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Xenobiotic Biodegradation Test Using Attached Bacteria in Synthetic Seawater

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The aerobic biodegradability of aniline, used as reference chemical, has been performed in synthetic seawater with attached biomass in a continuously fed reactor (biofilm chemostat reactor, BCR). Marine bacteria inocula came from local marine fish aquarium filters to limit the geographic and seasonal variations in quality. A pretreatment of these inocula combining 5-μm filtration and centrifugation was used to concentrate bacteria and remove organic carbon contamination of the test. The performances of the BCR were tested in comparison with simple shake flask tests. Among the different variables tested, the ratio S_0/X_0 (initial concentration of xenobiotic to initial density of the inoculum), the presence of dissolved oxygen, and the hydraulic residence time appear to be the key parameters controlling the length of the biodegradation process. On the other hand, the addition of a co-substrate (easily biodegradable compound) does not provide advantages. Thus, marine biofilm chemostat reactors with a high density of attached bacteria (around 10^7 cells cm^{-2}) and fed with synthetic seawater plus nitrogen provide good tools for screening biodegradability of chemicals in the marine environment. © 1995 Academic Press, Inc.

INTRODUCTION

The potential of a chemical substance to biodegrade in seawater must be assessed in order to protect the marine environment. Persistence of xenobiotics represents a real risk for ecosystems as well as for human beings. This risk can be reduced to an acceptable level by efficiently screening industrial chemicals and limiting the use of persistent ones. This type of strategy requires well-designed, standardized assay procedures to provide accurate information on the potential of a chemical to biodegrade. The Organization for Economic Cooperation and Development (OECD) adopted two tests for seawater (OECD 306) in 1992. These are adaptations of two standardized freshwater tests (OECD 301D and 301E) that are carried out under static fluid conditions with suspended microbial cultures. Biofilm cultures (Rittmann and Mc Carty, 1980; Bryers, 1987; Banks and Bryers, 1991; Oga *et al.*, 1991) have frequently been used in parallel for testing the potential of xenobiotic compounds to biodegrade (Salkinoja-Salonen *et al.*, 1983; Bouwer, 1989; Bergen *et al.*, 1993) and/or their toxicity (Block *et al.*, 1989) in freshwater. There are several reasons for using biofilm cultures in chemical biodegradation testing. Adherent bacteria are frequently more prevalent (Wuhrmann, 1972; Costerton and Boivin, 1987) and sometimes more active than free planktonic cells (Bourquin and Pritchard, 1979; Van Loosdrecht *et al.*, 1990). Biofilms can establish specific microenvironments by allowing the development of sequential zones where aerobic respiration, denitrification, and sulfate reduction are observed within the biofilm community (Cobb and Bouwer, 1991). In thick biofilms, bacteria are trapped within a polymeric matrix which provides an effective protection against toxic materials (Dwyer *et al.*, 1986; Hoyle and Costerton, 1991). More cells can accumulate in a biofilm and their spatial positioning may promote interactions and gene exchange between different microbial species (Angles *et al.*, 1993). It is now well established that the majority of microbial activity in any natural ecosystem is associated with an interface.

Biofilm reactors have been used for domestic and industrial wastewater treatment for several years and their control is well understood from an engineering point of view (Jördening, 1992; Martin, 1992; Mendez and Lema, 1992; Santos *et al.*, 1992). But, to the knowledge of the authors, such systems have never been used to establish the potential of a xenobiotic to be degraded under seawater conditions. The main objective of this paper is to provide operational parameters for chemical biodegradation tests with biofilm marine bacterial cultures developed under controlled laboratory reactors. The biofilm chemostat reactor (BCR) reported in this study was tested in comparison with the shake flask method using suspended marine bacteria (adapted from OECD 306) in order to evaluate the advantages and the limits of each system.

Mixed cultures of bacteria, extracted from marine aquarium filters and resuspended in synthetic seawater, were used as inocula. The salinity tolerance and the total number of cells and

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viable bacteria were determined as well as organic carbon contamination. Parameters considered were (a) the influence of inoculum density on any lag time and outcome of the biodegradation test, (b) the possible advantage of a cosubstrate (sodium benzoate) in accelerating biodegradation, (c) the use of a pregrown biofilm for the biodegradation test, and (d) the hydraulic residence time (HRT) in the biofilm chemostat reactor. Aniline was chosen as the biodegradable reference compound (AFNOR, 1985; OECD, 1981).

