Biodegradation (2005) 16: 237–252

Biodegradation of naphthalene-2-sulfonic acid present in tannery wastewater by bacterial isolates *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC

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Accepted 23 June 2004

Key words: Arthrobacter, biodegradation, *Comamonas,* kinetics, naphthalene-2-sulfonic acid, tannery wastewater

Abstract

Two bacterial strains, 2AC and 4BC, both capable of utilizing naphthalene-2-sulfonic acid (2-NSA) as a sole source of carbon, were isolated from activated sludges previously exposed to tannery wastewater. Enrichments were carried out in mineral salt medium (MSM) with 2-NSA as the sole carbon source. 16S rDNA sequencing analysis indicated that 2AC is an *Arthrobacter* sp. and 4BC is a *Comamonas* sp. Within 33 h, both isolates degraded 100% of 2-NSA in MSM and also 2-NSA in non-sterile tannery wastewater. The yield coefficient was 0.33 g biomass dry weight per gram of 2-NSA. A conceptual model, which describes the aerobic transformation of organic matter, was used for interpreting the biodegradation kinetics of 2-NSA. The half-lives for 2-NSA, at initial concentrations of 100 and 500 mg/l in MSM, ranged from 20 h (2AC) to 26 h (4BC) with lag-phases of 8 h (2AC) and 12 h (4BC). The carbon balance indicates that 75–90% of the initial TOC (total organic carbon) was mineralized, 5–20% remained as DOC (dissolved organic carbon) and 3–10% was biomass carbon. The principal metabolite of 2-NSA biodegradation (in both MSM and tannery wastewater) produced by *Comamonas* sp. 4BC had a MW of 174 and accounted for the residual DOC (7.0–19.0% of the initial TOC and 66% of the remaining TOC). Three to ten percent of the initial TOC (33% of the remaining TOC) was associated with biomass. The metabolite was not detected when *Arthrobacter* sp. 2AC was used, and a lower residual DOC and biomass carbon were recorded. This suggests that the two strains may use different catabolic pathways for 2-NSA degradation. The rapid biodegradation of 2-NSA (100 mg/l) added to non-sterile tannery wastewater (total 2-NSA, 105 mg/l) when inoculated with either *Arthrobacter* 2AC or *Comamonas* 4BC showed that both strains were able to compete with the indigenous microorganisms and degrade 2-NSA even in the presence of alternate carbon sources (DOC in tannery wastewater = 91 mg/l). The results provide information useful for the rational design of bioreactors for tannery wastewater treatment.

Introduction

Aromatic sulfonates ($C–SO_3^-$) are produced in large quantities by the chemical industry and are widely applied in concrete finishing, industrial textile processing, tanning of hides, and in the manufacture of agrochemicals and pharmaceuticals (Riediker et al. 2000; Suter et al. 1999). The aquatic toxicity of aromatic sulfonates appears to be small and the risk of bioaccumulation is limited since the octanol–water partition coefficients (pK_{ow}) are typically <2 (Angelino et al. 1999; Chen et al. 2002; Greim et al. 1994). On the other hand, aromatic sulfonates are reported to exhibit

only limited biodegradability (Brilon et al. 1981; Wittich et al. 1988) and, as a result, would be expected to be discharged from wastewater treatment works into the aquatic environment (Alonso & Barceló 1999; Altenbach & Giger 1995). This makes them potentially hazardous with respect to contamination of groundwater and drinking water supplies (Reemtsma 1996).

There are many reports of the occurrence of aromatic sulfonates, especially naphthalenesulfonates, in waters, soils and sediments (Angelino et al. 1999; Reemtsma 1996). Benzene- and naphthalene sulfonates were identified in textile and tannery wastewaters (Klinkow et al. 1998; Reemtsma 1996; Song et al. 2003), river water (Alonso & Barceló 1999; Zerbinati et al. 1997), and in leachates and plumes from landfills (Riediker et al. 2000). Specifically, naphthalene-2-sulfonic acid (2-NSA), a major component of the condensation product of naphthalenesulfonic acid and formaldehyde (CNSF) and used as a retan agent, has been identified as a potential pollutant in effluent, soil and water (Song et al. 2003; Top et al. 2002; Zurrer et al. 1987).

The C–SO_3^- structure is highly polar and confers a high degree of stability to the aromatic ring (Brilon et al. 1981; Sheldon & Kochi 1981; Whitham 1995). Naphthalenesulfonates and their substituted analogs have been described as non-biodegradable (Zurrer et al. 1987) or poorly degradable (Angelino et al. 1999; Brilon et al. 1981; Reemtsma 1996; Stolz 1999). This may either be due to the molecular structure imparting resistance to degradation or because of direct toxicity to potential microbial degraders (Ruff et al. 1999). Although naphthalenesulfonates have a low acute toxicity (rat LD_{50}: 1400 mg/kg body weight) and show no mutagenic or carcinogenic effects, their discharge presents a risk because of their toxicity to fish (*Salmo gaidneri* LC_{50}: 100 mg/l) and other aquatic organisms (e.g. algae EC_{50}: 54.3 mg/l). The activity of unadapted (conventional) biological wastewater treatment plants may be inhibited during times of extreme peak loads (Greim et al. 1994).

The biodegradation of sulfonated compounds in wastewater has been studied (Reemtsma 1996) but most research has focused on surfactants (such as linear alkylbenzene sulfonate), that are common in municipal wastewaters (Altenbach & Giger 1995) and rapidly degraded (Perales et al. 1999; Zerbinati et al. 1997). However, there have been a few studies of the more hydrophilic naphthalenesulfonates with pK_{ow} values < 0.57 (Chen et al. 2002; Greim et al. 1994; Nörtemann et al. 1986; Zurrer et al. 1987). Several bacterial consortia are known to degrade naphthalenesulfonates (Nörtemann et al. 1986; Zurrer et al. 1987) but few individual species have been reported (Brilon et al. 1981; Stolz 1999; Wittich et al. 1988). Some green algae (e.g. *Chlamydomonas reinhardtii*) use NSA as a sulfur source (even in the presence of sulfate) but leave the naphthalene ring undegraded (Luther & Soeder 1987). Most xenobiotic organosulfonates which were examined as carbon or sulfur sources for the growth of aerobic bacteria, are subject to desulfonation (Ruff et al. 1999). Kertesz et al. (1994) and Cook et al. (1999) reviewed the varying stages at which the mechanism of microbial desulfonation of organosulfonates occurs, namely (I) before, (II) during or (III) after, ring cleavage. The type I mechanism involves destabilization of the C–SO_3^- bond by addition of an oxygen atom to the carbon, usually by a dioxygenase-catalysed reaction. This yields an unstable naphthalenesulfonate dihydrodiol (i.e. *cis*-1,2-dihydroxy-1,2-dihydro-2-naphthalene sulfonates) which then spontaneously rearomatizes to the corresponding catechol with the loss of the cleaving group, sulfite. This mechanism for reductive desulfonation of naphthalenesulfonate was described in *Pseudomonas testosteroni*, *Moraxella* and *Pseudomonas* sp. TA-2 species when sulfonates were provided as a carbon source (Brilon et al. 1981; Ohe et al. 1990; Wittich et al. 1988). A monooxygenolytic desulfonation of naphthalenesulfonates by *Pseudomonas putida* s-313 has been studied by Beil et al. (1996). Desulfonation of *ortho*-aminobenzene sulfonate by *Alcaligenes* sp., simultaneously with ring cleavage of the initial deamination product 3-sulfocatechol, is of type II mechanism (Thurnheer et al. 1990). Desulfonation after ring cleavage (type III) has been observed by Feigel & Knackmuss (1988) for *p*-aminobenzenesulfonic acid (sulfanilic acid). *Hydrogenophaga palleroni* and *Agrobacterium radiobacter* metabolize sulfanilic acid in a synthrophic association *via* 4-sulfocatechol and then *ortho*-cleavage of the aromatic ring to 3-sulfomuconate prior to desulfonation.

