 10% of the total ThOD of compound #22 (10% ThOD) was significantly greater after 24 h than that of the medium treated with 0% of ThOD (0% ThOD) ($p = 0.03$). Similarly, the mean turbidity measured in the medium treated with 25% of ThOD (25% ThOD) was significantly greater after 24 h than that measured in 10%ThOD-treated medium ($p = 0.001$).

In the three treatments in which growth was measured, biphasic growth profiles were observed, with initial growth phases up to 24 h and second growth phases beginning after 48 h (0% ThOD-treated medium and 10% ThOD-treated medium) and 72 h (25% ThOD-treated medium). The second phase was concurrent with the fall in NSA concentration from 48 h to 96h, to below detectable levels (Figure 7.9 b). The increase in the turbidity during the second growth phase was proportional to the concentration of NSA in the media; the largest increase in turbidity was measured in 0% ThOD-treated medium (31.86 ± 0.05 mg NSA L^{-1}) and the lowest increase measured in 25% ThOD-treated medium (5.92 ± 0.22 mg NSA L^{-1}). No NSA was detected in the 50% ThOD-treated medium. Thereafter, the turbidity in 0% ThOD-treated medium fell while the turbidity in both 10% ThOD-treated medium and 25% ThOD-treated medium continued to rise. After 192h, the turbidities of the media showed significant treatment-dependent variation (ANOVA, F = 1218, $p < 0.0001$) with the turbidities of both 10% ThOD-treated medium and 25% ThOD-treated medium being significantly greater than that of 0% ThOD-treated medium ($p = 0.00001$). The turbidity of 25% ThOD-treated medium was significantly greater than that of 10% ThOD-treated medium ($p = 0.00006$).

Between 48 h and 72 h, the COD in the 0% ThOD-treated medium fell significantly ($p = 0.01$) from the initial concentration and was concurrent with the fall in NSA (Figure 7.9 c). Thereafter, the COD did not change significantly. The COD in the 10% ThOD- and 25% ThOD-treated medium did not change significantly ($p > 0.05$) over the experimental period. The COD in the 50% ThOD-treated medium rose significantly ($p = 0.037$), after 24 h, from the initial concentration and remained significantly greater until the end of the experimental period. Similarly, excluding the reduction in NSA, there was no significant change in the concentration of compound #22 after 192 h in 0% ThOD- and 10% ThOD-treated medium, compared to the initial concentration (Figure 7.9 d).

However, the concentration of compound #22 in the 25% ThOD-treated medium fell significantly between 72 h and 96 h (p = 0.00002), and again between 96 h and 192 h (p = 0.00002). No compound #22 was detected in the 50% ThOD-treated medium.

Positive growth controls of 10%, 25% and 50% ThOD-treated medium plus 500 mg NSA L^{-1} were used to assess whether or not photochemical oxidation of compound #22 gave rise to intermediates that were toxic or inhibitory to *Sphingomonas* sp. strain RBN. After 72 h, the turbidity of the positive growth controls medium rose sharply, reaching a maximum OD_{600nm} of 0.573 (10% ThOD), 0.539 (25% ThOD) and 0.557 (50% ThOD) after 96 h. This increase was concurrent with a fall in NSA levels to below detectable levels. However, after 192 h, the concentration of compound #22 and COD (excluding NSA removal) in the positive controls had not changed significantly. The growth and utilisation of NSA in the positive controls suggests that none of the intermediate compounds, arising from the photochemical treatment of compound #22, were inhibitory or toxic to RBN.

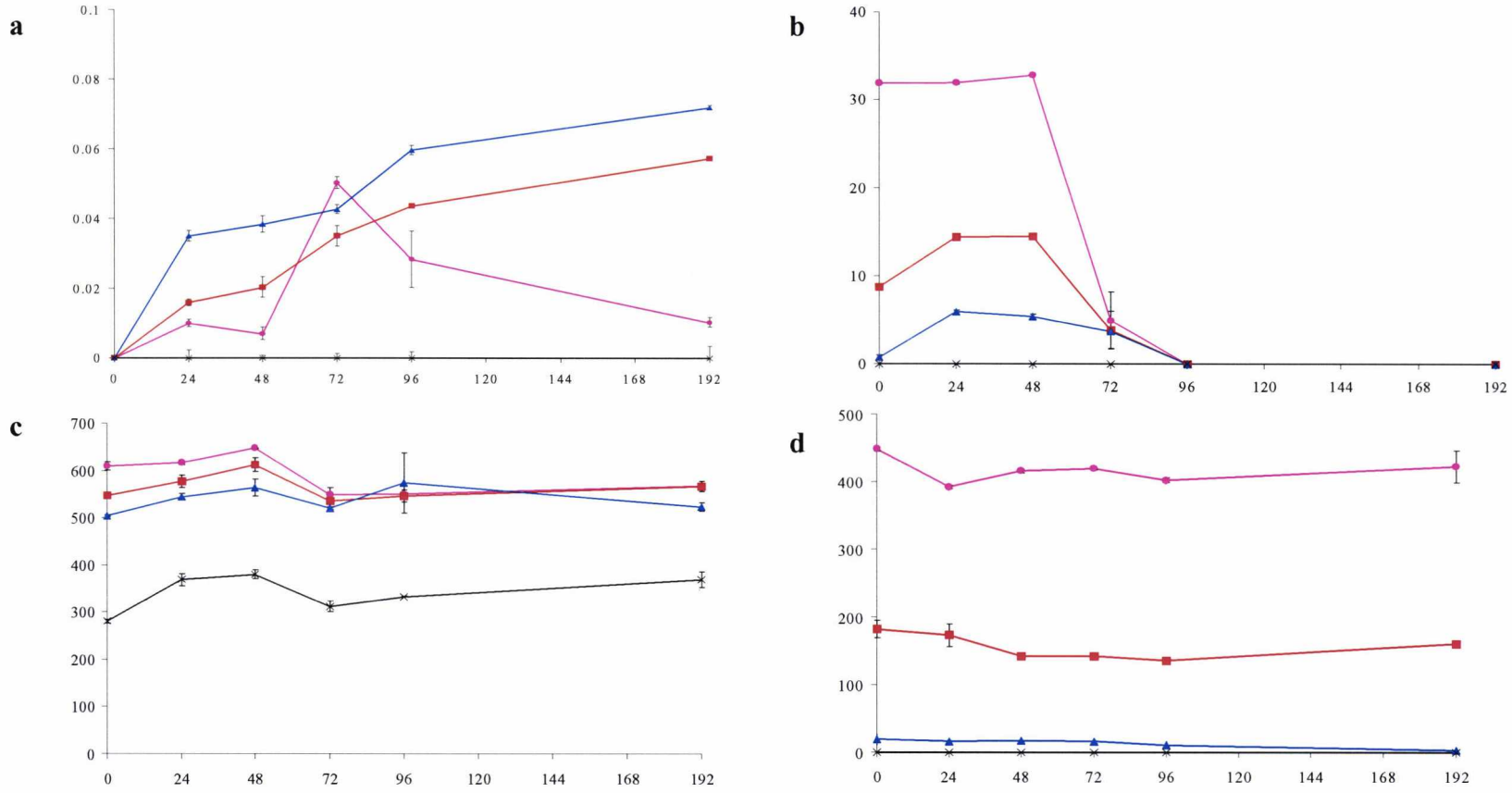

Figure 7.11. Influence of combined photochemical and biological treatment on the degradation of compound #22. ● - 0% ThOD-treated medium: x - 50% ThOD-treated medium; ▲- 25% ThOD-treated medium; ■ - 10% ThOD-treated medium; **a** – Turbidity (OD_{600nm}), **b** – NSA concentration (mg L^{-1}), **c** – COD (mg L^{-1}), **d** – compound #22 concentration (mg L^{-1}). Abscissa = time (h) on all plots. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol.

7.6. Evaluation of the influence of photo-Fenton treatment on the biodegradability (BOD_5) of compound #22

The influence of photo-Fenton UV-treatment on the BOD_5 of compound #22 was assessed (section 2.2.1.2), using sludge 4 as the inoculum (section 2.1.1). The results are shown in table 7.2. The BOD_5 of the 0%-ThOD treated medium (UV-treatment only) was higher than that previously measured (0 mg L^{-1}, section 3.3). The BOD_5 of the media, treated with photo-Fenton treatment at 10%, 25% and 50% of ThOD, were significantly reduced compared to the 0% ThOD treated medium (ANOVA, $F = 7.053$, $p = 0.012$). The mean BOD_5 values of the 10%, 25% and 50% treated media showed a trend of decreasing BOD_5 against increasing degree of photo-Fenton treatment. However, the differences between the means of those treated groups were not significant (ANOVA, $F = 0.3644$, $p = 0.71$). A similar trend is shown in the COD : BOD ratios. Overall, the results show a decrease in the degradability of compound #22 with an increasing degree of photo-Fenton treatment. However, the degradability of compound #22 was improved by all the treatments when compared to the BOD_5 measured for untreated compound #22 (section 3.3).

Table 7.2. Influence of photo-Fenton treatment on the biodegradability (BOD_5) of compound #22. SEM shown (n =3).

	Treatment (% of ThOD)			
	0%	10%	25%	50%
COD of treated media (mg L^{-1})	497.5	488.5	409	221
BOD_5 of treated media (mg L^{-1})	65.83 (± 4.17)	28.33 (± 8.33)	23.33 (± 13.33)	17.22 (± 2.78)
BOD : COD	0.13 (± 0.008)	0.05 (± 0.017)	0.05 (± 0.030)	0.07 (± 0.013)

7.7. Identification of the oxidation products of photo-Fenton treatment of compound #22

Provisional identification of the organic compounds present as residual COD after photo-Fenton treatment was attempted by GC-MS analysis, after solvent extraction of the reaction solution (section 2.13.1). Two distinct peaks were found in the solutions treated with 10%, 25% and 50% of ThOD with retention times of 9.92 min (peak A) and 10.25 min (peak B) (Figure 7.10). These peaks were provisionally identified using the NIST library. Peak A was identified as 1,1-(6-hydroxy-2,5-benzofurandiyl) bis-ethanone and peak B was identified as butylated hydroxytoluene or 4,6-di(1,1-dimethylethyl)-2-methyl phenol. However, the spectral match was poor in both cases. The area of both peaks increased with increasing strength of treatment (% ThOD) although the relationship between treatment strength and peak area was not linear with a large increase in the accumulation of both peaks at 50% of ThOD, compared with the other treatments (Figure 7.11). As such, the oxidation of the intermediates from which peak A and B were formed, may have represented a rate-limiting step in the photo-oxidation of compound #22. A number of other compounds were found to accumulate (t_R > 17 min). The chromatographic separation of these compounds was poor and they could not be identified by the spectral library. However, the mass spectra indicated that these compounds were oxidised aliphatic chains, including carboxylic acids or alcohols.

Further elucidation of the organic components was attempted after derivatisation of the solvent extracts with iodomethane. This derivatisation methylates hydroxyl groups on the organic components thus increasing their suitability for GC-MS analysis (Tuhkanen & Beltrán, 1995). After derivatisation, 4 peaks, peak C (t_R 4.6 min), peak D (t_R 4.99 min), peak E (t_R 5.67 min) and peak F (t_R 15.08 min) were apparent on the chromatogram at each level of treatment (Figure 7.12). These peaks were provisionally identified as 4-methyl-3-penten-2-one or 2,4-dimethyl-2-pentene (peak C), 4-hydroxy-4-methyl-2-pentanone (peak D), 2,6-dimethyl-4-phenyl pyrilium iodide (peak E) and butylated hydroxytoluene (peak F). Peak F was the most abundant on the basis of peak area. Unlike the underivatised samples, no relationship between peak areas and degree of treatment was apparent.

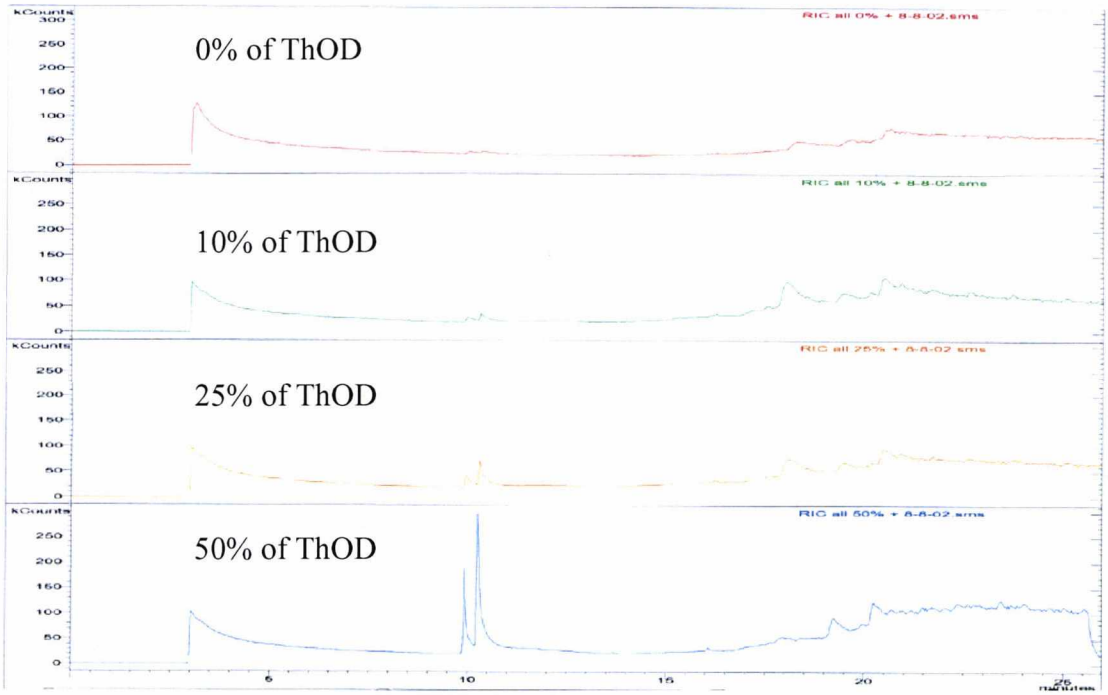

Figure 7.10. GC-MS spectra of solvent extracted solutions of compound #22 after photo-Fenton treatment showing accumulation of peak A (t_R 9.92 min) and peak B (t_R 10.25 min) with increasing treatment strength.

