4-Chlorophenol degradation by a bacterial consortium: development of a granular activated carbon biofilm reactor

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Abstract A bacterial consortium that can degrade chloro- and nitrophenols has been isolated from the rhizosphere of *Phragmitis communis*. Degradation of 4-chlorophenol (4-CP) by a consortium attached to granular activated carbon (GAC) in a biofilm reactor was evaluated during both open and closed modes of operation. During the operation of the biofilm reactor, 4-CP was not detected in the column effluent, being either adsorbed to the GAC or biodegraded by the consortium. When 4-CP at 100 mg l^{-1} was fed to the column in open mode operation (20 mg g^{-1} GAC total supply), up to 27% was immediately available for biodegradation, the rest being adsorbed to the GAC. Biodegradation continued after the system was returned to closed mode operation, indicating that GAC bound 4-CP became available to the consortium. Biofilm batch cultures supplied with 10–216 mg 4-CP g^{-1} GAC suggested that a residual fraction of GAC-bound 4-CP was biologically unavailable. The consortium was able to metabolise 4-CP after perturbations by the addition of chromium (Cr VI) at 1–5 mg l^{-1} and nitrate at concentrations up to 400 mg l^{-1}. The development of the biofilm structure was analysed by scanning electron microscopy and confocal laser scanning microscopy (CLSM) techniques. CLSM revealed a heterogeneous structure with a network of channels throughout the biofilm, partially occupied by microbial exopolymer structures.

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Introduction

A number of chlorinated aromatic compounds have been designated priority environmental pollutants by the US EPA and the EEC. Some are used extensively in several industries, including pulp and paper manufacture (Keith and Telliard 1979; Mason 1991). This has led to their widespread release into the environment, affecting both soil and groundwater. The degradation of these compounds under both aerobic and anaerobic conditions has been reported (Haggblom 1992; Puhakka et al. 1995; Sanford and Tiedje 1997) and degradation pathways have been elucidated (Reineke and Knackmuss 1989; Hollander et al. 1997). In recent years, the potential of biofilm reactors to remediate contaminated groundwater and toxic liquid effluents has been recognised. Biofilm reactors are advantageous in that the biomass is active even at very low concentrations of target organic chemicals, they are less sensitive to the presence of toxic and inhibitory materials, and are more resistant to shock loading than dispersed growth systems (Lee et al. 1994; Fauzi 1995; Shi et al. 1995). The microbial degradation of aromatic compounds adsorbed onto powdered activated carbon (PAC) has been shown to be effective at concentrations which caused a dramatic inhibition of degradation in suspended or sand-attached cultures (AbuSalah et al. 1996). Granular activated carbon (GAC) is a good bacterial immobilisation matrix as it is very adsorptive and has a very high surface-to-volume ratio, due to its large number of internal pores and rough surface texture (Weber et al. 1979; Christiansen and Characklis 1990). Immobilised bacterial GAC reactors have been assessed for the microbiological clean-up of water contaminated with BTX (Xing and Hickey 1994), halogenated aliphatics (Fauzi 1995), toluene (Shi et al. 1995) and chlorobenzene (Klecka et al. 1996).

Klecka et al. (1996) used a GAC fluidized-bed reactor for the degradation of chlorobenzene present in an influent stream at 125–145 mg l^{-1}, with more than 99%

efficiency. Adsorption effects were also noticed and the adsorbed chlorobenzene was available for later biodegradation. Fauzi (1995) developed a GAC reactor to degrade very low concentrations of dichloropropanol ($\mu g\ l^{-1}$ range) in a continuous flow system, and the operation was stable and effective. Treatment of groundwater contaminated with toluene at 2.7 mg l^{-1} in a GAC fluidized-bed bioreactor was achieved with 99.4% efficiency during steady-state operation, while during shock loading the GAC served to adsorb the compound for subsequent degradation (Shi et al. 1995).

Both biofilm composition and activity are important parameters for the successful operation of fixed film processes for water treatment (Moller et al. 1996). However, experimental information on biofilm microstructure has been limited until recently because of the lack of analytical techniques for observing fixed bacteria in situ. Techniques for biofilm analysis were reviewed by Costerton et al. (1995). Scanning electron microscopy (SEM) provides information on the number and distribution of bacteria within the biofilm and on exopolymer structures (EPS). But advances in non-destructive methods, principally confocal scanning laser microscopy (CLSM), provide a more detailed insight into the structural organisation of biofilms and allow the quantification of biofilm constituents (Caldwell et al. 1992; Fauzi 1995; Massol-Deya et al. 1995; Moller et al. 1996).