Inoculation of wastewater treatment systems with defined degradative microorganisms could be a suitable strategy in the removal of toxic or re-

calcitrant compounds (Bokhamy et al. 1997). However, the success of introduced microorganisms depends on their survival in competition with indigenous microorganisms and the expression of their degradative activity (Top et al. 2002).

The objectives of the present study were to: (A) isolate and identify microorganisms that degrade 2-NSA; (B) measure the biodegradation of 2-NSA when supplied as a sole carbon source in mineral salt medium (MSM); (C) investigate the survival and degradative capacity of the named isolates when added to a non-sterile tannery wastewater containing 2-NSA; and (D) evaluate the relationship between the removal of total organic carbon (TOC) and dissolved organic carbon (DOC) and the degradation of 2-NSA in tannery wastewater. This is the first report of the accelerated degradation of 2-NSA in tannery wastewater using defined bacterial inoculants.

Materials and methods

Chemicals

An UK tannery provided a condensation product of naphthalenesulfonic acid and formaldehyde (CNSF) commonly used as a synthetic tanning agent. All chemicals were analytical or equivalent grade and obtained from Sigma-Aldrich Co. Ltd (Gillingham, Dorset, UK) unless otherwise indicated. High performance liquid chromatography (HPLC)-grade solvents and 2-NSA ($C_{10}H_7SO_3Na$) were purchased from Fisher Scientific Co. Ltd. (Loughborough, UK). HPLC-grade water was prepared by purifying demineralized water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). The ion-pair reagents used to separate polar aromatic sulfonates were triethylamine (TEA) (Fisher Scientific UK Ltd, Loughborough, UK).

Tannery wastewater: properties and analysis

Wastewater samples were collected from a tannery in the UK, which uses CNSF in the preparation of hides, and stored at 4 °C. Solid phase extraction (SPE) of anionic compounds was performed as described by Song et al. (2003). COD (100–2000 mg/l) was measured with a spectrophotometer (*Dr. Lange* Lasa 50, Lange, Hampshire, UK) using LCK 514 cuvettes. BOD_5 (5-day biochemical oxygen demand) was measured using LCK555 cuvettes at concentration of 500 mg/l (using a standard inoculum supplied by Alcontrol, UK) (Song et al. 2001). TOC (the theoretical maximum amount of carbon that can be mineralized to carbon dioxide and water) and DOC (the carbon that may be most bioavailable for mineralization) was oxidized to carbon dioxide at 850 °C and measured by high temperature catalytic combustion using a Total Organic Carbon Infrared Analyser (DC-190, Rosemount Dohrmann Analytical Inc., Santa Clara, CA). TOC and DOC values were calculated from the difference between total carbon and inorganic carbon. The system was calibrated using potassium hydrogen phthalate (KHP, Sartec Group, Tenterden, UK). Prior to DOC analysis, samples were centrifuged at $1000 \times g$ for 10 min to remove solids. Due to the high sensitivity of the detector and the utilization of ultrapure water for preparation of solutions, TOC determination was accurate to within 0.1 mg/l C. Particulate organic carbon (POC) is defined as the difference between TOC and DOC. 2-NSA was quantified by a calibration plot and standard addition, and was present in the wastewater at 5.0 ± 0.5 mg/l. The contribution of 2-NSA in the tannery wastewater was 2–3% of the total COD and TOC and termed the hard (i.e. recalcitrant) COD (Song et al. 2003). This recalcitrant fraction cannot be removed by conventional wastewater treatment. Therefore, inoculation of wastewater treatment systems with defined degradative microorganisms should help to reduce hard COD discharges to surface waters and limit the accumulation of recalcitrant compounds in recycling treated effluent.

Sources of microbial inocula

Four sources of microbial inocula were used in enrichment experiments. Sludge 1 (S1) was a naïve sludge (i.e. one not previously exposed to tannery effluent) from a municipal sewage works. Sludge 2 (S2) was an activated sludge from an on-site conventional biological treatment plant at a tannery. Sludge 3 (S3) was an activated sludge from a membrane reactor used to treat tannery wastewater. Sludge 4 (S4) was an activated sludge from a municipal sewage works receiving tannery effluent. Inoculum, 20 ml, was centrifuged ($3000 \times g$, 10 min) and the supernatant fraction discarded.

The pellet was suspended in phosphate buffer (10 mM potassium phosphate, pH 7.0), centrifuged (13,000 × *g*, 10 min) and washed again. Phosphate buffer suspensions of the pellet (1% v/v) were used to inoculate enrichment cultures.

Growth media, enrichment cultures and culture storage

A MSM was used for all enrichments and biodegradation studies. It was prepared by the Tanner (1997) method but using 1 g/l of K_2HPO_4 as buffer (final pH 7.0). A 1 ml aliquot of inoculum was washed in PBS buffer (8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in 1 l) and introduced into 100 ml of MSM containing 500 mg/l CNSF (27% 2-NSA, Song et al. 2003). The culture was maintained at 25 °C on a rotary shaker (150 rpm). After 6–7 enrichment cycles (i.e. subculturing every 3–4 d), the dense culture broth was diluted (1 in 10) with sterile distilled water and spread onto MSM agar containing 2-NSA (100 mg/l) as the sole carbon source. After growth at 25 °C for 4–5 d, colonies were selected randomly, inoculated into 100 ml of fresh MSM with 2-NSA (100 mg/l) and incubated at 25 °C for 4 d. Four further subcultures were carried out.

Pure cultures were stored at −70 °C using bacterial preserver beads (Protect®, TSC Technical Service Consultants Ltd., Heywood, Lancashire, UK). Prior to each experiment, two beads were introduced into flasks containing MSM supplemented with 2-NSA and incubated at 25 °C. After 48 h cultures were resuspended in 5 ml PBS buffer (final $OD_{540\ nm} = 0.5$) and used to inoculate the experimental cultures.

DNA preparation, sequencing of PCR products and identification of bacterial isolates

Aliquots (2 ml) of 48 h cultures were centrifuged at 13,000 × *g* for 5 min. The total DNA was isolated from the pellets using a QIAGEN DNeasy® Tissue kit (QIAGEN GmbH, Hiden, US) in accordance with the manufacturers instructions.

The universal primers, Com1/Com2, were selected that targeted conserved regions of 16S rDNA coding sequences. The sequence of the upstream PCR primer (Com1), corresponding to nucleotides 519–536 according to *E. coli* position, is 5′-cagcagccgcggtaatac-3′ and that of the downstream primer (Com2), corresponding to nucleotides 907–926 of the *E. coli* sequence, is 5′-ccgtcaattcctttgagttt-3′. Genomic DNA, 1 μl, was used as a template for PCR amplification by means of Com1/Com2 primer set. For each template/primer pair combination, PCR amplification was performed in a total volume of 50 μl containing 1 μl of genomic DNA solution, 10 pmol of each primer, dNTPs (200 μM of each) (Roche Diagnostic Corporation, Indianapolis, USA), 1 U of Hotstar *Taq* DNA polymerase (QIAGEN GmbH, Hiden, US), 1 μl of bovine serum albumin (BSA), 5 μl of 1.0 mM $MgCl_2$ and 5 μl of 10 × reaction buffer (15 mM $MgCl_2$ pH 8.7).