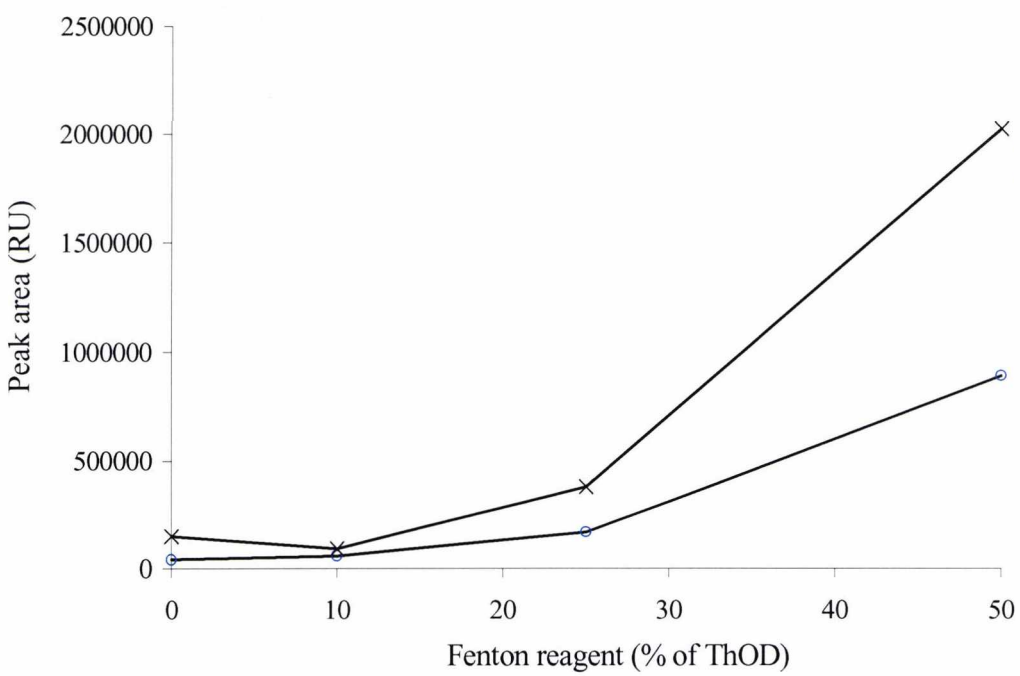

Figure 7.11. Relationship between the formation of peak A (o) and peak B (x) and strength of treatment (% of ThOD) after photo-Fenton treatment of compound #22

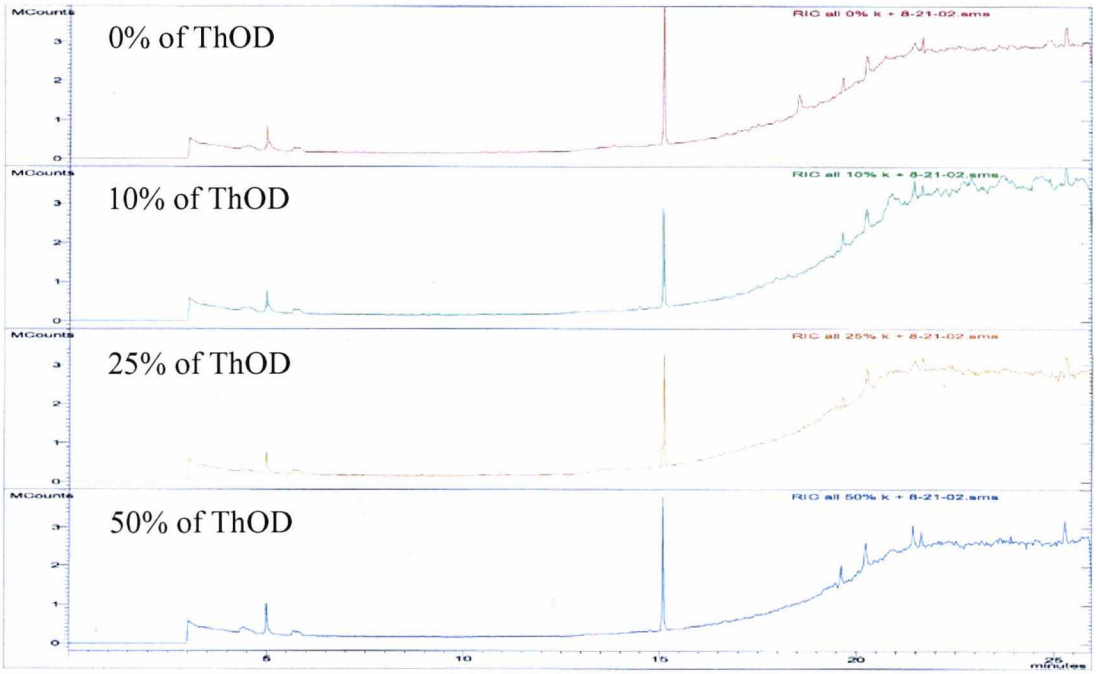

Figure 7.12. GC-MS spectra of solvent extracted, iodomethane-derivatised solutions of compound #22 after photo-Fenton treatment showing peak C (t_R 4.6 min), peak D (t_R 4.99 min), peak E (t_R 5.67 min) and peak F (t_R 15.08 min).

Discussion

The results suggest that neither *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS were capable of utilising compound #22 as sole source of carbon and energy (section 7.1), and that RBN did not utilise compound #22 as a source of carbon and sulphur under sulphur-limited conditions (section 7.2). The low levels of growth observed in both experiments can be attributed to the low concentration of monomeric NSA measured in compound #22 (section 3.1). The maximum turbidity of a batch culture of *Sphingomonas* sp. strain RBN in MSM plus 500 mg NSA L^{-1} and 500 mg #22 L^{-1} (section 7.3) was comparable to that in batch culture with 500 mg NSA L^{-1} as sole source of carbon and energy (section 4.5). This indicated that compound #22 was not toxic or otherwise inhibitory to the growth and/or metabolism of *Sphingomonas* sp. strain RBN. However, compound #22 was not degraded, either cometabolically during the growth of *Sphingomonas* sp. strain RBN on NSA, or as sole source of carbon and energy by the large, active population of *Sphingomonas* sp. strain RBN present after the utilisation of NSA. The recalcitrance of compound #22 was also highlighted by complete absence of degradation in the BOD_5 test (section 3.3).

Compound #22 is a condensation polymer of NSA and formaldehyde, consisting of monomeric NSA and NSA-formaldehyde condensate polymers ranging from 2- to 11-mer (Z. Song, personal communication). The putative structure of compound #22 is shown in Figure 7.13. The rate-limiting step in the degradation of naturally occurring polymers is the hydrolysis of the polymer to monomers (Ubukata, 1997). These compounds are too large to cross the bacterial cell wall and must be hydrolysed to monomers by extracellular enzymes, e.g. hydrolases, before assimilation. This rate-limiting effect has been demonstrated by comparing the degradation kinetics of dextran and peptone (polymers), with glucose and a mixture of free amino-acids (monomers), by activated sludge from a municipal sewage treatment plant. The substrate removal rate and oxygen uptake rate was higher for the monomers than the polymers, indicating that hydrolysis of the polymer to monomer was the rate-limiting step in their degradation. The limiting effect of polymerisation on the degradation of xenobiotic compounds has also been reported. Larson *et al.* (1997) showed that degradation of acrylic acid polymers decreased with increasing molecular weight. Only monomeric and dimeric acrylic acid were mineralised, with low weight oligmers (Mw 500 – 700) undergoing extensive but

incomplete degradation. The extent of degradation dropped sharply for polymers with a molecular masses of greater than 1000, indicating an inverse relationship between degree of polymerisation and susceptibility to bacterial degradation.

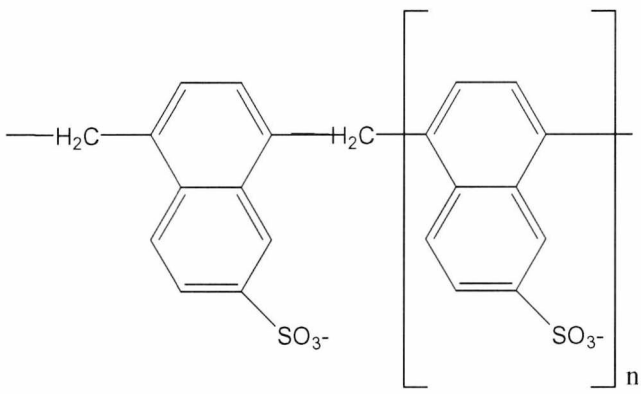

Figure 7.13. Putative structure of compound #22.

Other factors have also been shown to the limit the degradation of xenobiotic polymers. The degradation of polyurethanes by cholesterol esterase was reduced by presence of secondary structures and molecular orientation leading to reduced access of the enzyme to the readily hydrolysable ester sites within the polymer molecule (Santerre *et al.*, 1994). Pure aromatic polymers are also highly resistant to biodegradation and compounds such as poly(ethylene terephthalate) (Tokiwa & Suzuki, 1977) and poly(ethylene naphthalate) (Levefre *et al.*, 1999) have been reported to be resistant to enzymatic and microbial degradation, respectively.

Both *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS were shown to utilise NSA as a source of carbon and energy but neither isolate was able to use the NSA polymer, compound #22. The data also suggest that any degree of polymerisation rendered NSA resistant to degradation. No increased level of growth was observed during the batch growth of *Sphingomonas* sp. strain RBN with compound #22, after addition of NSA (section 7.3), compared to the growth of RBN during batch growth with NSA as sole source of carbon (section 4.5). The levels of growth observed with compound #22 as sole source of carbon and energy (section 7.1) were related to the NSA content of compound #22, suggesting that even dimers of NSA would be resistant to degradation by the isolates. However, further investigation of the quantities of oligomers before and after inoculation with the isolates would be required to confirm this.

Polyethylene 2,6-naphthalene dicarboxylate was found to be resistant to degradation by an undefined 2,6-naphthalene dicarboxylate-degrading bacterial consortium isolated from soil (Levefre *et al.*, 1999). A complete resistance (100%) to biodegradability, even by adapted monomeric NSA-degrading populations, was reported for SNFC used as plasticisers for concrete and found in the leachate from a construction site. No biodegradation of these components was observed in either the laboratory or *in situ*, after 195 d (Ruckstuhl *et al.*, 2002). SNFC would, therefore, appear to be entirely resistant to bacterial degradation. Compound #22 is a mixture of SNFC oligomers, and this would explain both its resistance to degradation by the isolates (section 7.1), and low BOD_5 values (section 3.4).

A number of chemical oxidation techniques, including ozonation, titanium dioxide (TiO_2)-catalysed photodegradation, and Fentons reagent, have been shown to degrade recalcitrant organic compounds. The use of Fentons reagent-mediated oxidation to degrade recalcitrant organic compounds, such as *p*-chlorophenol (Ghaly *et al.*, 2001) and anionic- and linear-alkylbenzene sulphonates (Lin *et al.*, 1999), has been reported. The reaction utilises the generation of hydroxyl free radicals (•OH) from H_2O_2 in the presence of an iron catalyst. These highly reactive free radicals will oxidise and may ultimately mineralise organic compounds (Walling, 1975). The rate of reaction is enhanced by illumination with UV-light (Chen *et al.*, 1997; Perez *et al.*, 2002). The degradation of compound #22 using Fentons reagent was investigated.

The concentration of H_2O_2 used for the photo-Fenton treatment of compound #22 was determined by calculation of the theoretical oxygen demand (ThOD) of the naphthalene sulphonate-formaldehyde condensate subunit (section 2.13.1). ThOD is the stoichiometric amount of oxygen required to oxidise a compound to end products, such as CO_2. ThOD may be predicted from the COD of the compound. However, the use of COD may significantly underestimate the oxygen required for complete oxidation of the compound (Baker *et al.*, 1999). Preliminary experiments indicated that samples of H_2O_2-treated compound #22 taken for HPLC analysis, were not stable in storage and that the degradation continued even when the samples were stored in the dark at 4°C. As such, the stability of the treated samples was investigated at 100% of ThOD after catalase treatment to remove residual H_2O_2. Samples were treated by buffering with phosphate

buffer, raising the pH to 7.0, and treatment with catalase at 37°C for 30 min. The use of phosphate buffer has a two-fold effect of chelating the iron catalyst and creating suitable conditions for subsequent enzyme treatment. The increase in pH will cause precipitation of the dissolved iron, as iron (III) hydroxide, leading to the decomposition of H_2O_2 to oxygen and water rather than •OH (Bishop *et al.*, 1968). Both treatments will effectively remove the iron catalyst from the reaction solution. As shown in Figures 7.6 and 7.7, the use of combined UV and H_2O_2 treatment in the absence of a catalyst had no effect on the concentration of either the COD or compound #22 and thus, removing the catalyst will halt the reaction. To ensure that no further degradation of the organic material in the reaction solution occurred, the solutions were treated with catalase to remove residual H_2O_2, eliminating the potential for free radical generation. The efficacy of the sample treatment is shown in Figure 7.4. Untreated samples continued to degrade even when stored in the dark at 4°C, as indicated by the complete removal of compound #22, whereas enzyme-treated samples were stable.

The presence of residual H_2O_2 may also have a detrimental effect on the accuracy of COD measurements. Lin *et al.* (1999) reported that residual H_2O_2 increased the measured COD by 0.26 mg COD L^{-1} mg H_2O_2 L^{-1} during oxidation of alkylbenzene sulphonates and linear alkylbenzene sulphonates with Fenton reagent. However, in that study the samples were not treated with catalase prior to measurement of the COD. The H_2O_2 was also supplied in excess, with up to a third of the H_2O_2 present in the reaction solution at the end of treatment. In this study, the concentration of H_2O_2 added was equal to, or less than, 100% of the amount theoretically required for complete mineralisation of compound #22. The samples were also treated with catalase to remove residual H_2O_2 prior to COD analysis. It was expected that the H_2O_2 would be either entirely utilised in the oxidation of compound #22 or degraded by catalase treatment. Where the samples were not catalase treated (0% ThOD), a significant increase in the COD of the reaction solution was measured, indicating that H_2O_2 was contributing to the COD. In the treated samples, the COD removal was found to be as predicted by the stoichiometry and as such, demonstrates that, subsequent to catalase treatment, the COD measurements were not affected by residual H_2O_2.