We have isolated chloro- and nitro-aromatic degrading bacterial consortia from the rhizosphere of *Phragmitis communis* colonising an industrial site contaminated with chloroorganics, heavy metals, nitrates and sulphates. The primary objective of our work was to establish a GAC biofilm reactor for the degradation of the aromatic pollutants. Here we report the efficiency of the bioreactor to degrade 4-chlorophenol (4-CP) during closed mode (recycle system) and open mode of operation; and the effects of hexavalent chromium (CrVI) and nitrate perturbations on consortium activity. The development and structure of the biofilm community was followed using microscopic techniques.

Materials and methods

Isolation of bacterial consortia

Rhizosphere samples (roots and associated soil) of the reed *P. communis*, collected from a chemically contaminated industrial site in Northern Portugal (Estarreja), were used to inoculate batch flasks containing 50 ml minimal salts medium (per litre: $Na_2HPO_4 \cdot 2H_2O$ 2.7 g; KH_2PO_4 1.4 g; $(NH_4)_2SO_4$ 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.2 g; yeast extract 0.02 g and 10 ml trace elements solution of the following final composition per litre: $Na_2EDTA_2H_2O$, 12.0 g; NaOH, 2.0 g; $MnSO_4 \cdot 4H_2O$, 0.4 g; $ZnSO_4 \cdot 7H_2O$, 0.4 g; H_2SO_4, 0.5 ml; Na_2SO_4, 10.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 2.0 g; $CuSO_4 \cdot 5H_2O$, 0.1 g; $CaCl_2$ 1.0 g). Aromatic compounds were supplied as the sole carbon and energy sources, at concentrations of 50–100 mg l^{-1}. Cultures were incubated aerobically at 25 °C at 200 rpm and the optical density was monitored at 600 nm. Cultures (5 ml) were transferred to 50 ml identical fresh medium at weekly intervals, at which time aliquots (0.1 ml) were plated onto minimal agar plates containing the relevant substrate. Enrichments were established using 4-CP, 4-nitrophenol (4-NP), nitrobenzene, chlorobenzene and various mixtures of these compounds.

Reagents

All chemicals were of the highest purity grade available (Sigma, St. Louis, USA; Aldrich, Dorset, UK). GAC (12–18 mesh) was obtained from BDH, Dorset, UK. Prior to use, GAC was washed several times with deionised water to remove carbon fines, dried in an oven at 105 °C, and sterilised by autoclaving.

Packed-bed biofilm reactor

The packed-bed reactor consisted of a tubular glass column (26 cm × 2.6 cm) with four side sampling ports, packed with 50 g GAC, with a bed volume of 110 cm^3 (Fig. 1). The reactor was maintained at 30 °C. The GAC column (B) was inoculated with 500 ml of the 4-CP batch-grown consortium EST2, introduced by peristaltic pump from an aerated feeding vessel (A). The reactor was operated initially in recycle mode (closed system) for 3 days, to allow the inoculum to attach to the GAC. The decrease (> 90%) in optical density of the circulating inoculum was used as an indication of biomass loading onto the GAC column. At three intervals over a 5-week period, spent medium was removed from the reactor and replaced with identical fresh medium supplemented with 4-CP (1 l at 45 mg l^{-1}), with a hydrodynamic residence time (HRT) of 26.1 min. In open mode operation, feeds of 10 l minimal medium containing different concentrations of 4-CP were passed through the reactor. These feeding periods occurred from day 41 to 43, 91 to 93 and from day 100 to 107, with HRTs of 17.6 min, 12.8 min and 70.4 min, respectively. Chlorophenol and chloride concentrations in the recirculating vessel and in the column effluent were analysed. Throughout the lifetime of the reactor samples of the GAC were taken for microscopic analysis.

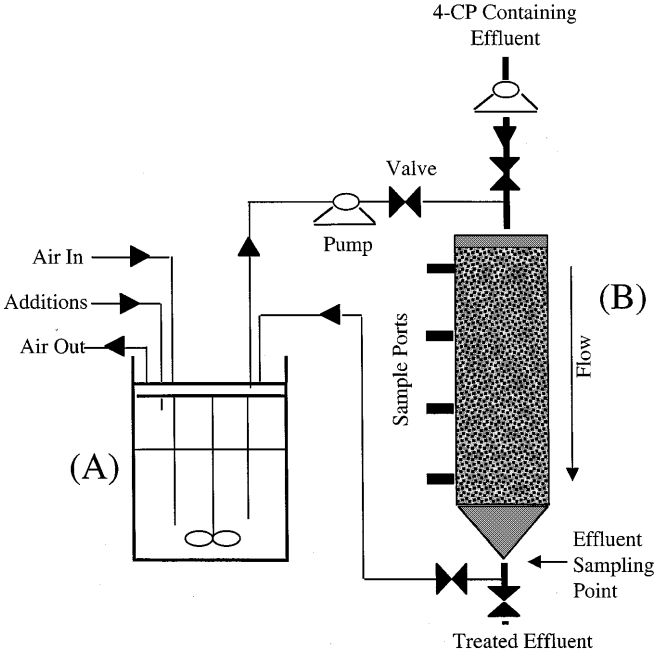

Fig. 1A, B Configuration of packed-bed biofilm reactor, showing the culture vessel (**A**) and granular activated carbon (GAC) reactor (**B**). *Thin line* represents recirculation through the feeding vessel (closed mode operation), *thick line* represents circulation through the GAC column only (open mode operation). Both vessel and reactor were maintained at 30 °C