Thermal cycling was performed on a Techne Elite thermal cycler with an initial denaturation at 95 °C for 4 min followed by 15 cycles consisting of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min, then 15 cycles of 94 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 1.5 min, and a single final extension at 72 °C for 4 min (Stach et al. 2001).

The purified PCR product was ligated into a pGEM-TEasy™ vector (Promega Corp. Madison, USA) according to the vender's instructions and transformed into *E. coli* JMP109 chemically competent cells (Promega Corp., USA). Plasmid DNA was purified from the transformants using a QIAprep® Spin Miniprep kit (QIAGEN GmbH, Hiden, US) following the manufacturers instructions and used as a template for direct 16S rDNA sequencing (MWG AG Biotech, Ebersberg, Germany). The resulting sequences were aligned with published sequences from the Genbank database using the basic local alignment search tool (BLAST) network service. Phylogenetic analysis and tree construction was performed using PAUP (Swofford 1999) using UPGMA and BootStrap functions as described by Stach et al. (2001).

Degradation of 2-NSA in MSM and added to tannery wastewater

A 1 ml aliquot of a 48-h preculture was added to 250-ml Erlenmeyer flasks containing 99 ml MSM plus 100 or 500 mg/l 2-NSA as a sole carbon source. The flasks were sealed with foam bungs and incubated on an orbital shaker platform at 120 rpm and 25 °C. Samples were taken at designated time intervals and centrifuged (10 min,

13,000 × *g*) to collect the supernatant fraction. Aliquots (2 ml) of the supernatant were kept at 4 °C prior to HPLC, TOC and DOC analysis. Microbial growth was assayed by measuring the optical density at 540 nm ($OD_{540\ nm}$) (Wittich et al. 1988) using a Unicam 5625 UV/vis spectrophotometer (Unicam, Cambridge, UK). Biomass dry weight (DW) was determined by centrifugation of the broth in a pre-weighed centrifuge tubes at 8000 × *g* for 30 min, washing the sediment with distilled water (twice), and drying at 80 °C to a constant weight.

In order to investigate the degradation of 2-NSA in tannery wastewater in the presence of additional carbon sources and the ability of the inoculated bacteria to compete successfully with indigenous microorganisms, the following treatments and controls were designed: (1) non-sterile and non-inoculated wastewater [designated TW]; (2) non-sterile and non-inoculated wastewater + 2-NSA (100 mg/l) [TW + NSA]; (3) non-sterile wastewater + NSA (100 mg/l) + 2AC or 4BC [TW + NSA + 2AC or 4BC]; (4) sterile and non-inoculated wastewater + 2-NSA (100 mg/l) [STW + NSA] and (5) sterile wastewater + 2-NSA (100 mg/l) + 2AC or 4BC [STW + NSA + 2AC or 4BC].

A 1 ml aliquot of the inoculants was added to 250-ml flasks containing 99 ml tannery wastewater. All treatments were incubated at 25 °C as described above. Samples were taken at designated time intervals for HPLC, TOC, DOC analysis and microbial enumeration. Colony forming units (CFUs) were counted by standard dilution plating techniques on R2A agar (Life Technologies, Paisley, UK). The plates were incubated at 37 °C for 72 h and those containing 20–200 CFUs were used for the calculation of microbial numbers. Inoculant concentrations were $1.7 \pm 0.17 \times 10^7$ cfu/ml for *Arthrobacter* sp. 2AC and $8.0 \pm 0.17 \times 10^6$ cfu/ml for *Comamonas* sp. 4BC. All experiments were performed in triplicate.

Kinetic modelling

Microbial growth models, based on those of Monod, have been studied by many (Hirata et al. 2000; Peyton et al. 2002). However, they have generally not described biodegradation and have not considered the influence of the initial substrate concentration on the growth rate. Furthermore, Monod's model does not take full account of all phases of microbial growth (i.e. the lag phase and the death phase). More specifically, there is little kinetic information concerning naphthalenesulfonate biodegradation. In this study, a degradation kinetic model proposed by Quiroga et al. (1999) was used to fit the experiment data and predict the biodegradation of 2-NSA.

In this model, substrate concentration (S) varies with time (t) according to the following equation:

$$S = \frac{h \cdot (S_0 - q) - q \cdot (S_0 - h) \cdot e^{p \cdot t}}{(S_0 - q) - (S_0 - h) \cdot e^{p \cdot t}}, \quad (1)$$

S_0 is the initial concentration of the 2-NSA (mg/l); S is the concentration of the 2-NSA at the instant t (mg/l); t is the degradation time in hours.

The derived regression parameters, p, q and h (Perales et al. 1999; Quiroga et al. 1999), are defined as:

$$p = \sqrt{K_1^2 - 4 \cdot K_2 \cdot K_0},$$

$$q = \frac{(-K_1 + p)}{2 \cdot K_2},$$

$$h = \frac{(-K_1 - p)}{2 \cdot K_2},$$

where p is the substrate degradation rate (h^{-1}), i.e. the velocity or the rate at which the process takes place; h is the maximum quantity of substrate available to form biomass and includes a measure relating to the initial microorganism concentration (mg/l) and q is the level of non-biodegradable substrate (mg/l). K_2 is the coefficient of S^2 in the second degree polynomial, K_1 is the coefficient of S in the second degree polynomial and K_0 is an independent term of the second degree polynomial.

To predict biodegradation rate constants, non-linear regression (SigmaPlot for Windows® V1.02) was used to fit kinetic parameters to the experimental data.

HPLC-UV-ESI-MS and determination of 2-NSA concentrations

High-pressure liquid chromatograph (HPLC) (Agilent 1100), with a variable-wavelength UV detector and a electrospray ionization mass spectrometry (ESI-MS) detector (LCQ™, Thermo-Finnigan Ltd, Herts, UK), was used to identify the

sulfonic compounds. Chromatographic and MS conditions were as described by Song et al. (2003).

2-NSA analysis was conducted with an HPLC system (Kontron instruments, Watford, UK) equipped with an UV Diode Array Detector (UV-DAD) 440, Autosampler 465, gradient pump and SP 4600 Data Integrator. Data storage and quantification was carried out using KromaSystem 2000 software (Kontron instruments, Watford, UK). A 250 × 4.6 mm Econosi C-18 (5 μm) reverse-phase column (Alltech Associates Applied Science Ltd, Carnforth, UK) was employed for the separation. An isocratic mobile phase was used consisting of methanol: H_2O:orthophosphoric acid (50:49.7:0.3). The injection volume was 20 μl and the flow rate was 0.6 ml/min; column temperature was maintained at 35 °C. UV-DAD was set at the range of 200–400 nm and elution was monitored at 280 nm. Negative ion (NI) ESI-MS mass spectrum of the peak confirmed the presence of 2-NSA with the parent ion at m/z 207 and a [SO_3^-] radical at $m/z = 80$ (Song et al. 2003). A standard curve was determined by plotting the peak area obtained from the integrator versus known standard concentrations of 2-NSA injected.

Results and discussion

Identification of bacteria

After many subcultures, two microbial strains, designated 2AC and 4BC, were isolated from sludges S2 and S4, respectively. Both isolates produced round and smooth colonies on MSM agar. After 96 h at 25 °C, 2AC colonies were 1 mm in diameter and cream in colour while 4BC colonies were <0.5 mm diameter and white. Microscopic observations of Gram-stained cultures (both 0.8–2.0 μm long × 0.2–0.5 μm wide) showed that strain 2AC was Gram-variable and strain 4BC was Gram-negative.