The reaction was characterised by development of colour and the depth of colour increased with increasing concentration of H_2O_2. This is typical of the Fenton reaction

and the colour normally disappears once the reaction reaches completion, i.e. complete oxidation of the organic compound(s) (Walling, 1975). Tuhkanen and Beltrán (1995) also reported colour development when comparing the decomposition of naphthalene by UV or UV plus an excess of H_2O_2. A colour change from clear to yellow to brown was also observed in the samples treated with UV alone. The development of colour was due to the formation of UV-absorbing organic intermediates which reduced the rate of UV-mediated naphthalene degradation. Less colour development was observed in the UV/ H_2O_2-treated samples. In this study, a marked colour change was observed but the measured COD removal was as predicted by ThOD, indicating that the formation of the coloured intermediates had not inhibited the degradation of compound #22.

Photo-Fenton treatment reduced the COD of the treated compound #22 solution in direct proportion to the concentration of H_2O_2, indicating that the oxidative reactions were occurring as predicted by the calculated stoichiometry (section 2.13.1). The concentration of compound #22 was significantly reduced by all the treatments, although the residual COD indicated that compound #22 was not completely mineralised and that photo-Fenton treatment of compound #22 under H_2O_2-limited conditions led to the formation of intermediate compounds. These compounds were not detected using HPLC. Tuhkanen and Beltrán (1995) reported development of intermediates during UV-H_2O_2 degradation of naphthalene. These intermediates could only be detected by GC-MS, and were identified as mono- and dicarboxylic acids, arising from the rupture of the benzene ring of the initial monoaromatic intermediate degradation products. Similarly, Bolduc and Anderson (1997) also reported an accumulation of intermediates that were not detectable by UV-HPLC, with increasing reaction time, during the TiO_2-catalysed photodegradation of *m*-dinitrobenzene. The concentration of these intermediates declined after extended treatment. Here, treatment of compound #22 at 100% of ThOD led to a 96.6% (± 0.6) reduction in COD, indicating that extensive photo-Fenton treatment also reduced the concentration of intermediates.

The results show that photo-Fenton treatment of compound #22 significantly reduced both the concentration of compound #22 and COD in the reaction solutions. However, photochemical degradation techniques may not be economically viable as a treatment at industrial scale. Batch treatment by photo-Fenton treatment of *p*-nitrotoluene sulphonic acid (*p*NTS), a compound found to be entirely undegradable in biological treatment

systems, was reported as the most efficient method to achieve complete mineralisation of *p*NTS in effluents (Pulgarin *et al.*, 1999). Complete removal of *p*NTS, and total degradation of the aromatic intermediates, were achieved after 5 min and 20 min of photo-Fenton treatment, respectively. The cost of complete removal of *p*NTS was estimated as between US$150 (for batch treatment) to US$800 (for continuous treatment) per m^3 of wastewater containing 1g *p*NTS L^{-1}, making the process uneconomical. However, the use of limited photo-Fenton treatment in combination with biological treatment, achieved 100% removal of *p*NTS and nearly 80% removal of DOC at a cost of US$ 71 per m^3 of wastewater containing 1 g pNTS L^{-1}. Therefore, the use of photo-Fenton pretreatment of compound #22 to increase the biodegradability of compound #22 was examined.

The use of limited photo-Fenton treatment (10%, 25% and 50% of ThOD), to increase the biodegradability of compound #22 by *Sphingomonas* sp. strain RBN was investigated. A small amount of growth of RBN was observed in the 0%, 10% and 25% treated media and, in all cases, was directly proportional to the extent of photo-Fenton treatment (Figure 7.11 a). However, there was no growth in the 50%-treated medium. Where growth occurred, the profiles showed biphasic curves; the second phase was concurrent with degradation of the residual NSA in the media (Figure 7.11 b). This biphasic growth curve is typical of diauxic growth (MacGregor *et al.*, 1991) and suggested that photo-Fenton treatment had generated an intermediate(s) that was more readily degradable than NSA. The greater growth measured in the treated media, compared to that measured in the 0% treated medium, suggested that photo-Fenton treatment had improved the degradability of compound #22 by the formation of biodegradable oxidised intermediates. The lack of growth in the 50%-treated medium may indicate that these intermediates were further oxidised to compounds that could not be utilised by RBN. Further investigation of identity and degradability of these compounds would be required to confirm this. However, the levels of turbidity in the treated solutions were low. The growth in the positive controls indicated that the oxidation products of compound #22 were neither inhibitory nor toxic to RBN and, therefore, would not limit growth if utilisable carbon sources were available. As such, it would seem probable that any degradable intermediates formed from the photo-Fenton treatment of compound #22 were only present at low concentrations.

Similarly, while growth was measured in the treated samples, there was no significant reduction in the COD, except after the utilisation of the NSA in the 0%-treated samples (Figure 7.11 c). There was also no significant reduction in the concentration of compound #22 in the solutions. Therefore, photo-Fenton treatment would not appear to have significantly enhanced the susceptibility of compound #22 to degradation by *Sphingomonas* sp. strain RBN.

Partial oxidation of *m*-dinitrobenzene decreased its BOD, indicating that the oxidation intermediates were toxic, inhibitory or recalcitrant. Similarly, photooxidative degradation of diphenylamine, by 50 min treatment with TiO_2, reduced the BOD : TOC, even after removal of the majority of the parent compound. Further treatment increased the BOD : TOC but only after 85% of the initial carbon had been removed (Bolduc & Anderson, 1997). The biodegradability of a number of substituted NSA compounds was increased by ozonation. Reductions in COD and TOC of 20 – 84% and 7 – 50%, respectively, lead to an increases in BOD: COD from 0 – 0.2 to 0.27 – 0.48. However, there was an initial reduction in the BOD : COD in a number of the compounds, which was reversed by further treatment. Furthermore, the biodegradability of some of the compounds, such as 2,3-dihydroxy-6-NSA, did not increase after ozonation, in spite of significant reductions in COD (Shiyun *et al.*, 2002). Clearly, the chemistry of the intermediates produced by physicochemical treatments will determine their subsequent biodegradability. Consequently, while the structure and concentration of compound #22 may have been significantly altered by photo-Fenton treatment, the resulting intermediates were no more biodegradable than the parent compound. This supposition is supported by the experimental evidence (section 7.5).

Five of the oxidised intermediates were identified by GC-MS, before and after derivatisation by methylation. Only two distinct peaks were observed in the underivatised samples and these were identified provisionally as 1,1-(6-hydroxy-2,5-benzofurandiyl) bis-ethanone (peak A) and butylated hydroxytoluene or 4,6-di (1,1-dimethylethyl)-2-methyl phenol (peak B). The other compounds in the samples were tentatively identified from their chromatography as short oxidised aliphatic chains. The formation of peak A and B was dependent on the concentration of H_2O_2, although the relationship between H_2O_2 and peak area was not linear (Figure 7.11). Derivatisation of the reaction solution gave rise to four peaks, which were provisionally identified as 4-methyl-3-penten-2-one

or 2,4-dimethyl-2-pentene (peak C), 4-hydroxy-4-methyl-2-pentanone (peak D), 2,6-dimethyl-4-phenyl pyrilium iodide (peak E) and butylated hydroxytoluene (peak F).

A mechanism for the degradation of naphthalene by UV or UV plus H_2O_2 was studied and found to begin with the sequential formation of naphthol and naphthoquinone (Tuhkanen & Beltrán, 1995). These intermediates were highly reactive and rapidly decomposed to monoaromatic structures including benzaldehyde, benzoic acid, phthalic acid, cyclic structures such as 2,3-dihydroxybenzofuran, and aliphatic mono- and dicarboxylic acids. Ozonation of 1-NSA, 1,3-diNSA and 3-nitrobenzene gave rise to aliphatic acids including maleic, fumaric, oxalic and formic acids (Calderara *et al.*, 2002). A number of products were identified during the sunlight-mediated photocatalysis of naphthalene, including 1-napthol and coumarin, via 1,2-naphthoquinone, and isobenzofuranone, via 1,4-naphthalene endoperoxide (McConkey *et al.*, 2002). The biodegradability of organic compounds was modelled on the basis of compounds of known biodegradability (Loonen *et al.*, 1999) This revealed that the presence of carboxylic acids in aromatic compounds, and hydroxyl groups in aliphatic compounds, enhanced their biodegradability. Therefore, it would be expected that the reported carboxylic acid and hydroxylated products of photo- and photo-chemical oxidation treatments would be readily degradable.

A similar range of oxidised monoaromatic and aliphatic organic compounds were detected after photo-Fenton oxidation of compound #22. However, the subsequent biodegradability of compound #22 was not significantly increased. These compounds were also detected in the 0% ThOD-treated samples, indicating that they may have arisen either as products of UV treatment of compound #22 or have been present as contaminants in compound #22. With the exception of butylated hydroxytoluene (in the underivatised samples), there was no relationship between the degree of photo-Fenton treatment and concentration of detected intermediates. Therefore, the oxidation of compound #22 may have given rise to undegradable intermediates that were not detectable by GC-MS.

The reactions of the hydroxyl radical with organic compounds are predominantly of two types: i) addition, leading to the formation of an organic radical; and ii) hydrogen abstraction (Bishop *et al*, 1968):

i) Addition $\bullet OH + C_6H_6 \longrightarrow (OH)C_6H_6\bullet$ (organic free radical)

ii) Hydrogen abstraction $\bullet OH + CH_3OH \longrightarrow \bullet CH_2OH + H_2O$

Photocatalysis of aromatics is reported to proceed primarily by the formation of •OH radicals (addition reaction), leading to the formation of unstable intermediates and rapid cleavage of the aromatic ring to aliphatic alcohols, ketones and acids (Pichat, 1994). The photooxidation of naphthalene was reported to be initiated by either a 2,2- or a 2,4-hydroxyl-cycloaddition (McConkey *et al.*, 2002). Similarly, ozonation of three different NSAs was initiated by an attack on bonds with the highest electron density via a dipolar cycloaddition. This resulted in the liberation of the sulphonate group (Rivera-Utrilla *et al.*, 2002).

If the proposed structure of compound #22 (Figure 7.13) is assumed to be correct, and the hydroxyl radical attack on the structure was initiated in the region of the sulphonate group (as has been previously described for other photooxidative chemical degradation techniques), degradation would have begun on the unpolymerised ring of the NSA subunit of compound #22. This would have been mechanistically similar to the pathway for biodegradation of NSA. Therefore, photo-Fenton oxidation of compound #22 under H_2O_2 limited conditions may have led to oxidation of the naphthalene ring subunit of compound #22 but not to disruption of the polymeric structure of the compound. A possible mechanism for this reaction is shown in Figure 7.14. As is apparent, even extensive oxidation, such as occurred following treatment with 50% of ThOD, could have given rise to a polymeric intermediate compound which, due to its polymeric nature, would remain undegradable by RBN or sludge 4. The polymeric nature of such a product would also render it undetectable by GC-MS. However, further analysis of the products of photo-Fenton oxidation of compound #22 is required to confirm this mechanism of degradation.

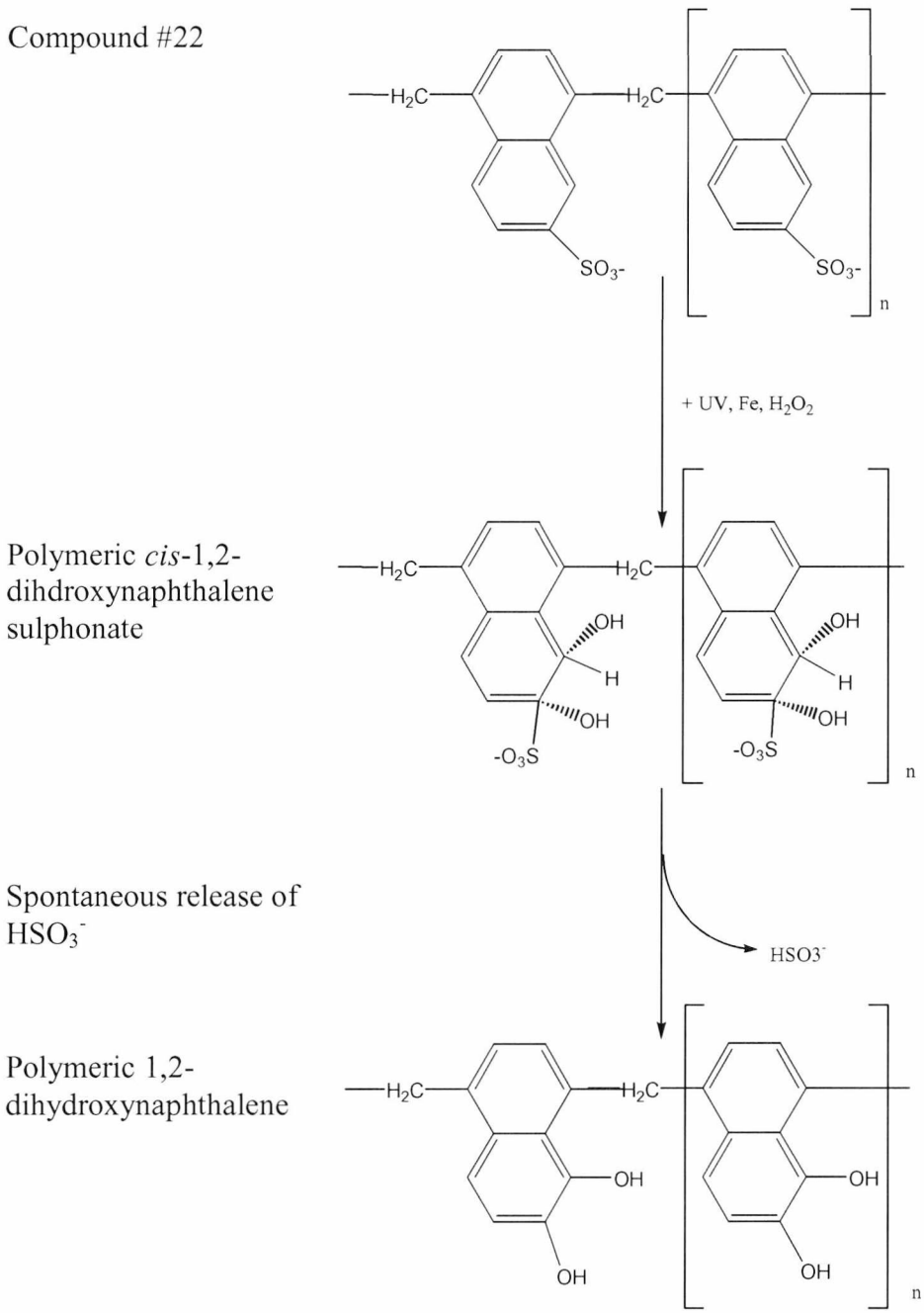

Figure 7.14. A proposed mechanism for the hydroxyl free radical attack during photo-Fenton degradation of compound #22, showing initiation of ring cleavage with retention of the condensation polymer backbone.