Chloride analysis

Chloride concentrations were determined by the method of Bergmann and Sanik (1957). To 1 ml culture sample, 0.1 ml of reagent A (0.25 M ferric ammonium sulphate in 9 M nitric acid) and 0.1 ml of reagent B (mercury thiocyanate saturated in absolute ethanol) were added, mixed and centrifuged for 5 min. The absorbance at 460 nm was read within 1 h and the chloride concentration determined against a standard curve of NaCl (0.1–1 mM).

Chromium analysis

Chromium was quantified using a previously described method (Vogel 1978). To 1 ml of culture supernatant, 0.1 ml H_2SO_4 (1 M) and 20 µl 1,5-diphenyl carbizide solution (1% w/v in 70:30 ethanol: H_20) were added. After mixing, the absorbance at 540 nm was measured and compared to a standard curve of $K_2Cr_2O_7$ (0.1–1 mg l^{-1}).

Analysis of haloaromatics

Mono- and di-chlorophenols and nitrophenol were analysed by high performance liquid chromatography (HPLC), on a Perkin Elmer Series 4 Liquid Chromatograph, fitted with a reversed phase C18 column (Sphereclone ODS, Phenomenex) with a mobile phase of methanol/H_20 (70:30) at a flow rate of 1 ml min^{-1} (Kiyohara et al. 1992). Peaks were detected by UV at 290 nm and identified by comparing UV spectra and retention times with those of reference compounds. Samples were prepared by centrifugation for 5 min prior to HPLC analysis.

Adsorption of 4-CP to GAC and bioavailability studies

The adsorption of 4-CP to GAC was determined by incubating a series of 50 ml Falcon tubes containing 0.2 g GAC with 30 ml minimal medium containing 4-CP at 200–2,000 mg l^{-1}. Tubes were placed in a rotary shaker at 30 °C. After 24 h samples were taken, centrifuged at 13,000 rpm and the amount of unbound 4-CP in the supernatant analysed by HPLC. To determine the bioavailability of 4-CP after adsorption to GAC, a series of five flasks containing 10 g GAC in 600 ml of minimal medium containing different concentrations of 4-CP (0–3,600 mg l^{-1} 4-CP) were incubated for 24 h at 30 °C with gentle shaking, before adding an inoculum (5% v/v) of a pre-grown 4-CP culture. The flasks were incubated statically to allow biofilm development, which was visualised by CLSM. Chloride release and 4-CP in culture samples were measured periodically.

Scanning electron microscopy

Biofilm development was examined by SEM. Samples of GAC were removed from the reactor column and biofilms fixed overnight with 5 mM phosphate buffer (pH 7) containing 2.5% glutaraldehyde, dehydrated with a water-acetone mixture (graded series of 25, 50, 75 and 100% acetone) and dried with CO_2 using a Polaron Critical Point Drying Apparatus (Model E3000), before being coated with gold for 4 min using a SEM coating unit (Polaron Model E5000). SEM micrographs were obtained with a Phillips SEM 525 microscope (Phillips, Eindhoven, The Netherlands).

Confocal laser scanning microscopy

GAC particles were taken from the column reactor and double-stained with acridine orange, 0.005% (w/v) in phosphate-buffered saline (pH 7.4), and Nile red, 5 µg l^{-1} in 50% glycerol, in order to visualise the biomass and EPS, respectively. Samples were also stained with a single dye, as required. Video images of the biofilms were generated by CLSM (Leica LaserTechnik, Germany). Samples were examined in horizontal (x,y) and sagittal (x,z) planes as optically thin sections (0.5 µm). Images were recorded on Kodak Ektachrome 100 ASA film by means of a red, green, blue (RGB) video-camera (FMV 1702) which received signals directly from the CLSM.

Results

Chloroaromatic-degrading consortia

Several bacterial consortia were isolated from enrichments inoculated with rhizosphere samples of *P. communis*. Consortium EST2 isolated on 4-CP was chosen for further study, as it was able to metabolise a range of haloaromatic compounds. Preliminary microbiological characterisation of this consortium showed that it consisted predominantly of a Gram-positive bacterium that produced opaque cream colonies and a Gram-negative bacterium that produced mucoid colonies. These two organisms were present in similar proportions in minimal medium containing 4-CP. EST2 was able to grow on and degrade 4-CP when the compound was supplied as sole source of carbon and energy. Growth was optimum at 30–40 mg l^{-1} 4-CP, although growth still occurred at concentrations up to 200 mg l^{-1}.