The isolates were further characterized by 16S rDNA sequence analysis. Partial sequences of the 16S rDNA from strains 2AC (551 nucleotides) and 4BC (541 nucleotides) were found to show a high degree of similarity ($>90\%$) with the gene sequences of the genera *Arthrobacter* and *Comamonas*, respectively. Figure 1 shows the two 2-NSA degrading isolates located among *Arthrobacter* (2AC) and *Comamonas* (4BC) type strains in a phylogenetic dendrogram based on 16S rDNA gene sequence. *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC showed the highest degree of similarity to *Arthrobacter globiformis* (99%) and *Comamonas testosteroni* (96%), respectively. A comparison of the two strains, based on 16S rDNA homology for the region amplified, indicated that they were not closely taxonomically related to each other (~10%). The sequence from a 2-NSA degrading strain, *sphingomonas* sp. BN6, showed 96% identity with all type-strains of *Sphingomonas yanoikuyae* as reported by Stolz (1999). There have been reports of *Pseudomonas testosteroni* A3 degrading 2-NSA (Brilon et al. 1981; Stolz 1999) but 2-NSA degrading *Arthrobacter* species have not been described previously.

Biodegradation of 2-NSA by Arthrobacter *2AC and* Comamonas *4BC*

Bacterial growth and rates of degradation of 2-NSA by *Arthrobacter* 2AC and *Comamonas* 4BC were examined under aerobic conditions. The results using two different initial concentrations of 2-NSA (100 and 500 mg/l) are shown in Figures 4–6. The growth of the two strains during the period of 5 d began with a lag phase (8–12 h) followed by a rapid exponential phase and decline phase (Figure 2). We observed that in the second phase of substrate degradation, $OD_{540\ nm}$ increased to 0.18 (100 mg/l 2-NSA) and 0.48 (500 mg/l 2-NSA), then decreased when the substrates were degraded due to floc formation. There is close correlation ($r > 0.95$) between the microbial growth phase and 2-NSA degradation (Figures 2 and 3). The biomass concentration was also determined spectrophotometrically by reading the culture absorbance at 540 nm. A calibration curve established the following dependence between the optical density ($OD_{540\ nm}$) and the dry biomass concentration (DW, mg/l): $DW = 327.2 \times OD_{540\ nm}$ ($r^2 = 0.972$).

Arthrobacter sp. 2AC had the shorter lag phase (8 h) and the subsequent rate of 2-NSA degradation was relatively low, with 49.1 ± 5.7% (100 mg/l) and 23.1 ± 2.1% (500 mg/l) reductions after 24 h (Figures 4A and 5A). 2-NSA degradation by *Comamonas* sp. 4BC began after a 12 h lag phase but degradation after 24 h was much higher (80.1 ± 6.1%, 100 mg/l) than or similar (27.3 ± 5.7%,

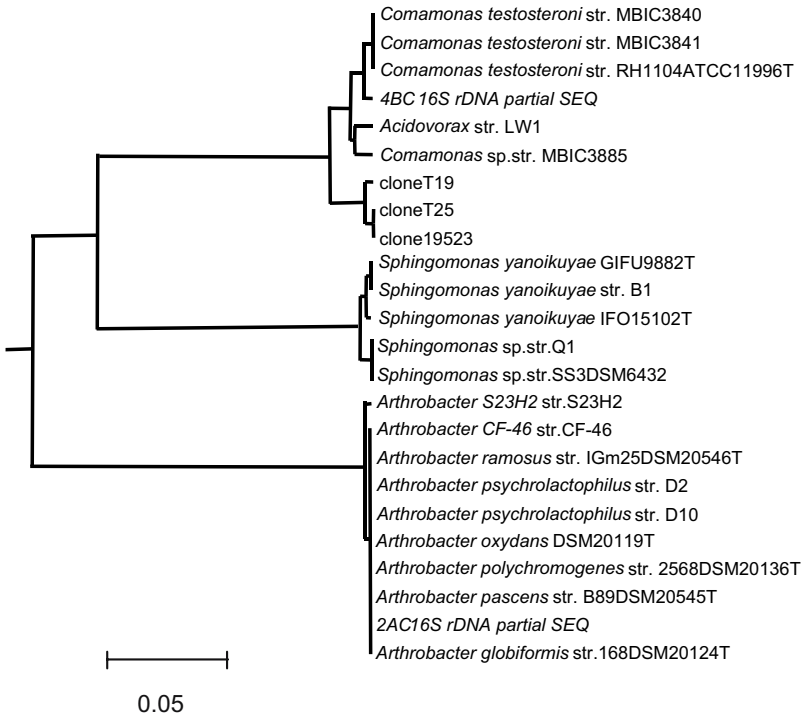

Figure 1. Cluster analysis showing the phylogenetic relationship of the two NSA degraders 2AC and 4BC (bold) derived from rDNA and based on 16S rDNA gene sequence analysis. The scale bar represents 5% difference in nucleotide sequences.

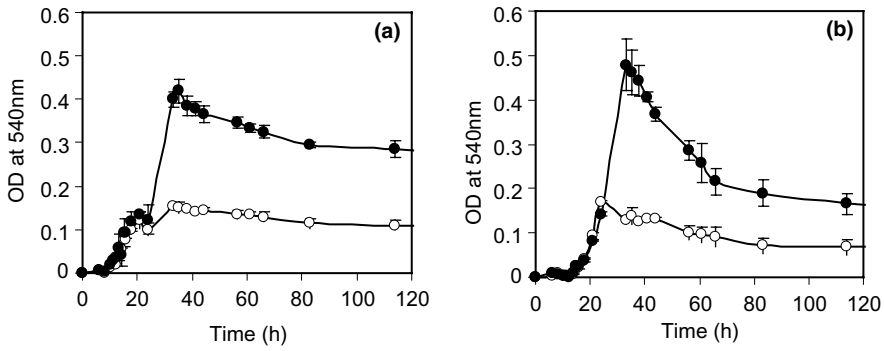

Figure 2. Growth of (A) *Arthrobacter* sp. 2AC and (B) *Comamonas* sp. 4BC in MSM containing 100 mg/l (○) or 500 mg/l (•) 2-NSA. (The data are means ± standard deviation for triplicate incubations. When the error bar is not visible it is within the data point).

500mg/l) to *Arthrobacter* sp. 2AC (Figures 4B and 5B). The length of time required for *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC to achieve a maximal rate of biodegradation was independent of the initial 2-NSA concentration in the range tested. The rate of degradation, excluding the lag phase (8 h for 2AC and 12 h for 4BC), was similar for both strains and increased with an increase in the initial 2-NSA concentration (14.7 ± 0.4 mmol/l/h, 100 mg/l; 72.8 ± 0.5 mmol/l/h , 500 mg/l). By the end of the experiment, 100% of 2-NSA was degraded and the biomass concentration was determined at 34.2 mg_{DW}/l (100 mg/l 2-NSA) and 157.0 mg_{DW}/l (500 mg/l 2-NSA). The biomass yield coefficient averaged 0.33 ± 0.02 g of biomass dry weight per gram of 2-NSA.