MATERIALS AND METHODS

Synthetic Seawater

The synthetic seawater used in this study was obtained by dissolving 33 g of powdered mixed salts (Reef Crystals, Aquarium Systems, Sarrebourg, France) in 1 liter of deionized pyrogen-free (MilliQ-filter system) water. The resulting salinity was around 26‰ (measured by densitometry). Major ions (in grams per liter) were, according to the manufacturer, Cl^-, 19.60; Ca^{2+}, 0.42; K^+, 0.36; Mg^{2+}, 1.40; Na^+, 10.40; P, 0.0002; and pH ≈ 8.2. Dissolved organic carbon never exceeded 2 mg liter^{-1}. Ammonium chloride (Prolabo) was added to the synthetic seawater samples to give a C/N ratio of 10 (C, organic carbon from test chemicals; N, nitrogen from ammonium chloride). All solutions were autoclaved at 121°C for 20 min prior to use.

Chemicals and Analytical Methods

Two test chemicals were used: aniline (Prolabo) and sodium benzoate (Merck). All analyses were carried out on samples filtered through membrane filters (Millipore Millex-GS, pore size 0.22 μm). Concentrations of dissolved aniline and sodium benzoate were measured by high-performance liquid chromatography (HPLC) or as dissolved organic carbon (DOC).

HPLC analyses were carried out on a Kontron chromatograph equipped with a 5-μm C_{18} Nucleosil column (Macherey–Nagel), a uv detector (Model 432), and an integrator (Tandon 286/N). The mobile phases (water + methanol) were slightly different for aniline (70:30) and sodium benzoate (60:40 + 0.5% H_3PO_4). HPLC flow rate was 0.7 ml min^{-1}. Injected volume was 20 μl and uv detection was carried out at 234 nm. Results are expressed as milligrams of test chemical (using external standard and calibration curves) or as milligrams of test chemical carbon per liter.

DOC was determined on Dohrmann DC-80 carbon analyzer using wet persulfate/uv oxidation and subsequent quantification of evolved CO_2 by infrared spectrometry as described in the *Standard Methods for the Examination of Water and Wastewater* (1992). Samples were filtered (Millipore Millex-GS, pore size 0.22 μm) before acidification through filters washed with 80 ml MilliQ water to provide carbon contamination. To reduce interference from chloride, sodium persulfate at 100 g liter^{-1} was used, and only 0.5 ml of each sample was injected, allowing better oxidation. All vessels were heated at 550°C for 3 hr prior to use, and samples were deep-frozen until analysis.

Bacterial Inocula

The bacterial suspensions used as inocula were obtained from fiberglass or polyurethane filters of seawater aquariums (Aquarium Tropical, Nancy, France, or Faculté des Sciences, Montpellier, France). Water was extracted by wringing out the filters. Liquid was allowed to settle for 30 min to remove the largest particles. The supernatant was used as an inoculum in the biodegradation assays (a few milliliters was placed in each flask) or as a test medium (dissolving the test chemicals directly with the inoculum). The total numbers of cells (cells ml^{-1}) and viable bacteria (CFU ml^{-1}) in each inoculum were measured before starting the assays.

Counting Methods

The total numbers of cells were counted by epifluorescence microscopy and viable bacteria were enumerated on marine agar medium (Difco). The techniques were used for water samples and for attached biomass on glass beads. In the latter case, the attached biomass was removed from the glass beads by 30 sec sonication (Labsonic U 2000 sonicator, probe diameter 19 mm, power 37 W) in 20 ml bacterium-free synthetic seawater.