The consortium also grew on other halogenated phenols and 4-NP. In batch suspension cultures, when EST2 was supplied with a mixture of 4-CP (50 mg l^{-1}) and 4-NP (50 mg l^{-1}), the latter compound was utilised first (Fig. 2). Significant 4-CP degradation was evident only after the majority of the 4-NP had been removed, although the culture did not appear to exhibit diauxic growth. 4-NP was metabolised preferentially, despite the inoculum being grown on 4-CP. When EST2 was grown on a mixture of three chlorophenols, 2-chlorophenol and 2,4-dichlorophenol were utilised first, followed by the

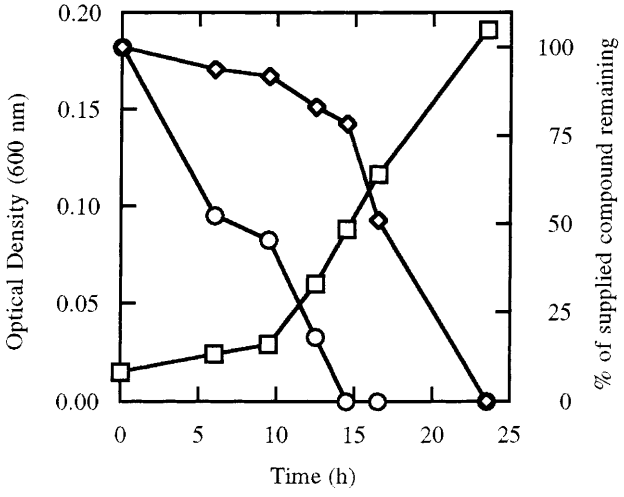

Fig. 2 Degradation of 4-chlorophenol (4-CP) and 4-nitrophenol (4-NP) (50 mg l^{-1}) by consortium EST2. 4-CP (◇); 4-NP (○); consortium (□). Inoculum supplied to an initial optical density of less than 0.02, from a culture pre-grown on 4-CP (50 mg l^{-1})

metabolism of 4-CP (Fig. 3). Again the supplied inoculum had been grown on 4-CP. A residual amount of 2-chlorophenol remained in the culture after extended incubation time. The EST2 consortium, pre-grown on 4-CP, was used to inoculate batch cultures which contained GAC particles (2 g 100 ml^{-1} medium). Cultures were incubated for 2 days prior to the addition of a mixture of 2-chlorophenol, 4-CP and 2,4-dichlorophenol (150 or 300 mg l^{-1} each). Over a 15-day period 35% and 25% respectively of the chlorophenol mixtures was degraded, based on chloride release (data not shown). HPLC analysis of residual chlorophenols, eluted from GAC particles with methanol, demonstrated that all three chlorophenols were metabolised by the immobilised consortium. Identical results were obtained when the chlorophenol mixture was adsorbed onto the GAC particles for 24 h prior to inoculation with the consortium.

Operation of the biofilm reactor

The consortium EST2 was used to inoculate a 4-CP-fed two-stage packed bed GAC biofilm reactor. Initially, the reactor was operated as a closed recirculating system (Fig. 1), in order to establish and maintain the biofilm on the GAC column. In this mode, the reactor was supplied at days 4, 15 and 35 with a fresh 1 l supply of minimal medium containing 45 mg 4-CP and at days 43 and 84 with 1 l minimal medium containing 90 mg 4-CP (Fig. 4), at a flow rate of 2.7 ml min^{-1}. Under these conditions biofilm development occurred on the GAC particles as visualised by microscopy. Stoichiometric release of Cl^- indicated that all the supplied 4-CP was dechlorinated.

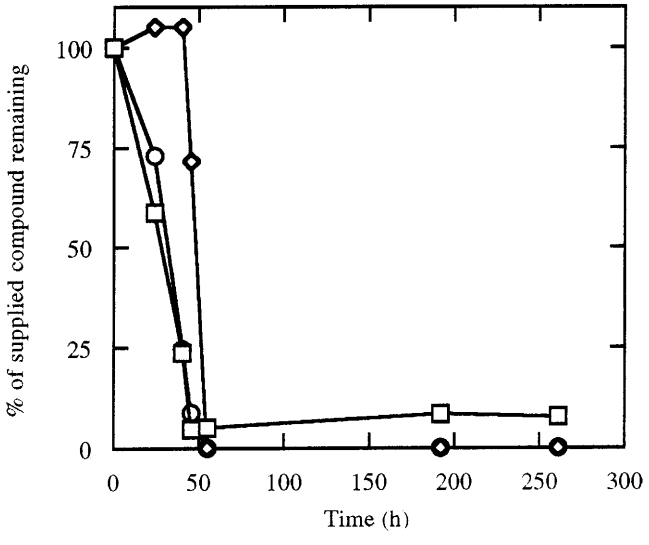

Fig. 3 Degradation of 2-chlorophenol (□), 4-CP (◇) and 2,4-dichlorophenol (○) (each at 25 mg l^{-1}) by consortium EST2. Inoculum supplied to an initial optical density of less than 0.02, from a culture pre-grown on 4-CP (50 mg l^{-1})