For modelling purposes, the initial 2-NSA concentration was the measured average value of

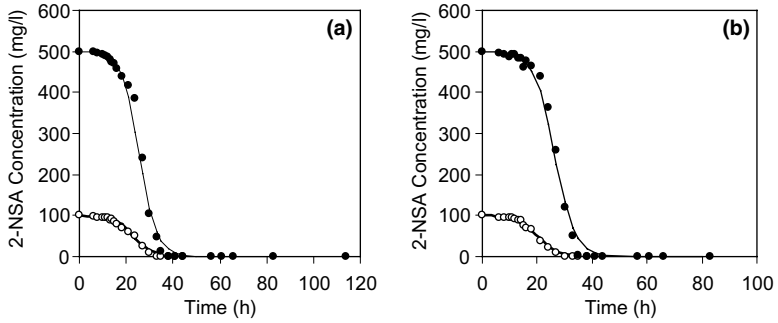

Figure 3. Biodegradation kinetics of 100 mg/l (○) or 500 mg/l (•) 2-NSA in MSM by (A) *Arthrobacter* sp. 2AC and (B) *Comamonas* sp. 4BC. Data points represent average measurement values for triplicate flasks, and each line is the theoretical curve derived from the Quiroga model (see Equation (1)).

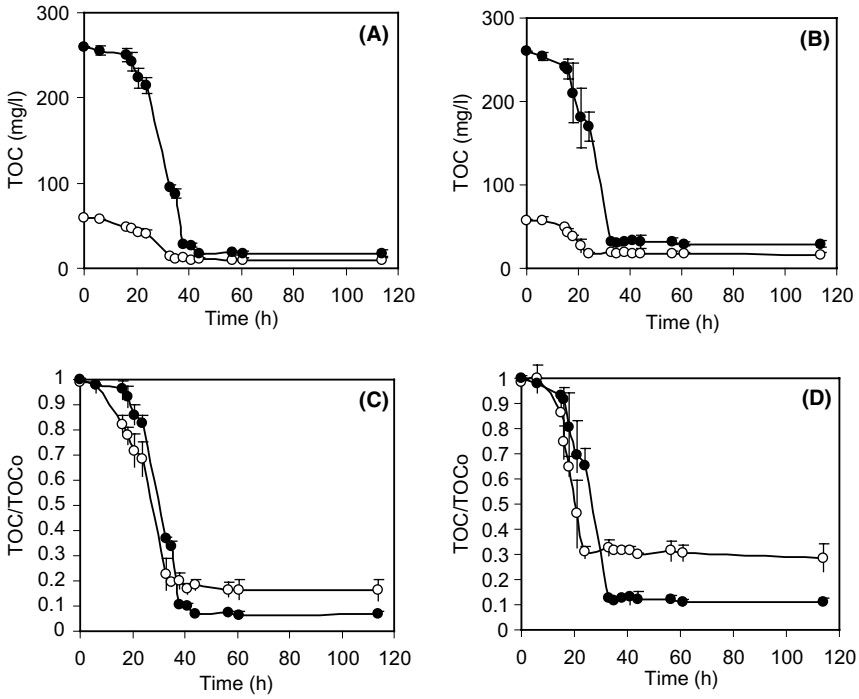

Figure 4. Reduction in TOC and the TOC/TOC_0 ratio in treatment of 100 mg/l (○) or 500 mg/l (•) 2-NSA in MSM by (A) and (C) *Arthrobacter* sp. 2AC, (B) and (D) *Comamonas* sp. 4BC; A and B show the relationship of TOC reduction to incubation time, and C and D show the relationship of TOC reduction ratio to time. (The data are means ± standard deviation for triplicate incubations. When the error bar is not visible it is within the data point.)

the non-inoculated control. The experimental data were fitted to the kinetic model (Equation 1) and are shown as solid lines in Figure 3. Kinetic parameters were estimated by simultaneously fitting the model to measured values of 2-NSA concentrations over time using a non-linear regression procedure (SigmaPlot for Windows® V1.02) (Table 1).

The agreement between the data and the modelled curve (Figure 3) is very good (see correlation coefficient values in Table 1). The lowest r^2 value for any experiment was 0.942, with most being >0.96. Similar 2-NSA biodegradation kinetics were noted for both *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC. The lag phase was the same for both 2-NSA concentrations and this

suggests there was no substrate inhibition during the degradation. However, with initial 2-NSA concentrations > 500 mg/l, lag phases of 40 h were recorded for unadapted bacteria.

Table 1 summarizes the kinetic parameters for the model (Equation (1)). The calculated values of h in the biodegradation experiment are close to the initial 2-NSA concentrations. Parameter q, which represents the concentration of non-biodegradable substrate, was close to zero, which confirms that all the 2-NSA is biodegradable. The substrate degradation rates (p) for the two strains were similar ($r = 0.98$) with a difference of $< 0.02\ h^{-1}$ at both initial 2-NSA concentrations of 100 and 500 mg/l. The parameter p for each strain increased with the increase of initial 2-NSA concentrations and ranged from 0.239 to $0.271\ h^{-1}$.

Rearrangement of Equation 1 enables calculation of the time taken for the residual 2-NSA concentration to fall to half its initial value (S_0), and these calculated half-lives are given in Table 1. The half-lives for 2-NSA at initial concentrations of 100 and 500 mg/l ranged from 20 to 26 h.

2-NSA concentrations of 2000 and 5000 mg/l depressed degradation rates by both isolates, and even after 96 h only 33% (33.1 ± 0.5 mmol/l/h) and 25% (62.6 mmol/l/h) of 2-NSA was degraded. This suggests that at initial concentrations of 2000 mg/l and greater, the 2-NSA (or its metabolites) may be directly toxic to the inoculants or repress the expression of the catabolic pathway. Stolz (1999) reported that the optimum substrate concentration for 2-NSA degradation by *Sphingomonas* sp. BN6 (originally identified as a *Pseudomonas* sp.) (Nörtemann et al. 1986) was ≤1mM (~200 mg/l). Above this concentration, metabolites (such as salicylate and salicylaldehyde) accumulated to toxic levels. Brilon et al. (1981) reported that 2-NSA (1 mM) was totally degraded by *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22 *via* 1,2-dihydrooxynaphthalene after oxygenolytic cleavage of the carbon–sulfur bond (i.e. 1,2-dioxygenation), subsequent spontaneous elimination of sulfite (which is readily oxidized to sulfate) and, finally, all organic sulfur was converted to sulfate.

Brilon et al. (1981) also reported that *Pseudomonas* sp. A3 and *Pseudomonas* sp. C22 can degrade 1-NSA after 2-NSA is depleted in a culture containing both 2-NSA and 1-NSA. However, in our experiments, 2-NSA-grown cells of *Arthrobacter* 2AC and *Comamonas* 4BC did not utilize 1-NSA as the sole source of carbon even after a prolonged adaption period (Wittich et al. 1988). This suggests that both *Arthrobacter* 2AC and *Comamonas* 4BC are nutritionally fastidious and cannot grow on 1-naphthalenesulfonic acids.