Epifluorescence counts were performed with extract from a 9-ml volume of each sample, diluted as required with bacterium-free synthetic seawater in sterile plastic tubes (Laboexpress) plus 1 ml of diamidinophenylindole (DAPI) used as fluorochrome (Sigma, 25 μg ml^{-1}). Samples were mixed, left to stand for 15 min, and filtered through black polycarbonate filters (Millipore GTBP 04700; pore size 0.22 μm), and then filters were examined under an epifluorescence microscope (Leitz microscope; excitation 340 nm; emission 465 nm) with an oil immersion (Merck) objective (100X). Bacteria were counted in 10 random fields per slide and results expressed as cells ml^{-1} or cm^{-2} of sample.

Viable bacteria were counted by spreading 0.1 ml of sample (or its dilution in sterile 9‰ NaCl) on sterile marine agar (Difco, 55.1 g liter^{-1}) and by incubating for 48 hr at 20 ± 2°C. Colonies were counted and expressed as colony forming units (CFU) per milliliter.

Reactor Procedures

Shake flask method. A 450-ml solution of test chemical(s) prepared in synthetic seawater plus inoculum or directly prepared in inoculum suspension (supplemented with nitrogen) was incubated with agitation (rotary shaker; 150 rpm) in 500-ml glass shake flasks closed with rubber caps. Flasks were incubated in the dark or in diffuse light at 18 ± 2°C for 28 days. The disappearance of the test compound(s) was followed by DOC analysis or by HPLC. Results are expressed as (i) percentages of the chemical removed at the end of the test, (ii) maximum rate of chemical removal, and (iii) length of the lag

phase prior to the onset of degradation, determined graphically according to Spain *et al.* (1984).

Biofilm chemostat reactor. The biofilm reactor was constituted by a glass column filled with 4-mm glass beads, providing a total bacterial support area of approximately 5500 cm^2 (Fig. 1). A peristaltic pump provided a high recycling continuous flow rate (Q_r = 12 liters h^{-1}) to ensure complete mixing of the aqueous phase in the reactor without fluidizing the glass beads. Nutrient/contaminant solution was continuously pumped into a mixing vessel by means of a peristaltic pump. Effluent from the system left by overflow to ensure constant volume. With $Q_r \gg Q$, the nutrient influent flow rate, the system can be mathematically treated as a chemostat. This mixing vessel was continuously aerated with humidified air, dried on silica gel, and filtered (Millipore Millex-FG, pore size 0.22 μm) to provide oxygen without external contamination by microorganisms or organic molecules. The tubes were Teflon or Norpren (for the peristaltic pump head). Dissolved oxygen concentrations in the recycling line were measured daily using an oximeter equipped with a Clark-type probe (Oxi-96, Bioblock) and were recorded automatically and then corrected for temperature, atmospheric pressure of the day, and salinity of the water.

The chemostat experiments were started by filling the acid-washed and autoclaved reactor with the undiluted inoculum suspension plus the test chemical ($V_{interstitial}$ = 290 ml). Initially, only the recirculating pump was turned on (*i.e.*, no continuous feeding for 5 hr). This step allows the suspended bacteria from the inoculum to attach to the glass beads and it minimizes the washout of the initial inoculum. After 5 hr continuous feeding of the synthetic seawater solution with test chemicals was started. Chemicals were measured daily at the inlet and outlet of the biofilm chemostat reactor by HPLC. Dissolved oxygen in the recycle loop was continuously recorded. As with the shake flasks, all operations were carried out in the dark or in diffuse light at 18 ± 2°C. Five different hydraulic residence times (HRT = V/Q), *i.e.*, 2, 4, 8, 16, and 32 hr, were tested in chemostat reactors by selecting different nutrient feed flow rates (Q).

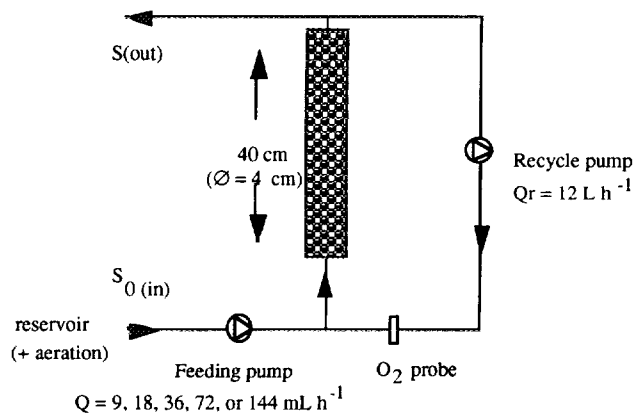

FIG. 1. Diagram of the biofilm chemostat reactor (BCR).