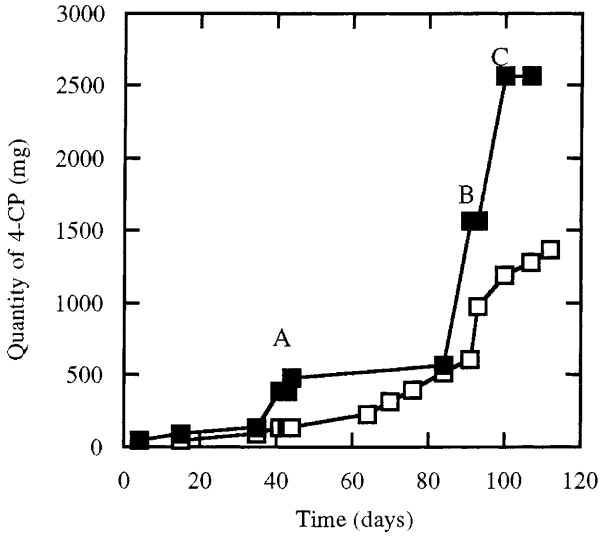

Fig. 4 Degradation of 4-CP in biofilm reactor during open and closed modes of operation. Cumulative supply of 4-CP (■); cumulative 4-CP degraded (□) based on chloride release. 4-CP was not detected in column effluent. *A* (Day 41–43), *B* (day 91–93), *C* (day 100–107) indicate feeds of 4-CP during open mode operation (see Table 1)

Adsorption, desorption and biodegradation of organic compounds are factors that influence the performance of a GAC column bioreactor. In order to quantify the degradation of 4-CP by the GAC biofilm in continuous mode, the reactor was also operated as an open system, with no recirculation to vessel A (Fig. 1). Changes between the two modes of operation were made as an attempt to better understand the contribution of each of the referred mechanisms at different stages during the operation of the system; and in order to ascertain whether all the 4-CP loaded into the column during open mode would be degraded after returning to closed mode. At days 41, 91 and 100 (Fig. 4), 4-CP in minimal medium was fed to the column (see Table 1 for operating conditions). 4-CP and Cl^- concentrations were monitored in the column effluent. The first feed of 4-CP, at day 41 (total 250 mg 4-CP, Fig. 4A), was completely adsorbed to the GAC particles, with no Cl^- or 4-CP detected in the column effluent. After the system was returned to closed mode, all 4-CP (including the two maintenance 90 mg 4-CP supplied at days 43 and 84) was degraded by day 91, as indicated by Cl^- release into vessel A. The second feed of 4-CP (total supplied 1,000 mg 4-CP, Fig. 4B) was on day 91 and again 4-CP was not detected in the column effluent. The Cl^- concentration in the column effluent showed that 27% of the 4-CP was degraded by day 93. The column was then returned to closed operation and a further 240 mg of 4-CP was degraded over the next 7 days. At day 100, a third feed of 4-CP was passed through the column at a slower rate (total supplied 1,000 mg 4-CP, Fig. 4C). Again, 4-CP was not detected in the column effluent, and 9.2% was degraded as indicated by Cl^- release. Degradation of 4-CP continued after the system was returned to closed mode.

Table 1 Degradation of 4-CP in granular activated carbon (GAC) reactor during open mode operation. Percentage of 4-CP degraded is based on Cl^- release in column effluent (*4-CP* 4-chlorophenol)

Feed period (days)	Total 4-CP supplied (mg)	Flow rate (ml min^{-1})	4-CP in effluent (mg)	4-CP degraded during feed period (%)
41–43	250 (10 l at 25 mg l^{-1})	4	0	0
91–93	1,000 (10 l at 100 mg l^{-1})	5.5	0	27
100–107	1,000 (10 l at 100 mg l^{-1})	1	0	9.2