Reduction of TOC during 2-NSA degradation by Arthrobacter *2AC and* Comamonas *4BC*

The reduction in TOC is closely related to the biodegradation of 2-NSA and could be used as an index of the biodegradation. Figure 4 shows the relationship between 2-NSA biodegradation (initial concentrations, 100 and 500 mg/l) and TOC (52 and 260 mg/l). TOC/TOC_0 is the ratio of remaining TOC to initial TOC (TOC_0). However, by comparing Figure 3 with Figure 4, it can be seen that the percentage reduction of TOC was always less than that of the reduction in 2-NSA concentration for both *Arthrobacter* 2AC and *Comamonas* 4BC. Thus, Figure 4 shows that both strains reduced TOC by 75% (± 5%) and 90% (± 2.5%) at initial 2-NSA concentrations of 100 and 500 mg/l, respectively, while 2-NSA was 100% degraded in both cases. Even after 120 h there is a TOC of 10–30 mg/l at initial 2-NSA concentrations of 100 and

Table 1. Kinetic parameters obtained using the Quiroga et al. (1999) model which best fits the experimental data for 2-NSA biodegradation

Parameters	*Arthrobacter* sp. 2AC		*Comamonas* sp. 4BC	
2-NSA (mg/l)	100	500	100	500
p (h^{-1})	0.239	0.269	0.252	0.271
q (mg/l)	0.121	0.498	0.112	0.532
h (mg/l)	100.39	500.51	100.68	500.41
r^2	0.978	0.983	0.966	0.942
$t_{1/2}$ (h)	23	25.5	20	26

500 mg/l. Table 2 shows the carbon mass balance at the end of the experiment. TOC concentrations in the culture containing *Arthrobacter* sp. 2AC (6.8–16.6% of the initial TOC) were lower than those containing *Comamonas* sp. 4BC (11.1–28.5%) at both 2-NSA concentrations. The results revealed that the proportion of carbon in biomass (biomass carbon) is approximately 33% of the remaining TOC (3–10% of the initial TOC) and DOC accounted for the remainder (5–19% of the initial TOC). The residual DOC may be due to 2-NSA metabolites (such as catechol), which were not detected by HPLC-UV using the standard conditions. The mechanism for the complete biodegradation of 2-NSA is complex since there are many different steps and intermediates (Nörtemann et al. 1986; Stolz 1999). In fact, the degradation of intermediates may require additional microbial species and, unless these are all present and active, the complete elimination of TOC may not be achieved.

A correlation analysis of the data points from Figures 2 and 4 shows that the average correlation between the reduction of TOC and microbial growth, for both *Arthrobacter* 2AC and *Comamonas* 4BC, was $r = 0.93 \pm 0.05$. This indicates that, not only does the initial 2-NSA structural breakdown (i.e. the cleavage of naphthalene and benzene rings) supply carbon and energy for microbial growth, but so also does further mineralization to CO_2.

MS analysis of by-products of 2-NSA biodegradation

In order to identify the degradation products responsible for the residual DOC, MS-MS analyses were carried out. Figure 5 shows the mass spectra of 2-NSA (MW 208, initial concentration 500 mg/l) and the by-products at various stages of degradation by *Comamonas* 4BC. The base peak at m/z 207 is the parent ion $[M–H]^-$ while $m/z = 143$ and 80 in the mass spectra are fragment ions $[M–SO_2H]^-$ and $[SO_3]^-$ (Figure 5A). Several new product ions in a treated sample (after 21 h degradation) are shown in Figure 5B. Those with ions of $m/z = 241$ and 239 (intensity, 30% and 15%, respectively) have a MW increase of 34 and 32 Da compared with the parent ion $[M–H]^-$ (m/z 207). This suggests that they are formed by oxidation of 2-NSA and the introduction of two hydroxyl groups (m/z 34) into the aromatic ring and/or on the sulfonate (m/z 80) bonded to the ring, resulting in production of sulfono *cis*-diol. The parent ion at m/z 241 corresponds to that of 1,2-dihydroxy-1,2-dihydro-2-naphthalenesulfonates. The formation of such hydroxylated by-products in the NSA degradation pathway by *Pseudomonas* sp. and *Sphingomonas* sp. has been proposed previously (Nörtemann et al. 1986; Ohe et al. 1990; Stolz 1999). Therefore, we suggest that *Comamonas* 4BC initially attacked 2-NSA by a selective 1,2-dioxygenase resulting in desulfonation and further metabolism of the desulfonated product, 1,2-dihydroxy-1,2-dihydronaphthalenesulfonate. The sulfite is spontaneously eliminated with formation of 1,2-dihydroxy-1,2-dihydronaphthalene. This is analogous to the known degradation pathway of naphthalene (Brilon et al. 1981; Wittich et al. 1988).

The presence in the mass spectrum of a by-product with an ion of $m/z = 185$ (intensity, 60%), which is 22 Da lower than 2-NSA, indicates that it

Table 2. Carbon balance at the end of treatment of 100 and 500 mg/l 2-NSA in MSM by *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC

Inoculants	Parameter (s)	% of the initial TOC (=DOC)	
		NSA 100 mg/l (TOC 52 mg/l)	NSA 500 mg/l (TOC 260 mg/l)
Arthrobacter sp. 2AC	TOC	16.6 ± 4.2	6.8 ± 1.4
	DOC	11.1 ± 2.8	4.8 ± 0.3
	Biomass carbon	5.5 ± 1.4	2.5 ± 1.1
Comamonas sp. 4BC	TOC	28.5 ± 5.3	11.1 ± 1.4
	DOC	19.0 ± 3.5	7.4 ± 1.0
	Biomass carbon	9.5 ± 1.7	3.7 ± 0.5

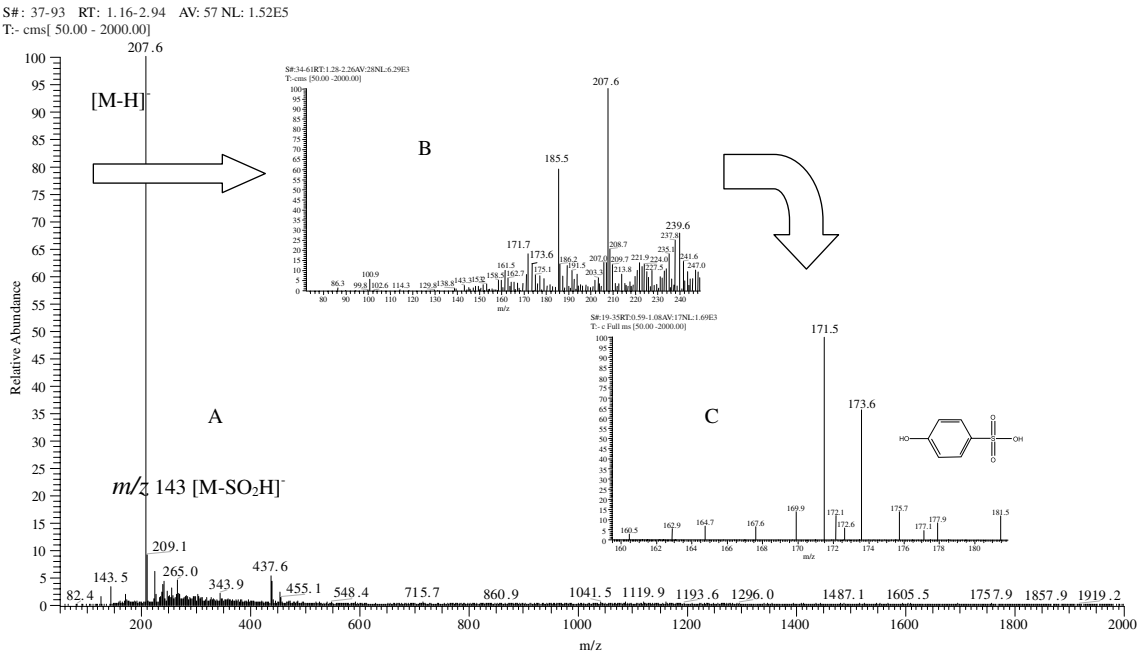

Figure 5. Negative ion ESI-MS mass spectra of 2-NSA and its by-products after degradation by *Comamonas* sp. 4BC: (A) 2-NSA (control); (B) after 21 h; and (C) after 66 h.

arises from some structural breakdown of the parent molecule. However, the structure of this by-product is unclear and subject to further investigation. As shown in Figure 5C, by-products with ions of $m/z = 185$, 239 and 241 (Figure 5B) undergo further degradation that lead to their complete disappearance, confirming that they are unstable intermediates. In the spectrum of the degraded 2-NSA, the molecular peak at 207 is almost indistinguishable from the background, whereas several peaks at lower masses are much more intense. It can be concluded that almost all the original 2-NSA has undergone chemical modification and that degradation has involved several stages.