RESULTS AND DISCUSSION

Inocula Characteristics

Water extracted from the aquarium filters and used as inocula contained a complex mix of microorganisms, aggregated or not, plus dissolved and particulate organic matter. This bacterial suspension used as inocula in this study contained 10^6–10^8 cells ml^{-1} with a variable proportion of viable bacteria (1–10% of total cells). Culture of these bacteria carried out at different salinities (0–129 g $liter^{-1}$) in nutritive agar medium revealed that the highest number of cultivable bacteria was obtained with nutritive medium containing 33 g $liter^{-1}$ Reef Crystals (Fig. 2). According to Ventosa *et al.* (1984) and Bertrand and Larsen (1989), bacteria that grow optimally under or around the salt concentration found in seawater are low halophiles and may be called marine bacteria.

The amounts of organic matter varied from 1 to 8 mg DOC $liter^{-1}$ in the bacterial suspension. Filtering the raw inoculum through a 5-μm mesh cloth removed some of the organic carbon suspended matters. The centrifugation at 8000*g* for 10 min of this filtered inoculum allowed recovery of the bacteria in the pellet and the removal of the supernatant containing contaminant DOC. Then, the pellet was resuspended in the same volume of synthetic medium (*i.e.*, MilliQ water + Reef Crystals 33 g $liter^{-1}$). Such treatment (filtration + centrifugation) permits removal of on average 80% of DOC from the raw inoculum.

Shake Flask Method

The shake flask biodegradation assay revealed that aniline was removed from the test medium in 8 to 12 days by a nonadapted suspended marine bacterial population, irrespec-

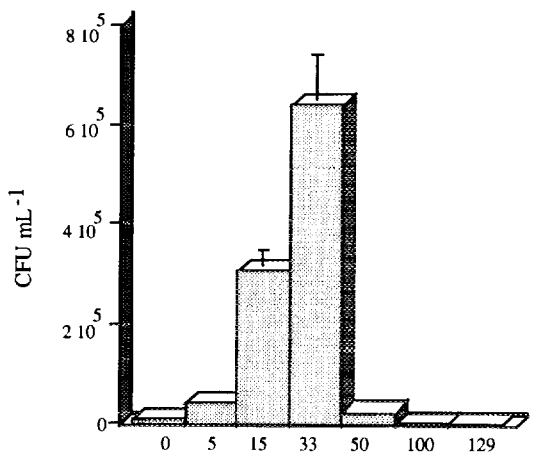

FIG. 2. Viable bacteria (CFU ml^{-1}) in six salt-increasing enrichments after incubation for 48 hr on nutritive agar medium (agar + m-Plate Counting Broth) at 20°C.

tive of the origin of the inoculum (Fig. 3a). The lag phase was slightly longer than that reported by Nyholm *et al.* (1992), perhaps because of the lower initial inoculum density. In both this report and that of Nyholm *et al.* (1992), aniline was totally degraded in 10–12 days. Inoculation of the aniline-degrading population at Day 14 into a new flask containing synthetic seawater plus aniline (6.8 mg C liter^{-1}) indicated that the adapted population rapidly degraded the test chemical (Fig. 3b). Upon reinoculation, the length of the lag phase (initially 5–10 days) was reduced to less than 1 day while the maximal rate of biodegradation remained identical at 150 μg C liter^{-1} h^{-1} when the density of the inoculum was adjusted to 6.2 $\times$ 10^5 cells ml^{-1}, irrespective of the inoculum origin.