Bioavailability of adsorbed 4-CP

Adsorption of 4-CP to the GAC column and subsequent Cl^- release after returning the reactor to closed mode suggested that all the 4-CP supplied was not immediately bioavailable. Therefore, the bioavailability of 4-CP adsorbed to GAC was investigated in batch systems. Concentrations of 4-CP (10.8–216 mg g^{-1} GAC, corresponding to 1.4–28 mM 4-CP) were added to flasks containing 10 g GAC suspended in 600 ml minimal medium. Flasks were incubated for 24 h, prior to being inoculated with 4-CP-grown EST2 consortium. There was no unbound 4-CP remaining in the medium prior to inoculation, as determined by HPLC. CLSM was used to follow the development of biofilms on the GAC in the flasks. The release of chloride to the medium was monitored to allow quantification of the amount of 4-CP that was degraded by the consortium (Fig. 5). An uninoculated flask used as a control showed no chloride release. In all the inoculated flasks, chloride release suggested that a residual amount of 4-CP remained adsorbed to the GAC (40–50 mg 4-CP per gram GAC for 54–216 mg g^{-1} supplied, and 8.5 mg g^{-1} for the lowest amount of 4-CP supplied: 10.8 mg g^{-1}); and was therefore biologically unavailable (Table 2). The ratio of 4-CP/GAC supplied to the column reactor in open system experiments was 5 mg g^{-1} for the first and 20 mg g^{-1} for the second and third 10 l feeds. Extrapolation from the batch data in Table 2 suggests that the amount of 4-CP that was biologically available in the open mode column experiments was approximately 21–24%. Therefore, based on the 24% upper limit, when the column was supplied with 100 mg l^{-1} feeds of 4-CP (Fig. 4B, C), only 250 mg 4-CP would have been immediately available to the biofilm consortium on the GAC column. Thus, approximately 100% (270 mg) and 37% (92 mg) of the bioavailable fraction was degraded under these two 4-CP feeding regimes, respectively.

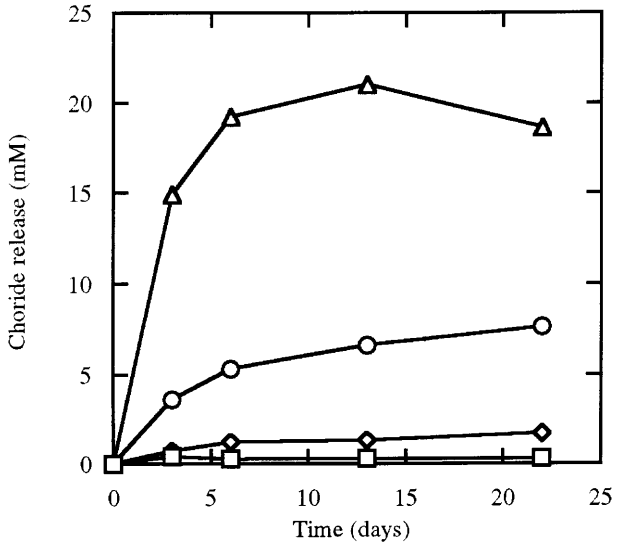

Fig. 5 Bioavailability of adsorbed 4-CP for biofilm development. 4-CP loading: (△) 216 mg g^{-1}; (○) 108 mg g^{-1}; (◇) 54 mg g^{-1}; (□) 10.8 mg g^{-1}. Inoculum supplied to an initial optical density of less than 0.02, from a culture pre-grown on 4-CP (50 mg l^{-1})

Effect of perturbations on 4-CP degradation

Chloroorganic pollution is often accompanied by inorganic pollution from heavy metals, nitrates, sulphates etc; and the effects of such compounds on the metabolism of organics need to be evaluated. Because of the heavy metal and nitrate contamination of the Estarreja site, the effects of Cr VI and nitrate on the degradation of 4-CP by consortium EST2 was investigated. Chromium (0–5 mg l^{-1} as $K_2Cr_2O_7$) was supplied to batch cultures grown on 50 mg l^{-1} 4-CP. After 24 h all the 4-CP had been degraded in cultures supplied with 0, 1, 3 mg l^{-1} chromium, while cultures supplied with 5 mg l^{-1} chromium degraded all the supplied 4-CP within 48 h. After 48 h all chromium supplied to the cultures had been removed from the culture medium. The adsorption of chromium to GAC was over 100-fold less than 4-CP adsorption and did not affect adsorption of the latter. Chromium adsorption was approximately 2-fold higher on colonised GAC, compared to uncolonised particles. Unlike 4-CP, chromium was desorbed from GAC particles when washed with fresh culture medium. Desorption was higher in uncolonised particles. Nitrate, supplied at concentrations up to 400 mg l^{-1}, did not affect biodegradation of 4-CP in batch cultures of EST2.

Table 2 Bioavailability of 4-CP after adsorption to GAC

4-CP loaded mg g^{-1} GAC	4-CP remaining adsorbed mg g^{-1} GAC	4-CP degraded %
10.8	8.5	21
54.0	40.9	24
108.0	47.8	56
216.0	54.0	75

Structural organisation of the biofilm

The development of the biofilm in the GAC column was monitored by SEM, using GAC samples taken from the top section of the GAC column. On day 7, microbial colonisation of the GAC particles was evident. After 14 days of column operation, aggregation of biomass (mainly rod-shaped bacteria) and formation of EPS was visible (Fig. 6). The GAC was colonised completely in approximately 4 weeks. A decrease in colonisation was observed down the length of the column.