The by-product with an ion of m/z 171, 173, and 174 is believed to be the end product of 2-NSA biodegradation since it is the sole product detected by LC-MS in the treated sample. This product must, therefore, be responsible for much of the remaining DOC (10–20 mg/l). The fact that product ions varied by 2 Da may be due to the degree of unsaturation of the substituent (Mascolo et al. 2001). The structure of this end product may be similar to phenolsulfonic acid (MW 174). However, it should be pointed out that at longer degradation times (i.e. >33 h) no by-products were detected using HPLC-UV. This is likely to be due to either aromatic ring cleavage or products at a concentration lower than the HPLC-UV limit (i.e. <1 mg/l). As discussed, it may be that by-products were formed sequentially rather than simultaneously (Figure 5). By-products with ions of $m/z = 185$ and 173 were not present in the proposed naphthalene and NSA catabolic pathway reported by Brilon et al. (1981).

In contrast to the hydroxylated by-products of 2-NSA generated by *Comamonas* sp. 4BC, there were no such by-products detected in the medium containing *Arthrobacter* 2AC. The remaining DOC (6–12 mg/l) was less than that in the medium containing *Comamonas* sp. 4BC (10–20 mg/l). This suggests that *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC have different catabolic pathways. The two strains do not degrade 1-NSA although it is widely regarded as having a similar metabolic pathway to 2-NSA (Brilon et al. 1981; Nörtemann et al. 1986; Ohe et al. 1990; Stolz 1999). This suggests that the initial attack on the naphthalenesulfonates is catalysed by highly specific enzymes.

Survival and activity of Arthrobacter *sp. 2AC and* Comamonas *sp. 4BC during the biodegradation of 2-NSA in tannery wastewater*

Biodegradation of 2-NSA in MSM by both *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC shows the potential application of these isolates to wastewater treatment. It is known that when microorganisms are provided with a mixture of possible energy sources (as in wastewater), many will degrade the lower molecular weight naturally occurring substrates in preference to the more complex xenobiotics. This is particularly true when carbon is in excess and easily degradable substrates are preferentially utilized (Egli 1995; Alexander 1999). At the very least, the presence of additional potential substrates may substantially alter the kinetics of mineralization of the target organic chemical (Scow et al. 1989). For example, addition of small amounts of sucrose had a negative influence on the anaerobic pre-treatment of petrochemical effluents containing terephthalate (Kleerebezem et al. 1997). Swindoll et al. (1988) observed that addition of easily degradable carbon sources, such as glucose, inhibited aerobic mineralization of phenol and toluene in subsurface soil samples. Glucose at high (3 g/l) concentrations was shown to inhibit phenanthrene mineralization in batch cultures of *Arthrobacter polychromogenes* (Keuth & Rehm 1991).

As described previously, 2-NSA is present in the tannery wastewater at a concentration of 5 mg/l. It is assumed that tannery wastewater contains a mixture of potential carbon and energy sources (it has a TOC of 110 mg/l and a DOC of 91 mg/l). If there is preferential metabolism of these carbon sources by either the indigenous bacteria or the inoculants, biodegradation of the 2-NSA will be reduced. In order to determine whether *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC degraded the target 2-NSA in tannery wastewater and to examine the growth of the two inoculants when in competition with indigenous bacteria and in the presence of alternative carbon sources, sterile and non-sterile tannery wastewater was used, spiked with 2-NSA and inoculated with *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC. The results are presented in Figures 6 and 7.

Bacterial counts on R2A plates show that the total indigenous population in tannery wastewater at the start of the experiment was $1.2 \pm 0.19 \times 10^6$ cfu/ml. This is likely to be an underestimate as inoculation volume was 1 ml: $1.7 \pm 0.17 \times 10^5$ cfu/ml for *Arthrobacter* sp. 2AC and $0.8 \pm 0.15 \times 10^5$ cfu/ml for *Comamonas* sp. 4BC. Thus, total microbes in inoculated flasks were $1.37 \pm 0.20 \times 10^6$ cfu/ml (*Arthrobacter* sp. 2AC) and $1.28 \pm 0.11 \times 10^6$ cfu/ml (*Comamonas* sp. 4BC) which represented less than 8% and 11%, respectively, of the total initial microbial population.

No degradation of 2-NSA took place in the sterile non-inoculated control [STW + NSA]. In the non-sterile and non-inoculated treatment [TW + NSA], the indigenous microbes, after a prolonged lag phase (~56 h), had degraded all the 2-NSA by 80 h (Figure 6). During the lag phase, no significant increase of the microbial numbers occurred and no 2-NSA degradation took place. The microbial population had increased 4.6-fold to 5.5×10^6 cfu/ml by 80 h (Figure 7).

In the non-sterile treatments inoculated with *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC

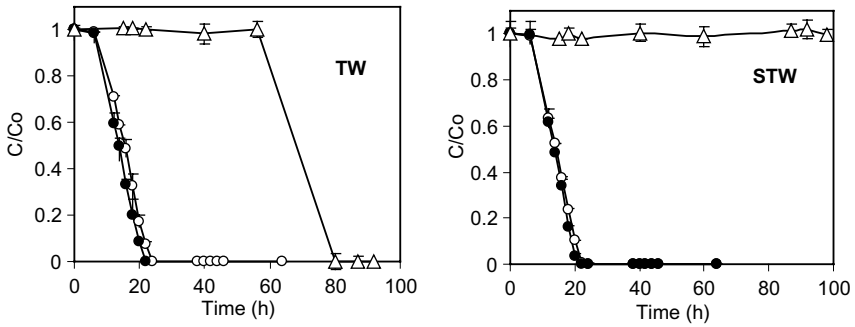

Figure 6. Biodegradation of 2-NSA (100 mg/l) added to non-sterile [TW] and sterile tannery wastewater [STW] by *Arthrobacter* sp. 2AC (○), *Comamonas* sp. 4BC (•) and control (Δ, non-inoculated). (The data are means ± standard deviation for triplicate incubations. When the error bar is not visible it is within the data point.)

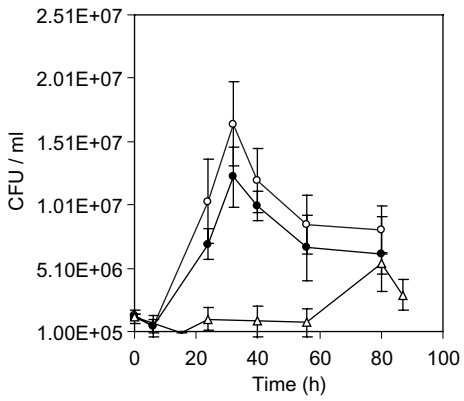

Figure 7. Total bacterial numbers (indigenous plus inoculated) in non-sterile wastewater containing 2-NSA (100 mg/l) inoculated with *Arthrobacter* sp. 2AC (○) or *Comamonas* sp. 4BC (•). Non-inoculated wastewater (Δ) contains only indigenous bacteria (The data are means ± standard deviation for triplicate incubations).