CHAPTER 8

CONCLUDING DISCUSSION

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Xenobiotic compounds are deliberately or inadvertently introduced into the environment by anthropogenic activities (Alexander, 1994). These xenobiotic compounds may be poorly degraded and, with frequent application or exposure, may accumulate in the environment. The presence of these compounds may also limit the treatment, reuse and discharge of industrial wastewaters (Janssens *et al.*, 1997). One group of xenobiotics, the aromatic sulphonates, are used in the synthesis of a diversity of compounds, such as syntans and concrete plasticisers. A number of aromatic sulphonates are resistant to biological degradation and, as a consequence, have been detected in landfill leachates, industrial effluents, the effluent from wastewater treatment plants, river water and seawater.

In the research reported in this thesis, significant concentrations of the aromatic sulphonate, naphthalene-2- sulphonate (NSA), were detected in two industrial effluents: the first from a chemical plant manufacturing naphthalene sulphonate-formaldehyde condensates (SNFC) including syntans (25.29 mg NSA $L^{-1} \pm 0.06$); and the second from a tannery, using syntans during leather manufacture (61.78 mg NSA $L^{-1} \pm 0.67$). The presence of NSA in the tannery effluent was of particular significance, as the effluent had been subjected to biological wastewater treatment prior to analysis. The presence of high concentrations of NSA in the biologically treated effluent suggested that either the biomass was under performing or that no NSA-degrading bacteria were present. Therefore, bioaugmentation of biological treatment plants with microbial isolates or communities specifically selected for the degradation of NSA was investigated.

Bioaugmentation may be defined as the inoculation of an environment with a specifically enriched microorganism(s). This has been used with varying degrees of success to clean up contaminated soil and to achieve degradation of xenobiotics in biotreament plants (Megharaj *et al.*, 1997; Selvaratnam *et al.*, 1997; Watanabe *et al.*, 1996). Two NSA-degrading isolates were enriched, one from the biomass of a reed bed treating the effluent

from a tannery utilising SNFC (isolate RBN), and one from an activated sludge plant treating effluent from a chemical plant manufacturing SNFC (isolate BAS). RBN was identified as a *Sphingomonas* sp. and BAS was identified as *Comamonas testosteroni*. *Sphingomonas* and *Comamonas* strains, isolated from different environments, have been previously reported to degrade a number of xenobiotic compounds (Locher *et al.,* 1989; Stolz, 1999; Boon *et al.,* 2000; Tiirola *et al.*, 2002).

Determination of the growth kinetics of the inoculant will provide valuable indicators of the success of bioaugmentation of a biotreatment plant, e.g. the growth rate of an inoculant in a biotreatment plant must be high enough to maintain the population mass balance against outflow from the system. Similarly, continued utilisation of the target compound by the inoculant in the presence of alternative carbon sources is a prerequisite for bioaugmentation. Both isolates had K_s values for NSA of 14 mg L^{-1}, which was below the concentrations of NSA measured in the effluents used in this study. Both isolates would be expected to continue degrading NSA, if inoculated into the biomass of an activated sludge plant treating effluent containing NSA at the concentrations previously measured. This hypothesis was supported by observation that both isolates completely degraded NSA (50 mg L^{-1}) supplied as approximately 10% of the total carbon in a medium containing a number of readily-degradable carbon sources. Additionally, neither isolate showed evidence of catabolite repression of NSA degradation in the presence of alternative carbon sources.

In *in vitro* experiments, both *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS converted indole to indigo (indicating expression of a dioxygenase) and utilised naphthalene as sole source of carbon and energy. Amplification products were detected after PCR amplification of the DNA from both isolates with primers for *nah*Ac, the large subunit in the terminal oxygenase of naphthalene dioxygenase. The degradation of NSA by RBN and BAS, would appear to be facilitated through the naphthalene catabolic pathway after liberation of the sulphonate moiety as sulphite, as reported for other NSA-degrading isolates (Brilon *et al.*, 1981; Ohmoto *et al.*, 1996). It has been suggested that the degradation of NSA may be initiated by a unique NSA-specific dioxygenase (Kuhm *et al.,*1991). The specificity of the NSA-dioxygenases of

Sphingomonas sp. strain RBN and *Comamonas testosteroni* strain BAS was not determined. Further investigation of the catabolic pathway(s) utilised by the isolates would, therefore, be required.

A characteristic desirable in an isolate for bioaugmentation, is the ability to disseminate plasmid-encoded catabolic genes by horizontal transfer (Burns & Stach, 2002). Plasmid encoding of pathways for the degradation of a number of environmentally significant compounds has been reported (Ka *et al.*, 1994; Feng *et al.*, 1997; Hayatsu *et al.*, 2000; Park & Kim, 2000), including the catabolic genes for naphthalene (Sayler *et al.* 1990). The transfer of catabolic genes from inoculated species to indigenous species may result in a xenobiotic-degrading species that outcompetes the inoculant, increasing the rate of removal of the target organism (Springael *et al.*, 2002). Horizontal transfer may also lead to the evolution of novel degradative pathways for xenobiotic compounds (Van der Meer *et al.*, 1998).

The capacity of both isolates to utilise NSA as sole source of carbon and energy was lost after curing in a non-selective medium, indicating that the NSA catabolic pathway was plasmid-encoded in both isolates. As such, both isolates may be capable of disseminating NSA catabolic genes to bacteria present in, and potentially better adapted to, biotreatment plants. Investigation of the promiscuity of these catabolic plasmids would, therefore, be pertinent to the utilisation of the isolates for bioaugmentation of biotreatment plants.

Inoculation of *Sphingomonas* sp. strain RBN into a suspended floc reactor, led to an increase in both the NSA removal rate (q_E) and total organic removal rate (k). Inoculation of the biomass of biological treatment systems with competent organisms has been previously shown to improve both q_E (Watanabe *et al.*, 1996; Bokhamy *et al.*, 1997) and k (Huang *et al.*, 1999; Huang *et al.*, 2001). Stephens & Stephens (1992) proposed that the success of bioaugmentation requires demonstration of the survival and growth of the inoculated strain(s), as well as demonstration of the desired degradative activity. *Sphingomonas* sp. strain RBN was chromosomally marked with *gfp* and inoculated into a suspended floc reactors treating a feed containing NSA. Although the indigenous

biomass was able to remove 100 % of the influent NSA (50 mg L^{-1}), both *Sphingomonas* sp. strain RBN*gfp* and plasmid-cured *Sphingomonas* sp. strain RBN*gfp*C (i.e. unable to utilise NSA) were shown to persist in the reactors for at least 13 d. Additionally, shock loading of the reactors with NSA (250 mg L^{-1}) led to a proliferation of *Sphingomonas* sp. strain RBN*gfp*, which subsequently reduced the recovery time (i.e. the time required to achieve pre-shock loading concentrations of NSA in the effluent) of the reactor. No proliferation was observed in the reactor inoculated with *Sphingomonas* sp. strain RBN*gfp*C. The numbers of *Sphingomonas* sp. strain RBN*gfp* declined after recovery of the reactor, indicating that the proliferation of *Sphingomonas* sp. strain RBN*gfp* was a direct response to the shock load of NSA and, therefore, a consequence of its capacity to degrade NSA. Proliferation of metabolically-competent isolates in response to shock loads has been previously reported. Shock loading of a phenol-digesting sludge inoculated with phenol-degrading *Pseudomonas putida* BH, lead to a 10-fold increase in the numbers of strain BH. The population rapidly decline to initial numbers when the shock loading ceased (Watanabe *et al*, 1998).

Sphingomonas sp. strain RBN was shown to persist after inoculation into suspended floc reactors, regardless of its metabolic capacity for the degradation of the target compound, and to improve both the steady state kinetics of NSA degradation and NSA shock load resistance of the reactors. As such, both the criteria for successful bioaugmentation proposed by Stephens & Stephens (1992) were satisfied. It is believed that this is the first report of the use of bioaugmentation of biotreatment plants for the remediation of NSA.

NSA is used widely in the synthesis of polymeric SNFC compounds, such as plasticisers for the production of high performance concretes (Piotte *et* al., 1995), and as synthetic tanning agents (syntans) in the manufacture of leather (Reemtsma & Jekel, 1994). For this reason, the concentration of SNFC in the tannery and chemical plant effluents was also determined by ion-pair UV-HPLC. Due to the absence of reference materials, a proprietary SNFC-based commercial product (compound #22) was used as a reference compound and peak profiles matching that of compound #22 were detected in both effluents. Although unequivocal determination of the SNFC concentration in the effluents could not be achieved, it was apparent from comparison of the peak areas in the

effluent with standard solutions of compound #22, that significant concentrations of these compounds were present in both effluents. As previously reported (Reemtsma & Jekel, 1994), the presence of SNFC in the tannery effluent, and their very low BOD_5 values, indicates that these compounds, along with NSA, are resistant to biological treatment. However, the concentration of SNFC in the effluents requires further investigation. Accurate quantification of SNFC is required in order to determine their contribution to the organic content of the effluent and the extent of their degradation in existing biotreatment plants. SNFC typically contain a number of oligomers of up to 90-mer (Miller, 1985). However, only oligomers of up to 4-mer have been detected in contaminated waters with larger oligomers thought to be adsorbed to organic material (Redín *et al.*, 1999; Crescenzi *et al.*, 2001; Menzel *et al.*, 2002; Ruckstuhl *et al.*, 2002). It is conceivable that alterations to the physical parameters of effluent treatment, such as filtration, may allow for the removal of SNFC from effluents and, therefore, the size distribution of the SNFC present should be determined.

Neither of the NSA-degrading isolates, *Sphingomonas* sp. strain RBN and *Comamonas testosteroni strain* BAS, was able to utilise compound #22 as sole source of carbon and energy in batch growth culture. *Sphingomonas* sp. strain RBN was also unable to cometabolise compound #22 in the presence of NSA. The recalcitrance of compound #22 to degradation by the isolates was probably due to its polymeric nature. The limiting effect of polymerisation on the degradation of xenobiotic compounds has been previously reported (Larson *et al.*, 1997). The use of photo-Fenton pretreatment to increase the biodegradability of compound #22, was investigated. Photo-Fenton treatment reduced the concentration and COD of compound #22. Analysis by HPLC of photo-Fenton treated solutions of #22 showed elimination of the associated peaks, suggesting that the structure of the compound had been degraded. However, there was no increase in the biodegradability by either *Sphingomonas* sp. strain RBN or in a standard BOD_5 test. It is proposed that, due to the nature of the oxidative attack on the NSA-subunit, that the compound may have undergone extensive oxidation but still retained the polymer backbone and hence, its recalcitrance to biodegradation. However, regardless of the mechanism of its persistence, it is clear that compound #22 and other SNFC are

highly resistant to bacterial biodegradation and are unlikely to be removed during effluent treatment while their polymeric structure remains intact.

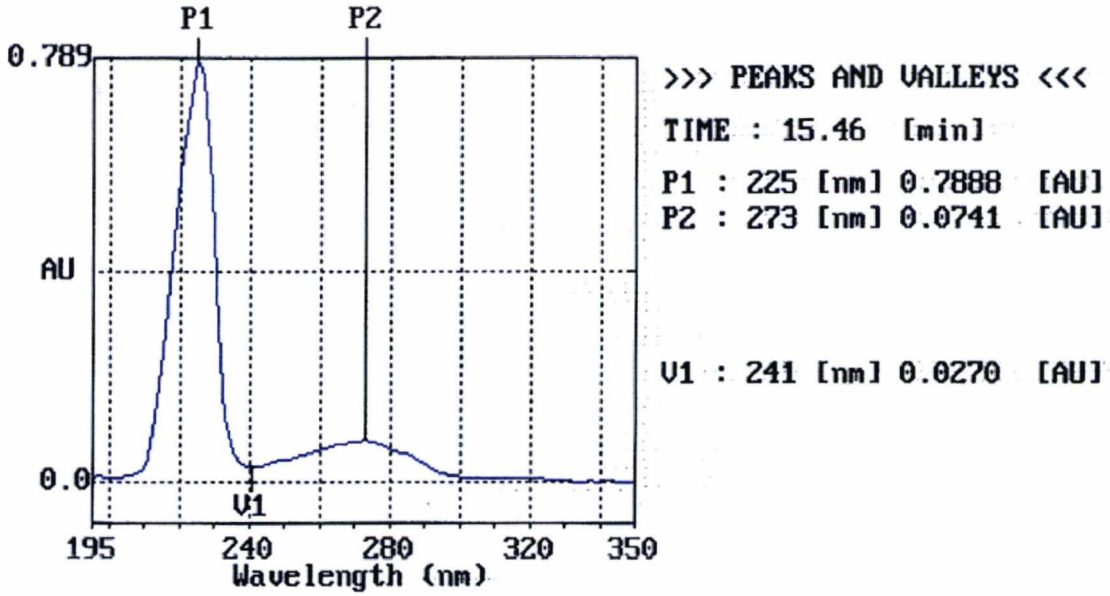
This study has shown that the microbial communities in biological treatment systems, such as suspended floc reactors, can be bioaugmented with selected NSA-degrading isolates. It also found that the predominant source of NSA in the environment, SNFC compounds, were resistant to biodegradation by the selected NSA-degrading isolates, *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS, and that photo-Fenton pretreatment of SNFC did not increase their biodegradability. As the effluent concentrations of SNFC (up to 838.77 mg L^{-1}) were greater than concentrations of NSA (up to 61.78 mg L^{-1}), the removal of SNFC warrants further investigation. A number of research areas are suggested by the work reported in this thesis:

- A more sensitive analytical technique, such as liquid chromatography with electrospray mass-spectrometry (Castillo *et al.*, 2001), is required in order to accurately determine the size and concentration of the SNFC oligomers present and hence, the true contribution of these compounds to pollutant loading of the effluents. Such a technique could also be used to assess the effect of Fentons treatment on the structure of compound #22, thus providing insight into its continued resistance to biodegradation.
- The biomass of the tannery biological treatment plant was not assessed for its capacity to degrade NSA. As such, it is not known whether the presence of NSA in the tannery effluent (section 3.2) was due to an absence of the requisite catabolic capacity, or a result of a limited or repressed capacity. It is conceivable that a desired catabolic capacity may already exist in a biomass but is subject to catabolite repression or other controlling factors. The same controlling factors may limit the efficacy of any inoculated organism. The diversity and expression of *nah*Ac and other PAH dioxygenase genes in the biomass must be assessed to determine both the innate potential for NSA degradation and the extent to which it is expressed.
- It has been proposed that the degradation of NSA is initiated by either a naphthalene dioxygenase with relaxed substrate specificity (Brilon *et* al., 1981) or by a unique aromatic sulphonate dioxygenase (Kuhm *et al.,* 1991). However, it

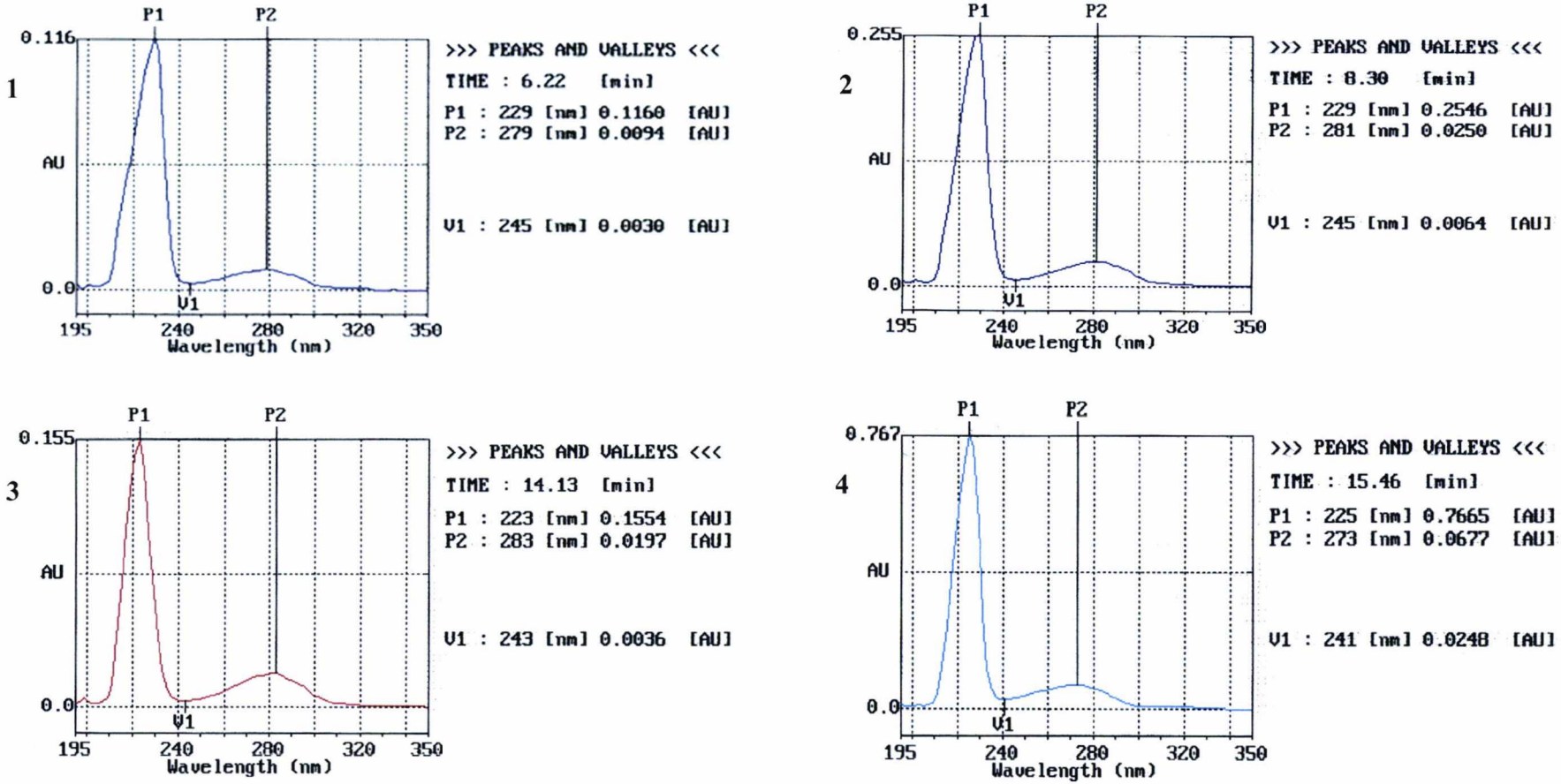
was not possible during this study to determine the nature of the enzyme(s) initiating catabolism of NSA by the isolates. Comparison of the sequence homology of the cDNA products of reverse-transcriptase-PCR with degenerate primers for *nah*Ac, encoding the region of the α subunit thought to determine substrate specificity (Parales *et al.*, 1998), of the enzyme(s) being actively transcribed during the degradation of NSA and naphthalene, may determine if the isolates are expressing a naphthalene dioxygenase with relaxed substrate specificity or by a unique aromatic sulphonate dioxygenase.

- The persistence of the isolates after inoculation into a biological reactor should be evaluated over an extended period. Inoculated populations typically decline with time with the consequence that the removal of the target compound may also decrease (Watanabe *et al.*, 1998; Boon *et al.*, 2000). In order for full-scale application of bioaugmentation to be successful, it will necessary to determine if and how often, repeat inoculations are required.
- The resistance to biodegradation by compound #22 was probably due to its polymeric structure. The degradation of xenobiotic polymers, including nylon (Deguchil *et al.*, 1997) and polyvinyl alcohol (Larking *et al.,* 1999) by lignin-degrading fungi expressing extracellular manganese peroxidases and laccases has been reported. The fungus, *Cunninghamella polymorpha*, degraded all of the oligomers of greater than 3-mer in compound #22 after 120 h in batch culture (Z. Song, personal communication). The sequential batch treatment of compound #22 with *Cunninghamella polymorpha* and the NSA-degrading bacterial isolates, *Comamonas* sp. 4BC or *Arthrobacter* sp. 2AC led to a total degradation of 65% of the added compound #22 (500 mg L^{-1}). Therefore, the application of fungal isolates able to degrade SNFC, either directly or by depolymerisation and subsequent bacterial mineralisation, to the treatment of SNFC-contaminated effluent represents a significant area for future investigation.

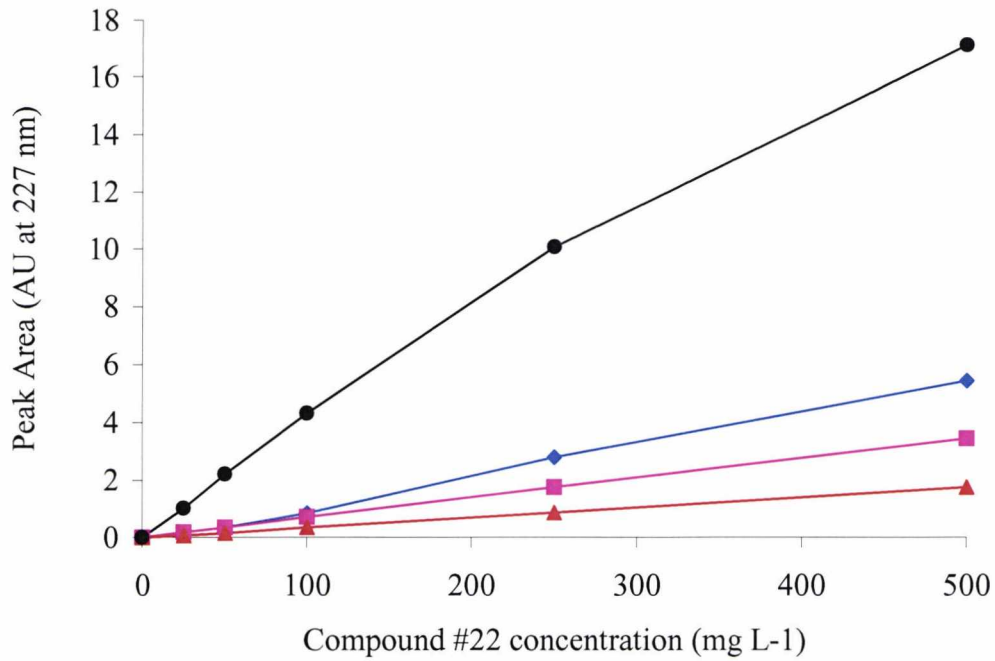
APPENDICES



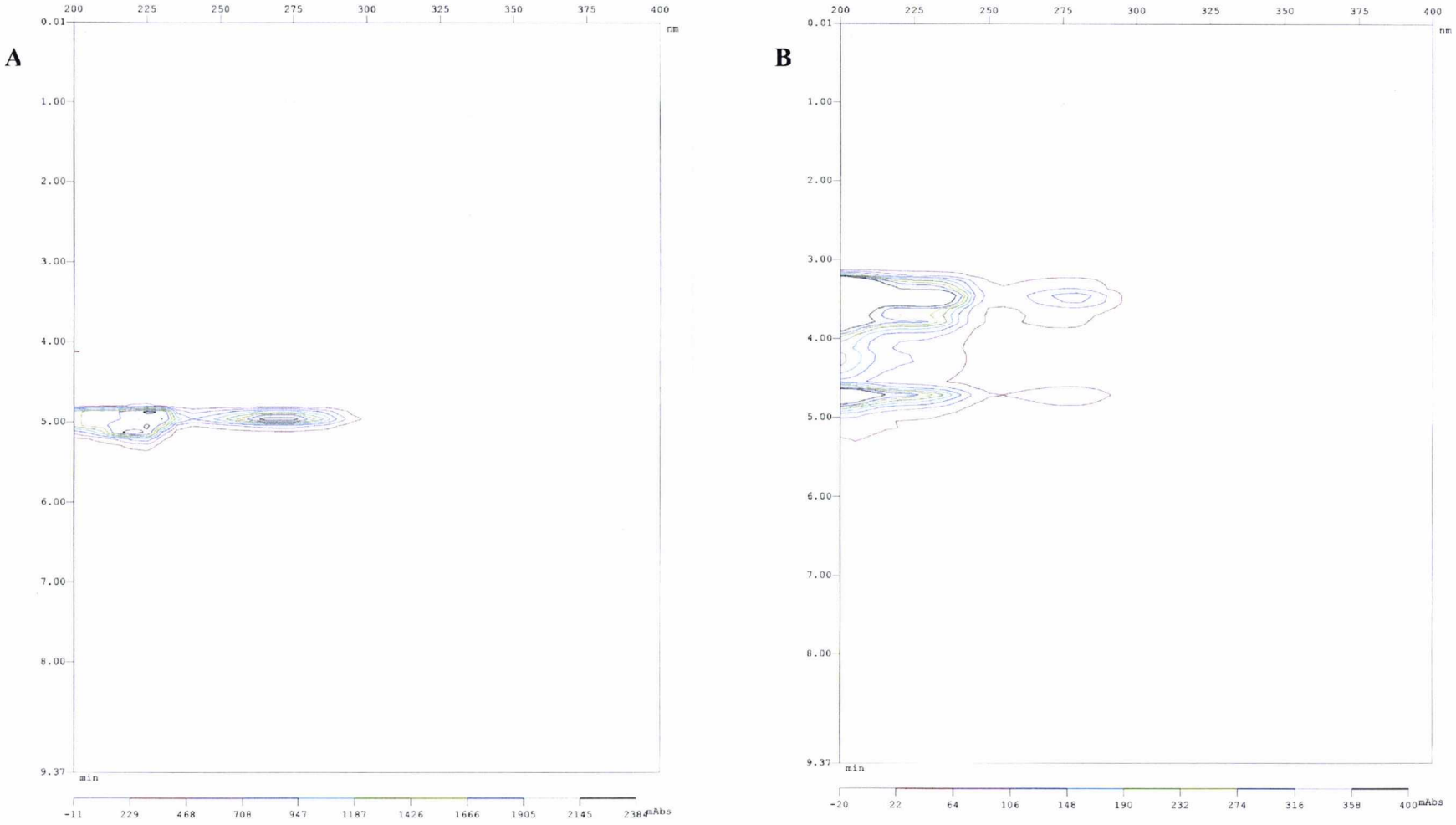
Appendix 1.1. IP-HPLC-UV profile of NSA (50 mg L^{-1})