The EPS, evident as strands in the electron micrographs, was also visualised by CLSM analysis. The biofilm was deposited on the top of the particle. The formation of EPS increased with time, as seen by CLSM (images not shown). Figure 7 shows a sagittal section of the biofilm. This mature biofilm has a network of channels evident either as long void spaces (black) or filled with EPS (red), as revealed by differential staining. The channels were approximately 2 μm wide. The total biofilm thickness on the colonised GAC surface was approximately 20 μm. Precise measurements of biofilm dimensions using this technique are complicated by apparent smearing of the confocal image in the vertical plane. This is an optical effect associated with the low magnification, and hence low numerical aperture (NA), lens necessary for getting a full view of the biofilm. This is because the axial resolution of the lens, and the related shallowness of the depth of field, rises with the square of the NA, whereas the lateral resolution rises with the first power of the NA (Inoue 1990).

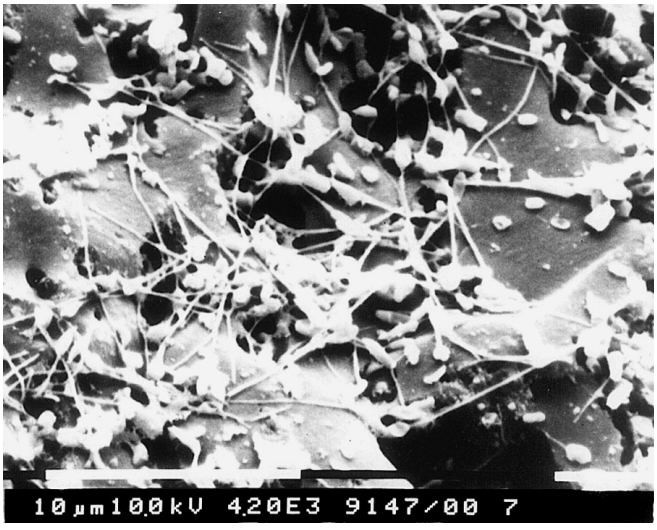

Fig. 6 SEM micrograph of the biofilm after 14 days of bioreactor operation

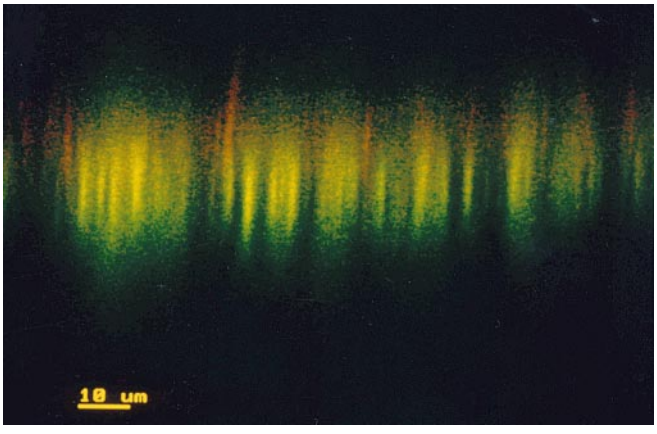

Fig. 7 CLSM image of biofilm attached to GAC particle in sagittal section (*x*, *z*). Bacteria can be seen as *green* and exopolymer structures as *red*

Discussion

It has been demonstrated previously that the degradation of haloorganic pollutants in rhizosphere soil can be faster than in non-vegetated soil (Walton and Anderson 1990; Reddy and Sethunathan 1994). Root exudates can stimulate aerobic microbial degradation of compounds which by themselves poorly support growth. Laboratory soil columns planted with ryegrass promoted the disappearance of aliphatic hydrocarbons (Gunther et al. 1996). Trichloroethylene mineralization experiments in non-vegetated soil and soil vegetated with *Lespedeza cuneata*, *Pinus taeda* and *Solidago* sp. showed that degradation rates were higher in vegetated soils. Similarly, alfafa growing in sandy soil promoted the bioremediation of phenol, toluene and TCE (Erickson et al. 1995). These reports suggest that the microbiota associated with plant roots may be a good source of microorganisms able to degrade haloaromatics. Therefore, rhizosphere/root samples from the contaminated site were selected as inocula for enrichment cultures.