[TW + NSA + 2AC, TW + NSA + 4BC], both strains began degrading 2-NSA after a lag phase of 10 h and there was no 2-NSA remaining after 24 h. Total bacterial counts (i.e. indigenous + inoculant) in these treatments after 24 h were: TW + NSA + 2AC = 1.1 ± 0.10 × 10^7 ml^{-1} and TW + NSA + 4BC = 0.70 ± 0.12 × 10^7 ml^{-1}. These values doubled between 24 and 32 h when they were approximately 10-fold higher than the initial total numbers. The increase in numbers after all 2-NSA was degraded is probably the result of growth on 2-NSA degradation products. Comparisons of numbers in TW + NSA + 2AC, TW + NSA + 4BC treatments with those in non-inoculated treatments [TW + NSA] shows that there was a significant ($p = 0.001$) increase. Although we were unable to grow *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC on selective media and count them independently of the indigenous population (attempts to introduce reporter genes are continuing), we estimated the population of the introduced strains by calculating the difference between the total counts in inoculated [TW + NSA + 2AC, TW + NSA + 4BC] and non-inoculated [TW + NSA] treatments (Figure 7) after 32 h. On this basis *Arthrobacter* sp. 2AC (1.56 × 10^7 cfu/ml) and *Comamonas* sp. 4BC (1.14 × 10^7 cfu/ml) had significantly increased from the initial inoculum of 1.7 × 10^5 cfu/ml (*Arthrobacter* sp. 2AC) and 0.8 × 10^5 cfu/ml (*Comamonas* sp. 4BC). Therefore, it is concluded that both inoculants survived and proliferated in the presence of the indigenous microbial species and retained their degradative activity.

The lag phases (10 h) and degradation rates of 2-NSA by *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC in spiked non-sterile wastewater [TW + NSA + 2AC, TW + NSA + 4BC] were similar to those in sterile tannery wastewater [STW + NSA + 2AC, STW + NSA + 4BC] suggesting that competition and production from the indigenous population was minimal (Figure 6). The results also demonstrate that alternate forms of carbon (including those that may be solubilized by autoclaving) are not used preferentially by the inoculated bacteria and have no effect on the rate and extent of 2-NSA metabolism. In other words, the defined strains are capable of degrading 2-NSA despite the presence of potential alternate carbon and energy sources (DOC in wastewater = 91 mg/l) and large numbers of other microorganisms.

COD, TOC and DOC values of the untreated tannery wastewater were 705 ± 15, 110 ± 5.0 and 91 ± 2.0 mg/l, respectively. Table 3 shows the removal of TOC and DOC from the tannery wastewater (with or without added 2-NSA) by *Arthrobacter* sp. 2AC, *Comamonas* sp. 4BC, and indigenous bacteria after 32 h. In the non-sterile tannery wastewater control [TW], 82.7% of the TOC (110.1 mg/l) was water-soluble (DOC = 91 mg/l) but only 9.2–9.8% of the TOC and 1.1–2.1% of DOC was removed by indigenous bacteria.

When the non-sterile tannery wastewater was spiked with 100 mg/l of 2-NSA [TW + NSA], the DOC/TOC ratio increased from 0.82 to 0.90 due to the 2-NSA which represented 36.2% (52 mg/l) of the total DOC (143.7 mg/l). After 32 h incubation, TOC was reduced by less than 10% of which only 1.1% was due to the decline in DOC. In fact, the reduction in TOC was due mostly to the reduction of the POC. After 80 h of incubation, all of the original and added 2-NSA was degraded and there was a 28% reduction in TOC.

The complete biodegradation of 2-NSA in the non-sterile spiked and inoculated wastewater [TW + NSA + 2AC, TW + NSA + 4BC] was accompanied by a 31–35% decline in TOC, of which 85% was due to the reduction in DOC. The DOC of the added soluble 2-NSA (100 mg/l) is the same as its TOC (52 mg/l), and this, together with the 2-NSA already in the wastewater, accounts for 33 ± 0.3% of the total TOC (165.8 mg/l). Therefore, the measured reduction in TOC must be due largely to

Table 3. TOC and DOC reduction after 48 h of biodegradation of 2-NSA (100 mg/l) added to tannery wastewater [TW] by *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC

Treatments	Initial samples		After 32 h incubation		TOC removal (%)	DOC removal (%)
	TOC (mg/l)	DOC (mg/l)	TOC (mg/l)	DOC (mg/l)		
TW	110.1	91.0	99.3	89.12	9.8	2.1
TW + NSA	165.8	143.7	150.6	142.1	9.2	1.1
TW + NSA + 2AC	162.0	141.5	105.5	92.4	34.9	34.7
TW + NSA + 4BC	162.0	139.1	110.6	96.1	31.7	30.9

the degradation of the added 2-NSA and that present prior to spiking. It was noted that, after the degradation of 2-NSA, the DOC in the spiked and inoculated wastewater treatments actually increased by 3–7 mg/l when compared with treatment TW. The increase could be due to two factors: the residual metabolites from 2-NSA degradation (Figure 4), and the gradual dissolution of some organic compounds (POC) present in the tannery wastewater. The remaining TOC (65–69%) is probably due to recalcitrant metabolites (e.g. the compound with m/z 173 described earlier) produced during biodegradation plus many non-degradable organics (such as 1-NSA) present initially in the tannery wastewater.

As stated previously, both isolates appear to be nutritional specialists and degrade the target compound 2-NSA rather than other organic compounds in tannery wastewater. Even related naphthalenesulfonates, such as 1-NSA and NSA polymers (Song et al. 2003), cannot be degraded by *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC. However, current research in our laboratory shows that a fungus, *Cunninghamella polymorpha*, does degrade NSA polymers (but not 2-NSA) and a mixed culture of *Cunninghamella polymorpha* and the two 2-NSA-degrading bacteria described here has been investigated.

Conclusions

2-NSA, one of the major contributors to the hard COD in tannery wastewater, can be identified and quantified using HPLC-MS in NI mode. Two bacterial strains, *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC, both capable of utilizing 2-NSA as a sole source of carbon and energy, even in the presence of other carbon sources and indigenous microorganisms, were isolated. The growth of both strains correlated with 2-NSA degradation. The lag-phase prior to degradation was independent of 2-NSA concentration in the range of 1–500 mg/l. Total biodegradation of 2-NSA (100–500 mg/l) was achieved within 33 h by both isolates, but between 10% and 30% of the TOC remained. This is assumed to be due to persistence of intermediates, in particular the product with an ion of m/z 171, 173 and 174. The biodegradation kinetics of 2-NSA is described using the Quiroga model and the r^2 values are close to unity. The determined degradation rate (p) is dependent on initial 2-NSA concentrations and p increased with initial 2-NSA concentrations (100–500 mg/l). Both *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC met three important criteria for successful bioaugmentation of wastewater treatment systems: (i) capability to degrade target pollutants; (ii) persistence of catabolic activity after inoculation; and (iii) compatibility with the indigenous microbial community (Yu & Mohn 2001). These properties suggest that *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC could become useful as inoculants in biotreatment processes targeted at polycyclic aromatic hydrocarbon derivatives.

Acknowledgements

This work was supported by the Engineering and Physical Sciences Research Council (EPSRC), through a LINK project 'Recycle and Reuse of Tannery Wastewater'. The suggestions and assistance of Drs. Kerry Senior, Liz Shaw and James Stach are gratefully acknowledged. The authors thank BLC Leather Centre Northampton for CNSF chemicals, tannery wastewater samples and scientific support.

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