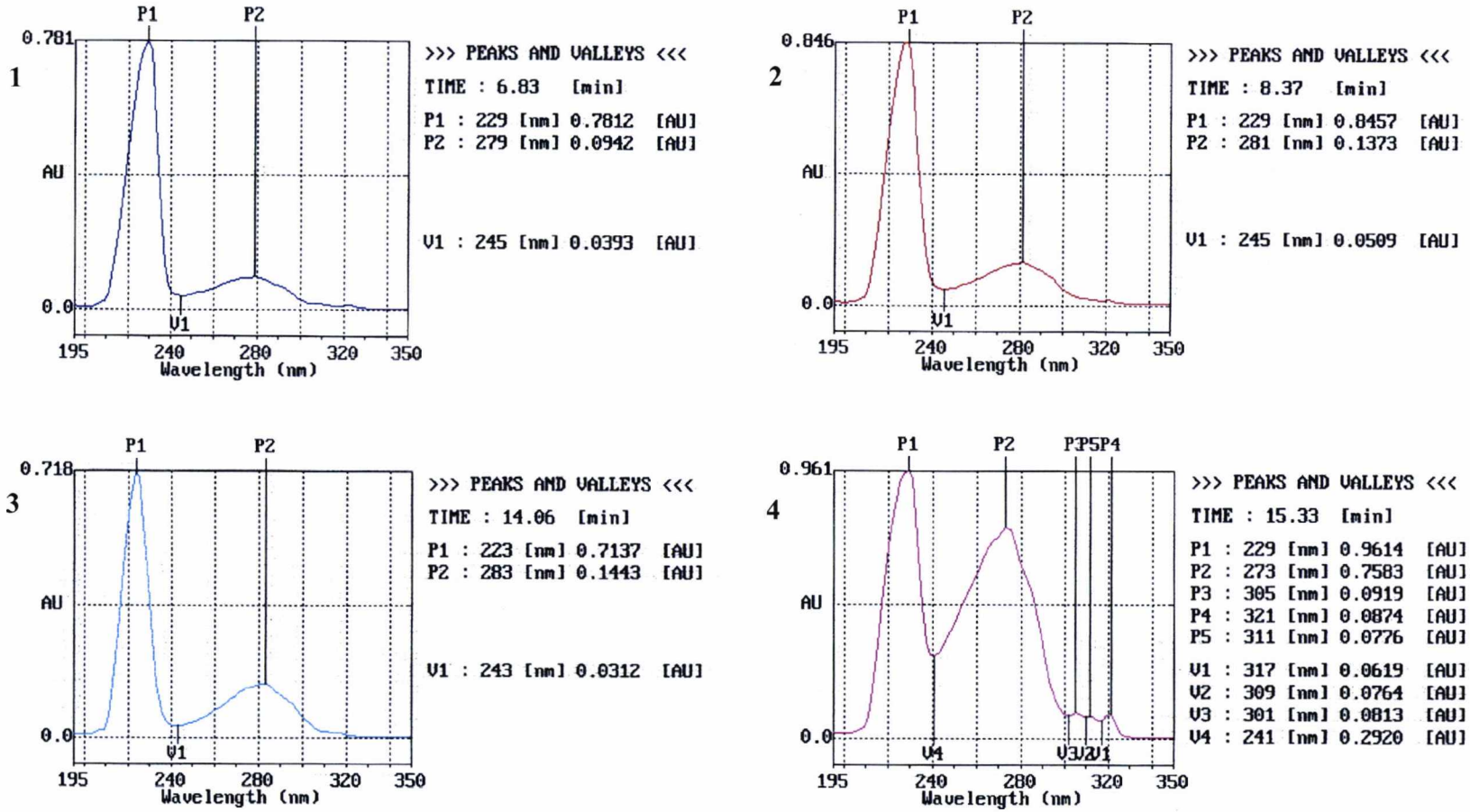
Appendix 1.2. UV profiles of the 4 peaks detected in compound #22 by IP-HPLC (section 2.2.1.5.1). Peak elution sequence indicated by numbers in bold.



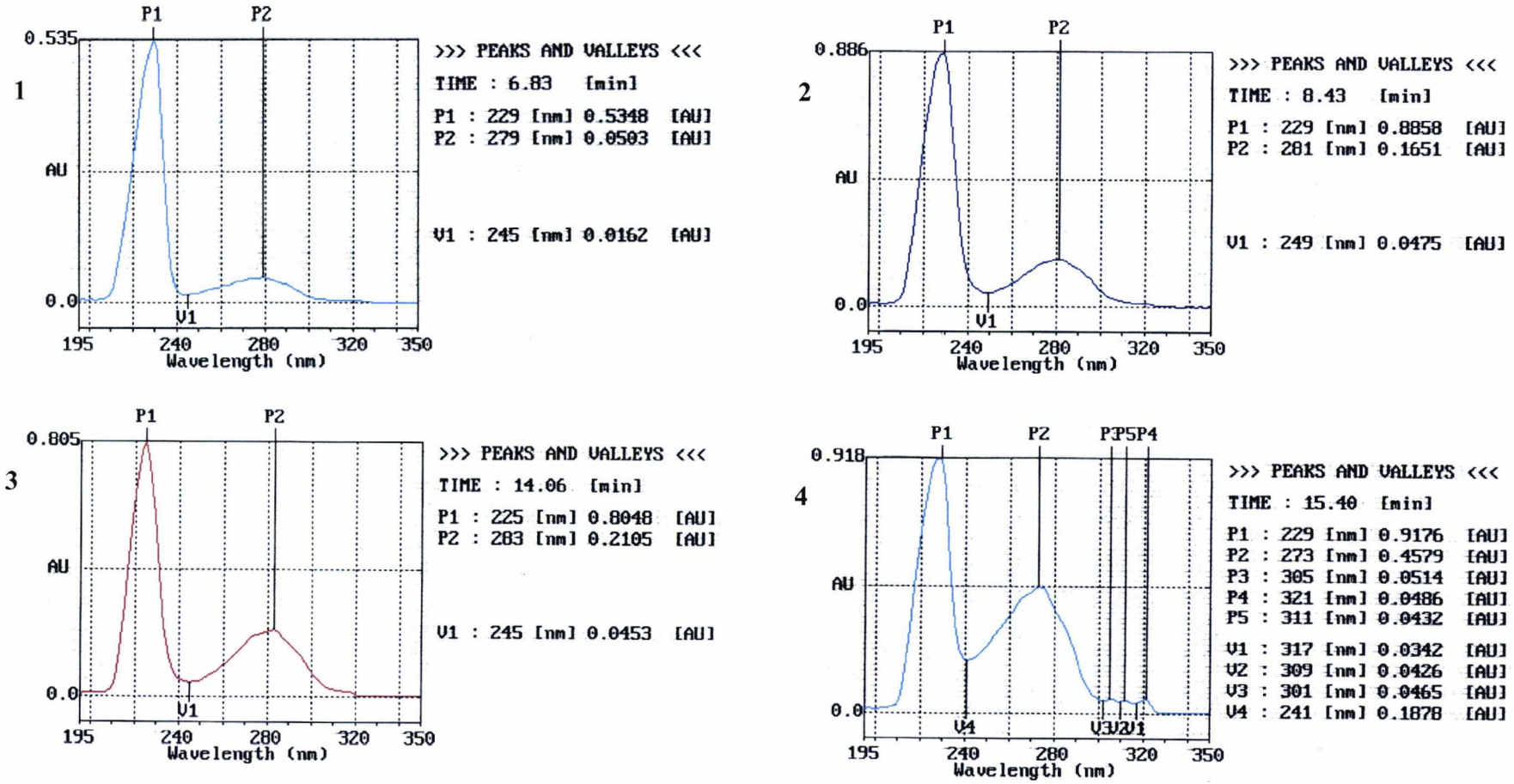
Appendix 1.3. Standard curves of the individual components of compound #22 at 227 nm; ♦ - t_R 6.2 min, ■ – t_R 8.3 min, ▲- t_R 14.1 min, ● – t_R 15.4 min. Best fit lines are shown, r^2 = 0.998, 0.999, 0.999 and 0.992, respectively. Vertical bars indicate S.E.M (n= 6); for symbols without bars, the limits of error were within the confines of the symbol.



Appendix 1.4. UV-HPLC profiles of 500 mg L^{-1} solutions of NSA (A) and compound #22 (B) (section 2.2.1.5.2)



Appendix 1.5. UV profiles of the 4 largest peaks detected in undiluted tannery biological treatment plant effluent by UV IP-HPLC (section 2.2.1.5.1). Peak elution sequence indicated by numbers in bold.



Appendix 1.6. UV profiles of the 4 largest peaks detected in undiluted (except peak 1, diluted 1 in 5) chemical plant effluent by UV IP-HPLC (section 2.2.1.5.1). Peak elution sequence indicated by numbers in bold.

Appendix 2.1. *nah*Ac sequences amplified from *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS.

1. *nah*Ac sequence of *Sphingomonas* sp. strain RBN (471 bp)

GCCTGATTCCATCCCCCGGCGACTATGTTACCGCAAAAATGGGTATTGACGAGG
TCATCGTCTCTCGGCAGAGCGACGGTTCGATTCGTGCCTTCCTGAACGTTTGTCG
GCACCGTGGCAAGACGCTGGTTAACGCGGAAGCCGGCAATGCCAAAGGTTTCGT
TTGCAGCTATCACGGCTGGGGTTTCGGCTCCAACGGTGAACTGCAGAGCGTTCC
ATTCGAAAAAGAGCTGTACGGCGAGTCGCTCAACAAAAAATGTCTGGGGTTGAA
AGAAATCGCTCGCGTGGAGAGCTTCCATGGCTTCATCTATGGTTGCTTCGATCAG
GAGGCCCCTCCTCTTATGNNCTATCTGGGTGACGCTGCTTGGTACCTAGGGCCCA
TCTTCAAACATTCAGGCGGTTTAGAACTGGTCGGTCCTCCAGGCAAGGTTGTGAT
CAAGGCCAACTGGAAGACACCCGAATCACTAGTGAATTC

2. *nah*Ac sequence of *Comamonas testosteroni* strain BAS (471 bp)

GCCTGATTCCATCCCCCGGCGACTATGTTACCGCAAAAATGGGTATTGACGAGG
TCATCGTCTCTCGGCAGAGCGACGGTTCGATTCGTGCCTTCCTGAACGTTTGTCG
GCACCGTGGCAAGACGCTGGTTAACGCGGAAGCCGGCAATGCCAAAGGTTTCGT
TTGCAGCTATCACGGCTGGGGCTTCGGCTCCAACGGTGAACTGCAGAGCGTTCC
ATTCGAAAAAGAGCTGTACGGCGAGTCGCTCAACAAAAAATGTCTGGGGTTGAA
AGAAGTCGCTCGCGTGGAGAGCTTCCATGGCTTCATCTATGGTTGCTTCGATCAG
GAGGCCCCTCCTCTTATGGACTATCTGGGTGGCGCTGCTTGGTACCTAGAGCCCA
TCTTCAAACATTCAGGCGGTTTAGAACTGGTCGGTCCTCCAGGCAAGGTTGTGAT
CAAGGCCAACTGGAAGACACCTGAATCGAATTCCC

Appendix 2.2. Partial 16S rDNA sequences amplified from *Sphingomonas* sp. strain RBN and *Comamonas testosteroni* strain BAS.

1. Partial 16S rDNA sequence of *Sphingomonas* sp. strain RBN (386 bp)

GGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGTTATTC
AAGTCAGGGGTGAAAGCCCAGTGCTCAACACTGGAACTGCCCTTGAAACTAGAT
AACTTGAATCCAGGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTA
GATATTCGGAAGAACACCAGTGGCGAAGGCGGCTCACTGGACTGGTATTGACGC
TGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGATAACTAGCTGTCTGGGCACATGGTGCTTAGGTGGCGCAGCTA
ACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGA
ATTGACGG

2. Partial 16S rDNA sequence of *Comamonas testosteroni* strain BAS (375 bp)

TTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTTGTAAGACAGTGG
TGAAATCCCCGGGCTCAACCTGGGAACTGCCATTGTGACTGCAAGGCTAGAGTG
CGGCAGAGGGGGATGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGCGG
AGGAACACCGATGGCGAAGGCAATCCCCTGGGCCTGCACTGACGCTCATGCACG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGA
TGTCAACTGGTTGTTGGGTCTTAACTGACTCAGTAACGAAGCTAACGCGTGAAGT
TGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGG

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	AF378796 *Sphingomonas alaskensis* AF01		1	0.934	0.934	0.934	0.934	0.926	0.942	0.950	0.934	0.930	0.939	0.934	0.928	0.934	0.934	0.928	0.928	0.934	*0.944*
2	AF145754 *Sphingomonas alaskensis* RB2510	0		0.934	0.934	0.934	0.934	0.926	0.942	0.950	0.934	0.930	0.939	0.934	0.928	0.934	0.934	0.928	0.928	0.934	*0.944*
3	AF161809 *Sphingomonas* sp. str. STS_R2A_43	0.066	0.066		1	1	1	0.992	0.981	0.963	0.973	0.997	0.984	1	0.995	1	1	0.995	0.995	1	*0.968*
4	AF181571 *Sphingomonas* sp. str. JRL-4	0.066	0.066	0		1	1	0.992	0.981	0.963	0.973	0.997	0.984	1	0.995	1	1	0.995	0.995	1	*0.968*
5	AF331661 *Sphingomonas yanoikuyae*	0.066	0.066	0	0		1	0.992	0.981	0.963	0.973	0.997	0.984	1	0.995	1	1	0.995	0.995	1	*0.968*
6	AB074685 α-proteobacterium D-N(2)-5	0.066	0.066	0	0	0		0.992	0.981	0.963	0.973	0.997	0.984	1	0.995	1	1	0.995	0.995	1	*0.968*
7	D13728 *Sphingomonas yanoikuyae* IF015102	0.074	0.074	0.008	0.008	0.008	0.008		0.973	0.955	0.966	0.995	0.976	0.992	0.997	0.992	0.992	0.997	0.997	0.992	*0.960*
8	AJ427917 *Sphingomonas* sp. JS1	0.058	0.058	0.019	0.019	0.019	0.019	0.027		0.971	0.955	0.981	0.971	0.981	0.976	0.981	0.981	0.976	0.976	0.981	*0.981*
9	AY026948 *Sphingomonas* sp. ML1	0.050	0.050	0.037	0.037	0.037	0.037	0.045	0.029		0.968	0.960	0.973	0.963	0.958	0.963	0.963	0.958	0.958	0.963	*0.973*
10	AY047219 *Sphingomonas yanoikuyae*	0.066	0.066	0.027	0.027	0.027	0.027	0.034	0.045	0.032		0.971	0.973	0.973	0.968	0.973	0.973	0.968	0.968	0.973	*0.947*
11	AY055774 *Sphingomonas* sp. str. EB01	0.070	0.070	0.003	0.003	0.003	0.003	0.005	0.019	0.040	0.029		0.981	0.997	0.997	0.997	0.997	0.997	0.997	0.997	*0.968*
12	AJ132471 *Sphingomonas* sp.	0.061	0.061	0.016	0.016	0.016	0.016	0.024	0.029	0.027	0.027	0.019		0.984	0.979	0.984	0.984	0.979	0.979	0.984	*0.963*
13	X87167 *Sphingomonas* sp. str. Q1	0.066	0.066	0	0	0	0	0.008	0.019	0.037	0.027	0.003	0.016		0.995	1	1	0.995	0.995	1	*0.968*
14	D84526 *Sphingomonas yanoikuyae* GIFU 9882T	0.072	0.072	0.005	0.005	0.005	0.005	0.003	0.024	0.042	0.032	0.003	0.021	0.005		0.995	0.995	1	1	0.995	*0.963*
15	U37524 *Sphingomonas yanoikuyae* B1	0.066	0.066	0	0	0	0	0.008	0.019	0.037	0.027	0.003	0.016	0	0.005		1	0.995	0.995	1	*0.968*
16	U37525 *Sphingomonas yanoikuyae* Q1	0.066	0.066	0	0	0	0	0.008	0.019	0.037	0.027	0.003	0.016	0	0.005	0		0.995	0.995	1	*0.968*
17	X85023 *Sphingomonas yanoikuyae*	0.072	0.072	0.005	0.005	0.005	0.005	0.003	0.024	0.042	0.032	0.003	0.021	0.005	0	0.005	0.005		1	0.995	*0.963*
18	D16145 *Sphingomonas yanoikuyae* GIFU 9882	0.072	0.072	0.005	0.005	0.005	0.005	0.003	0.024	0.042	0.032	0.003	0.021	0.005	0	0.005	0.005	0		0.995	*0.963*
19	X94099 *Sphingomonas* sp. B1	0.066	0.066	0	0	0	0	0.008	0.019	0.037	0.027	0.003	0.016	0	0.005	0	0	0.005	0.005		*0.968*
20	**Isolate RBN**	*0.056*	*0.056*	*0.032*	*0.032*	*0.032*	*0.032*	*0.040*	*0.019*	*0.027*	*0.053*	*0.032*	*0.037*	*0.032*	*0.037*	*0.032*	*0.032*	*0.037*	*0.037*	*0.032*	

Similarity

Divergence

Appendix 2.3 - Similarity and Divergence matrices of a partial 16S rDNA sequence from isolate RBN and closely related 16S rDNA sequences identified by Genbank. Values for RBN are shown in italics. Sequences aligned using MEGALIGN and matrices constructed using Similarity Matrices program on the Ribosomal Database website.