In the present study the selected consortium, EST2, degraded single and mixed haloaromatic compounds, including nitro- and chlorophenols, and mono- and dichlorophenols. The ability to degrade mixtures of compounds is crucial because groundwater is often contaminated with multiple pollutants. The ability to degrade mixtures of chloro- and methylphenols has been reported (Hollander et al. 1994) and the degradation of mixtures of compounds was reviewed by Arcangeli and Arvin (1995).

Consortium EST2 was used to establish a GAC biofilm column reactor. In such a system, both adsorption of target compounds to GAC and biotreatment occur simultaneously. In this study, 4-CP adsorbed to GAC, became slowly bioavailable and was mineralised by the biofilm, as indicated by Cl^- release, in both batch flask and reactor studies. In the column reactor a major advantage of 4-CP adsorption was holding the compound for longer than the HRT, allowing time for the biofilm consortium to effectively mineralise the compound. The studies of Shi et al. (1995), using GAC columns to treat toluene-contaminated water, also demonstrated that a major effect of adsorption of

compound to GAC is to stabilise the reactor, by dampening concentration changes in the influent stream. There are indications that the biodegradation of pre-adsorbed compounds is dependent on the adsorption and desorption characteristics of the target compounds in the matrix (Speitel et al. 1989). The biodegradation of *o*-cresol and 3-chlorobenzoic acid adsorbed to PAC varied with the type of carbon, the compound adsorbed and the time of contact between PAC and compound (Jonge et al. 1996a). It was suggested that desorption of the adsorbed compounds preceded biodegradation, based on the fact that the fraction biodegraded never exceeded the fraction which was leachable under the same conditions (Jonge et al. 1996b). During open mode operation of our reactor, all the 4-CP supplied was either adsorbed by the column or degraded by the biofilm. The apparent low efficiency of the biofilm reactor during open mode operation reflects the adsorption mechanism of 4-CP to GAC. It subsequently became available for biodegradation when the system was returned to closed operation. This demonstrates the advantages of using GAC biofilm reactors to accomplish bioremediation. Through a combination of adsorption and biodegradation mechanisms they give an increased stability to effluent quality and overall system efficiency. In the present study, the changes between open and closed mode operation were used for evaluating the efficiency of degradation of 4-CP in continuous mode at different stages of column operation and to show the bioregeneration of the 4-CP-loaded GAC column. Further experiments with these reactors will be made to determine the efficiency of 4-CP degradation under a range of steady-state conditions in order to provide design data for biofilm reactor scale-up.

Chromium, at concentrations as low as 0.5 mg l^{-1}, has been shown to severely inhibit the mineralisation of chlorophenol by anaerobic bioconsortia (Kuo and Genthner 1996). In contrast, challenging EST2 with 10-fold higher concentrations of chromium, or with nitrate, did not significantly impair its biodegradative ability. These data suggest that the biotreatment process is resistant to the typical perturbations that would be found in contaminated groundwater and is not limited to use with effluents containing single compounds.

SEM and CLSM enabled us to visualise the distribution of bacteria and EPS on GAC particles. Such knowledge is necessary for the modelling and scale-up of biofilm systems. Labouyrie et al. (1997) used different types of carbon for biofilm attachment and suggested that microorganisms are mainly fixed on the surface, and thus the porosity of the carbon particle would not be important. However, other studies have revealed that microorganisms also colonise GAC crevices (Fauzi 1995). EPS was produced in EST2 biofilms and it has been suggested that this binds bacteria together thus mediating aggregation and adhesion (Costerton et al. 1995). A channelling structure, partially filled with EPS, was also revealed by CLSM analysis. Other authors have reported such features in aerobic biofilm reactors (Wolfaardt et al. 1994; Fauzi 1995; Massol-Deya et al. 1995) and have suggested that mixed communities may have an organised distribution pattern within a biofilm. Factors which influence the production of EPS in biofilms were reported by Nielsen et al. (1997) and Samrakandi et al. (1997), but little is known about the function of EPS in biofilm reactors. The development of an extensive extracellular matrix and growth in attached communities may be important for the maintenance of optimum reactor operating conditions (Lawrence et al. 1994). Channel structures in biofilms may facilitate the transport of nutrients between the bulk solution and the deeper inner layers (DeBeer et al. 1994; Lawrence et al. 1994). The channels can supply as much as 50% of the oxygen consumed by the biofilm (DeBeer et al. 1994); hence the surface:volume ratio of the channels may be crucial. In our study, the channel structures were maintained throughout the operating time of the reactor. But there is a need to examine such structures over longer periods of time, in order to determine to what extent they become plugged with EPS. The latter may be important for modelling the biofilm process, as microbial exopolymers may hinder the diffusion of nutrients.

The results presented in this paper are a further contribution for the understanding of the operation of biofilm reactors established on GAC. The efficiency of biological removal and the positive protection offered by activated carbon adsorption are combined in such systems. Further, the biofilm communities established on GAC seem to have a structural organisation, which may be important for its function.

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