		1	2	3	4	5	6	7	8	9	10	11	12
1	M11224 *Comamonas testosteroni*		1	0.986	0.995	1	0.986	0.995	1	1	0.997	1	*1*
2	AB007997 *Comamonas testosteroni* M BIC 3840	0		0.986	0.995	1	0.986	0.995	1	1	0.997	1	*1*
3	AB008429 *Comamonas testosteroni* M BIC 3845	0.014	0.014		0.981	0.986	0.984	0.986	0.986	0.986	0.984	0.986	*0.986*
4	AB066235 *Comamonas* sp. PJ111	0.005	0.005	0.019		0.995	0.981	0.989	0.995	0.995	0.992	0.995	*0.995*
5	AF172067 *Comamonas testosteroni*	0	0	0.014	0.005		0.986	0.995	1	1	0.997	1	*1*
6	AF188304 *Comamonas testosteroni* D22	0.014	0.014	0.016	0.019	0.014		0.981	0.986	0.986	0.984	0.986	*0.986*
7	AF336312 *Comamonas testosteroni* M BIC 3841	0.005	0.005	0.014	0.011	0.005	0.019		0.995	0.995	0.992	0.995	*0.995*
8	AF408328 uncultured Comamonas clone KL-27-1-15	0	0	0.014	0.005	0	0.014	0.005		1	0.997	1	*1*
9	AY050494 *Comamonas testosteroni* SB2	0	0	0.014	0.005	0	0.014	0.005	0		0.997	1	*1*
10	D87101 *Comamonas testosteroni*	0.003	0.003	0.016	0.008	0.003	0.016	0.008	0.003	0.003		0.997	*0.997*
11	AB007996 uncultured *Comamonas testosteroni* M BIC 3840	0	0	0.014	0.005	0	0.014	0.005	0	0	0.003		*1*
12	**Isolate BAS**	*0*	*0*	*0.014*	*0.005*	*0*	*0.014*	*0.005*	*0*	*0*	*0.003*	*0*	

Similarity

Divergence

Appendix 2.4 - Similarity and Divergence matrices of a partial 16S rDNA sequence from isolate BAS and closely related 16S rDNA sequences identified by Genbank. Values for BAS are shown in italics. Sequences aligned using MEGALIGN and matrices constructed using Similarity Matrices program on the Ribosomal Database website.

PUBLICATIONS

The articles presented here are directly related to the subject of this thesis and were written by Kerry Senior. They were published in the BLC Journal, a monthly magazine produced by BLC, Leather Technology Centre, Northampton, as a means of disseminating relevant research to the leather industry.

Bioremediation of Syntans in Tannery Effluents

Report by Kerry Senior

As has been previously reported, tannery effluents may contain a variety of compounds that are resistant to conventional biological effluent treatment and which contribute to a fraction of the chemical oxygen demand (COD) of the effluent described as the 'hard' COD (BLC Journal October 2001). These compounds are typically aromatic and/or substituted organic compounds. BLC is currently involved in a DTI-funded LINK project with the University of Kent at Canterbury, investigating the recycling and reuse of effluents from tanneries. The project aims to investigate engineering and biological options for the treatment of tannery effluent. A part of the project deals with the identification of recalcitrant compounds in tannery effluent streams and the isolation of bacteria capable of degrading these compounds.

One group of compounds thought to be present in the hard COD are the syntans. Syntans are typically condensation polymers of phenol-, naphthalene- or cresol-sulphonates, or melamine-based compounds. The research described here focuses on naphthalene sulphonate-based compounds.

The simplest naphthalene sulphonate (NSA) syntans are formed by a condensation polymerisation of naphthalene sulphonate with formaldehyde. The putative structure of a simple commercially available syntan, designated #22, is shown in Figure 1. The structure is thought to be linear and between 2 and 10 mer in length (personal communication, Z. Song, University of Kent at Canterbury).

The presence of syntans in tannery effluent has been reported. Concentrations of up to 40 mg/l in tannery effluent have been detected after combined aerobic and anaerobic biological treatment[1]. Monomeric NSA has also been detected in the final effluents from tanneries[2]. Analysis at BLC of aerobically treated tannery effluent, by UV-diode array HPLC after ion-pair solid phase extraction (IP-SPE), has also tentatively identified syntans as a component of the residual COD.

A complicating factor for the analysis of syntans is the absence of reference materials. Syntans are commercial products which, in addition to the active component, will contain axillary compounds, the identity of which is often unknown. The active component itself will also vary in terms of both polymer size and structure. Syntan #22 was used as a

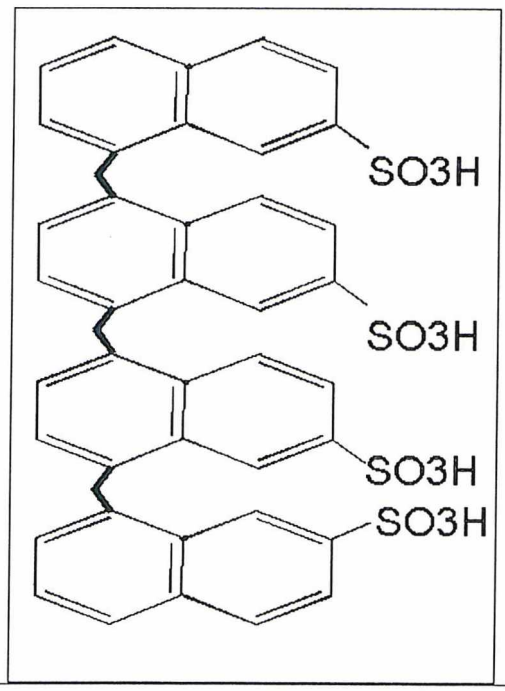


Figure 1 The putative structure of a commercially available syntan #22

reference compound for the analysis carried out at BLC. The UV spectra of the detected peaks was also compared with that of NSA, the monomeric sub-unit of NSA-based syntans. HPLC chromatograms of syntan #22 and the biologically treated effluent are shown in Figures 2 and 3. The peaks observed in the effluent were found to be closely matched in terms of peak number and retention time of those observed for #22. The peak spectra were also found to be very similar to that of NSA (not shown) in terms of absorbence maxima (the wavelength at which the molecule has its maximum UV absorption) and peak

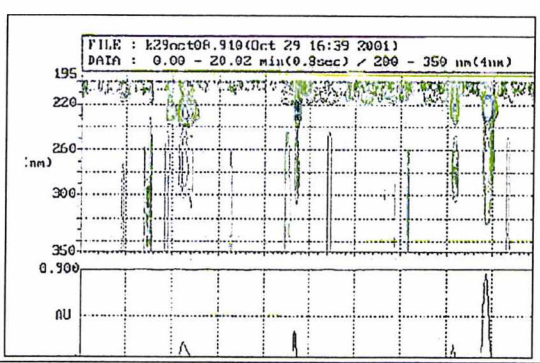


Figure 2 HPLC chromatogram of syntan #22

shape. The last peak on both chromatograms was found to be very closely matched to that of NSA, with the same peak shape and retention time, indicating that monomeric NSA is both a contaminant of syntan #22 and will remain in the effluent after biological treatment. Quantitatively, the analysis indicated the presence of 50 – 200 mg/l of syntan and 50 mg/l of NSA but it should be noted that, in the absence of defined reference materials, accurate quantification was not possible.

NSA and syntans have been shown to be significant contaminants in tannery effluent. The presence of these compounds may have implications for the recycling and reuse of these effluents. As such, it is necessary to improve the treatment of tannery effluents in order to remove these compounds. The bioaugmentation of biological treatment processes with micro-organisms capable of degrading specific recalcitrant compounds has been shown to enhance the removal of those compounds, eg, the bioaugmentation of an activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain[3].

A NSA-degrading isolate, RBN, was isolated from an activated sludge. Figure 4 shows the degradation of NSA by RBN in both batch and continuous culture. As is shown, RBN achieved 100% NSA removal at a range of hydraulic retention times (6 – 48 hours). Inoculation of an activated sludge with RBN was also found to enhance the rate of NSA removal. However, as shown in figure 5, RBN was found to be unable to degrade syntan #22. Even after the establishment of a large, metabolically active population, by the addition of NSA, RBN was unable to degrade syntan #22. As the growth of RBN on NSA was not inhibited by the presence of syntan #22 (ie, it was not toxic to RBN), it is probable that the degradation of syntan #22 was prevented due its polymeric structure. This may be due to the size of the molecule preventing its absorption by RBN and subsequent exposure to the degradative enzymes.

The very low potential for biodegradation of syntan #22 and other syntans has been previously reported

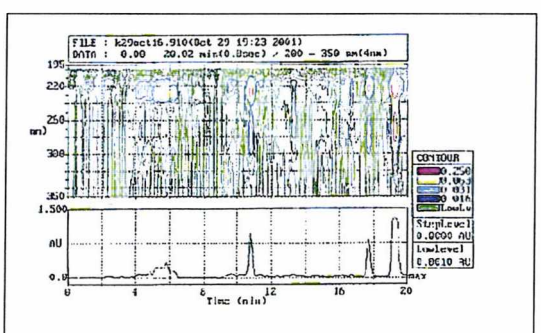


Figure 3 HPLC chromatogram of biologically treated effluent after IP-SPE fractionation

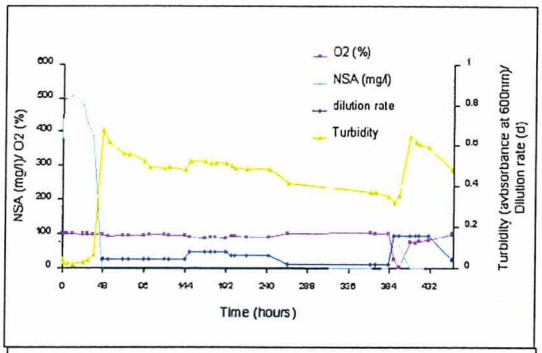


Figure 4 The degradation of NSA by strain RBN in batch and continuous culture in a chemostat reactor. The reactor was run in batch mode for 48 hours to establish the biomass, and then in continuous mode at a range of hydraulic retention times.

(BLC Journal, July/August 2001). The biological recalcitrance of these compounds is likely to be due to their polymeric nature. The results presented here would appear to support that hypothesis. Similar results have been found with polyacrylic compounds, where increasing levels of polymerisation resulted in a decreasing potential for biodegradation. It is clear that in order to facilitate the biodegradation of these compounds in effluents, they will have to be depolymerised. Research is currently being undertaken at BLC and the University of Kent at Canterbury, to investigate physico-chemical and biological options fo the depolymerisation of syntans and their subsequent biodegradation. However, the results also show that the removal of one component of the hard COD, NSA, can be enhanced by bioaugmentation with a specified micro-organism, RBN. As such, a combined physico-chemical (depolymerisation) and biological (biodegradation) treatment for the removal of syntans may be a viable proposition.

References

[1] *Reemstma T & Jekel M. (1994), Journal of Chromatography A, 660 (1-2), 199 - 204*

[2] *Castillo M et al (1999), Environmental Science & Technology, 33 (8), 1300 - 1306*

[3] *Boon N et al (2000), Applied and Environmental Microbiology, 66, 2906 - 2913*

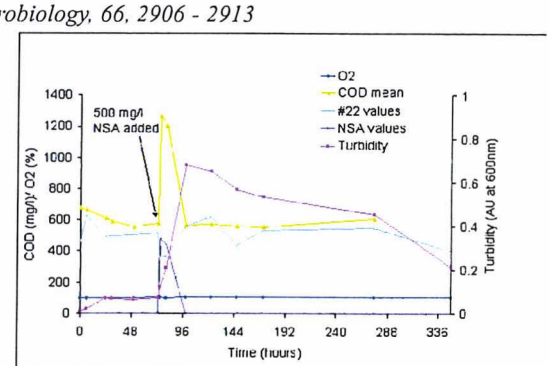


Figure 5 The degradation of syntan #22 by strain in batch culture in a chemostat reactor

Bioaugmentation of Activated Sludges Treating Effluents Contaminated With Recalcitrant Organic Compounds

Report by Kerry Senior

Tannery effluents may contain a variety of compounds that are resistant to conventional biological effluent treatment and which contribute to a fraction of the chemical oxygen demand (COD) of the effluent described as the hard COD[1]. These compounds are typically aromatic and/or substituted organic compounds. An example of a recalcitrant organic compound found in tannery effluents is naphthalene sulphonic acid (NSA). NSA is used in the synthesis of a number of leather process chemicals, including syntans and dyes. Concentrations of up to 50 mg NSA L^{-1} have been detected in the effluent from an on-site biological treatment plant of a tannery processing from raw hide to finished leather[2]. NSA may therefore, significantly contribute to the hard COD discharged by tanneries.

The presence of NSA and other recalcitrant compounds may have implications for the discharge, or recycling and reuse, of tannery effluents. As such, it is necessary to improve the treatment of tannery effluents in order to remove these compounds. The bioaugmentation of biological treatment processes, with microorganisms capable of degrading specific recalcitrant compounds, has been shown to enhance the removal of those compounds. The bioaugmentation of an activated sludge with an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain significantly improved 3-chloroaniline removal.[3] The isolation of a NSA-degrading isolate, RBN, has been previously reported[2]. The use of RBN for the bioaugmentation of biological treatment systems to enhance the degradation of NSA, was investigated as part of a DTI-funded LINK project currently underway at BLC.

Bioaugmentation of contaminated environments may fail if the selected isolates, found to utilise xenobiotic compounds as a sole source of carbon in the laboratory, preferentially utilise other carbon sources when inoculated into the contaminated environment, leaving the target compound undegraded. Similarly, in order for bioaugmentation to succeed, the inoculated organism(s) must continue to degrade the target compound at the concentrations at which it is expected to occur in the inoculated environment. The degradation by RBN of 50 mg NSA L^{-1} in a medium containing a number of readily-degradable carbon sources (CM) was assessed. The growth of RBN was assessed by measuring the turbidity of medium (OD_{600nm}) and NSA removal was measured by HPLC. The results are shown in Figure 1.

The concentration of NSA in the CM media inoculated with RBN, was reduced to 1.6% (± 0.5%) of the initial concentration after 24h, and below detectable levels after 48h. A concurrent increase in turbidity to a maximum OD_{600nm} of 0.572 (± 0.004) after 48h was measured. The complete removal of NSA showed that NSA degradation by RBN was not affected by the presence of alternative carbon sources.

In order for an isolate used for bioaugmentation to succeed, it must also be capable of survival and degradative activity in the presence of the diversity of other microorganisms in the bioaugmented environment. Therefore, use of RBN for the bioaugmentation of suspended floc reactor was investigated.

A suspended floc reactor was inoculated with activated sludge to give a MLSS in the aeration tank of 175 mg L^{-1}. The reactor was fed continuously with CM media and operated with a hydraulic retention time of 16h. The reactor feed and effluent were sampled for COD, and the aeration tank liquor was sampled for MLSS. The reactor was allowed to

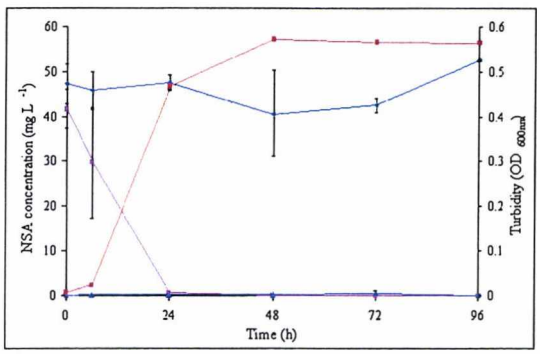


Figure 1 Utilisation of NSA and growth profile of isolate RBN in CM plus 50 mg NSA L^{-1}; ■ -RBN NSA, ● -RBN turbidity, ◆ -uninoculated NSA control, ▲ -uninoculated turbidity control. Vertical bars indicate S.E.M (n=3); for symbols without bars, the limits of error were within the confines of the symbol

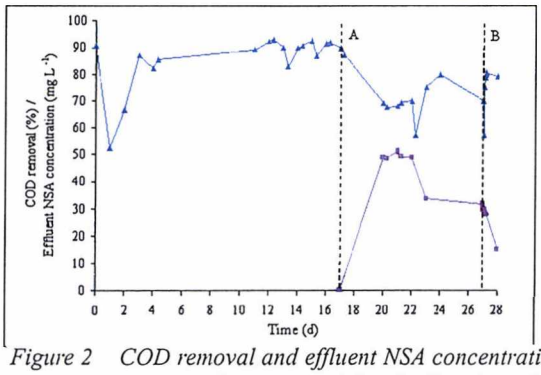


Figure 2 COD removal and effluent NSA concentration of a suspended floc reactor fed with CM after addition of NSA (A) and inoculation with RBN (B); ▲ -COD removal, ■ -effluent NSA concentration

equilibrate for 17 d. After 17 d, the reactor feed was supplemented with 50 mg L^{-1} of NSA and compound #21, a phenol sulphonic acid-based syntan, to provide a mixture of biogenic and recalcitrant organic compounds in the feed, as would be expected in an industrial effluent. Thereafter, the effluent was also sampled for NSA analysis by HPLC. The reactor was equilibrated for 10 days and inoculated with RBN. The feed, effluent and aeration tank liquor were sampled at hourly intervals for 6 hours, and then at 24h intervals, after inoculation. The COD removal (%) and NSA effluent concentrations are shown in Figure 2.

A mean COD removal of 85.9% (± 2.3%) was measured in the 17 d period after start-up of the reactor. The mean COD removal fell to 66.9% (± 1.7%) after the addition of NSA and compound #21 to the reactor feed. During this period, the NSA levels in the effluent rose to a mean concentration of 49.37 mg NSA L^{-1} (± 0.47 mg L^{-1}). Six days after the addition of NSA and compound #21, the mean COD removal had increased 77.5% and the mean NSA concentration in the effluent had fallen to 32.66 mg L^{-1}, indicating that the biomass had adapted to facilitate limited degradation of NSA. After inoculation of the reactor with RBN, the mean COD removal fell again to 74.4% (± 3.2%). The effluent NSA concentration fell during the 6h after inoculation and was reduced to 15.26 mg L^{-1} after 24h, indicating that addition of RBN to the reactor had enhanced the removal of NSA.

Gross COD removal may not give a true representation of the efficiency of effluent treatment in the reactor. The efficiency of the biological treatment of a complex effluent can be described by determining *k*, the maximum organic removal rate. The *k* values for the reactor were determined using the equation:

$k = Se(So-Se)/XvT$

where So = COD of the influent (mg L^{-1})

Se = COD of the effluent (mg L^{-1})

Xv = MLSS in the aeration tank (mg L^{-1})

T = HRT (d^{-1})

The calculated *k* values are shown in Table I. The addition of the recalcitrant organic compounds to the reactor feed led to fall in *k*, ie, a fall in the maximum organic removal rate. Augmentation of the biomass with RBN increased *k*, indicating that the addition of a metabolically competent organism had increased the biological treatment efficiency of the reactor biomass. This was concurrent with a reduction in the NSA concentration measured in the reactor effluent, suggesting that increase in *k* was, in part, due to the degradation of NSA by RBN.

The data show that inoculation with RBN improved the treatment efficiency (*k*) of the reactor, and that this improvement was due to degradation of NSA. Therefore, targeting a specific recalcitrant organic pollutant, NSA, by inoculation of the reactor with a NSA degrading isolate, RBN, was shown to be a successful strategy for improving the treatability of the feed. However, inoculation of the reactor with RBN did not return *k* to the value calculated prior to the addition of the recalcitrant compounds to the reactor feed. The relatively reduced value of *k* after inoculation may have been due to the presence of compound #21 in the effluent from the reactor. The reduced levels of COD removal (%) and reduced value of *k*, concurrent with the increased removal of NSA, before and after inoculation of the reactor, suggest that this compound was not degraded to any significant extent.

While it was possible to conclude empirically, from observed improvements in the total organic removal rate (*k*), that the inoculation of the biomass with RBN had improved the biological treatment of the NSA-containing effluent, it was impossible to draw any conclusions from these data on the fate or persistence of RBN. In order to examine its

Table I Maximum organic removal rate, *k*, for a suspended floc reactor fed with CM, after addition of NSA and compound #21, and inoculation with RBN

Reactor condition	k (d^{-1})
Naive biomass plus CM	39.41
Naive biomass plus CM plus recalcitrant organic compounds	15.62
RBN-augmented biomass CM plus recalcitrant organic compounds	20.30

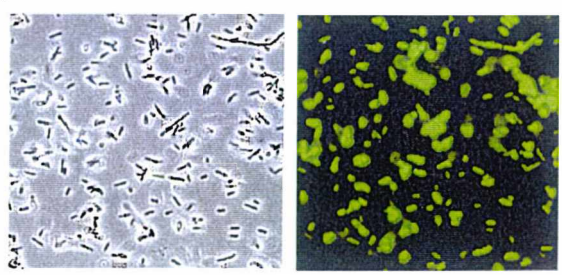


Figure 3 Fluorescence of bacteria marked with gfp viewed by normal phase contrast microscopy (left) and with UV illumination (right)

persistence when inoculated into a biomass, RBN was chromosomally marked with green fluorescent protein (*gfp*) by plate-mating with the donor strain *Escherichia coli* C118λpir. Bacteria expressing *gfp* will fluoresce under UV or blue light illumination (Figure 3). *Gfp* also provides a unique molecular marker which can be used to detect *gfp*-marked bacteria (Figure 4).

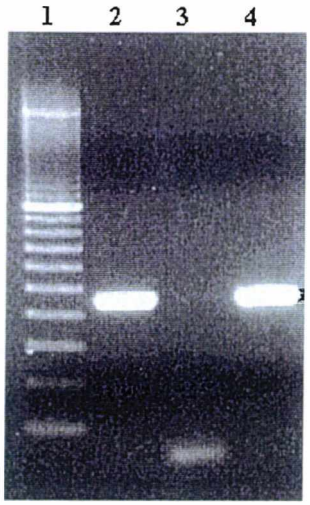


Figure 4 Molecular assessment of gfp-marked bacteria. Lanes: 1, molecular ladder; 2, gfp-marked RBN; 3, unmarked RBN; 4 gfp-marked Escherichia coli strain C118λpir

Gfp-marked RBN was inoculated into an activated sludge treating a feed of CM plus 50 mg NSA L^{-1}. Its survival was assessed by measurement of *gfp*. Plate-mating with *Escherichia coli* C118λpir also confered resistance to the antibiotic kanamycin on to RBN, and estimates of RBN numbers in the reactor were made by plate counts on agar containing kanamycin. Data for NSA removal, kanamycin-resistant cell counts and *gfp* in the treated activated sludge reactor are shown in figures 5, 6 and 7, respectively.

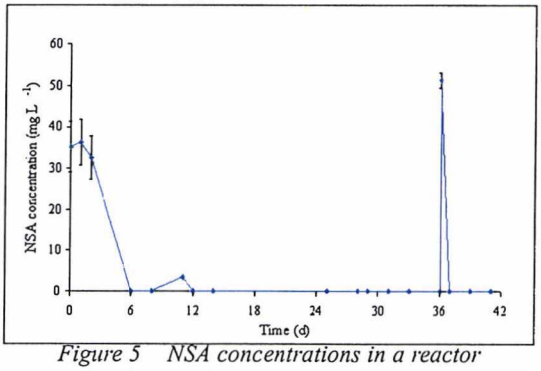


Figure 5 NSA concentrations in a reactor inoculated with gfp - marked RBN after 28 d and shock loaded with NSA after 36 d

The reactor was operated for 28 d before inoculation with RBN. The biomass in the reactor was able to degrade 100% of the added NSA prior to inoculation with RBN. Eight days (day 36) after inoculation with RBN, the reactor was given a shock load of NSA. As can be seen, the numbers of kanamycin-resistant cells and the amount of *gfp* measured in the reactor rose sharply, indicating a proliferation of RBN numbers in response to the shock load. The NSA concentration in the reactor effluent was reduced to undetectable levels 24h after the shock load. Conversely, no increases in kanamycin-resistant cell numbers and *gfp* were measured in a reactor inoculated with a strain of RBN which could not degrade NSA, and NSA remained detectable in the reactor effluent for 48h after the shock load (data not shown). The results show that RBN was able to persist after inoculation into an NSA-degrading reactor and increased the resistance of the reactor to shock loadings.

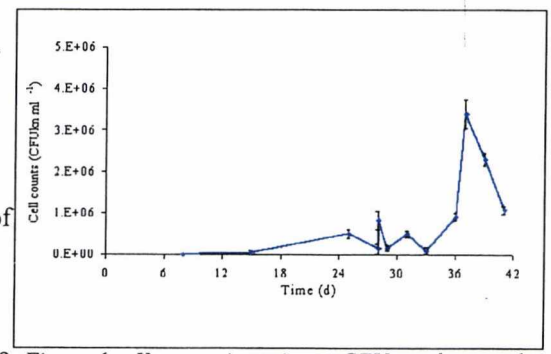


Figure 6 Kanamycin-resistant CFU numbers in the biomass from a reactor inoculated with gfp-marked RBN after 28 d and shock loaded with NSA after 36 d.

The success or failure of bioaugmentation depends on two conditions: the survival of the isolate and the demonstration of its degradative activity. Inoculation with RBN was shown to improve both the kinetics of NSA degradation, and NSA shock load resistance of activated sludges. RBN was also shown to persist in activated sludge regardless of its metabolic capacity for the degradation of the target compound. Therefore, these results show that the bioaugmentation of biological wastewater treatment system for the degradation of NSA was successful. It is possible then, that the degradation of other recalcitrant compounds in tannery effluents could be facilitated by bioaugmentation of biological wastewater treatment systems with specific microorganisms.

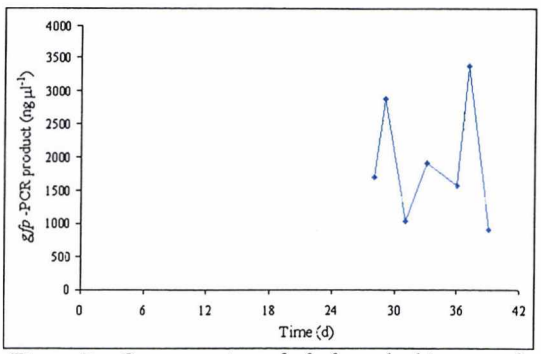


Figure 7 Concentration of gfp from the biomass of a reactor inoculated with gfp-marked RBN after 28 d and shock loaded with NSA after 36 d.

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