Thus, adaptation of an inoculum by preculture with the target xenobiotic decreased the lag phase and improved the precision of the test (Nyholm *et al.*, 1992; Thouand and Block, 1993). As reported by several authors (Wiggins *et al.*, 1987; Simkins and Alexander, 1984; Chudoba *et al.*, 1992; Thouand *et al.*, 1995), preculture increases the number of biodegrading organisms in the inoculum and can induce enzymes or increase the degradative enzyme level of existing cells. Such a lag phase reduction may also be obtained directly by increasing the initial raw inoculum density. The effect of the inoculum density on the length of the lag phase was tested with three different bacterial suspensions (samples taken from the filters of the marine aquarium at Nancy, France, in October 1992 with X_0 = 3.2 $\times$ 10^6 CFU ml^{-1} and 2.7 $\times$ 10^7 cells ml^{-1}, November 1992 with X_0 = 7.3 $\times$ 10^6 CFU ml^{-1} and 1.8 $\times$ 10^7 cells ml^{-1}, and December 1992 with X_0 = 1.5 $\times$ 10^5 CFU ml^{-1} and 4.0 $\times$ 10^7 cells ml^{-1}), diluted as required with synthetic seawater. Assays were carried out with the same concentration of aniline ($S_0 \approx$ 4 mg C liter^{-1}), giving an initial range of S_0/X_0 (mg C aniline/number of bacteria) of 10^{-10} to 10^{-7}. There was a significant linear relationship (r^2 = 0.73) at the 5% confidence limit between the lag phase (in days) and the logarithm of S_0/X_0, when the bacteria were counted as CFU (Fig. 4). The absence of a significant relationship for total cell counts (not provided) may be due to variations in viable cell proportions (1–10%) in the inocula used. A linear extrapolation of the results indicates that the higher the concentration of inoculum, the better the chance of a rapid positive response in the test. Using aniline as a model compound, this study demonstrates that starting the assay at $S_0/X_0 \approx 5 \times 10^{-11}$ (4 mg C liter^{-1} aniline/8.0 $\times$ 10^{10} CFU liter^{-1}), the lag phase may be reduced to below 1 day, even with a nonadapted bacterial population. Thus, properly designed biodegradation assays require both monitoring the initial xenobiotic concentration, S_0 (as generally suggested in mandatory tests), and the initial viable cell numbers, X_0, using a rapid method (such as culture on agar medium or preferably counting respiring bacteria using the INT or the CTC method) (Rodriguez *et al.*, 1992).

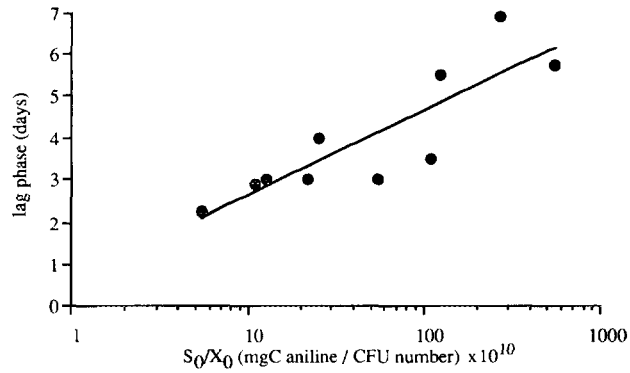

FIG. 4. Influence of S_0/X_0 on the lag phase during biodegradation of aniline in shake flask tests (aniline = 4 mg C liter^{-1}).

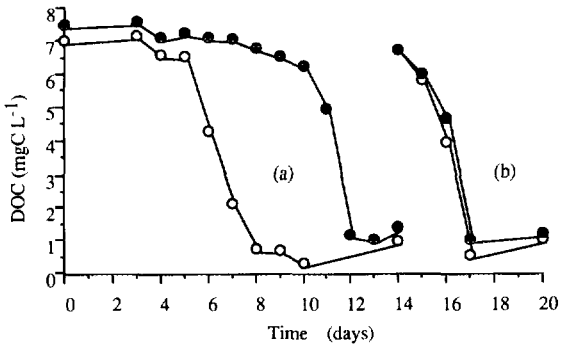

FIG. 3. Biodegradation of aniline by two marine inocula (●, Nancy, and ○, Montpellier) in shake flask cultures at 18° ± 2°C. (a) (d_0 to d_{14}) with initial bacteria concentration X_0 = 6.2 $\times$ 10^5 cells ml^{-1}; initial aniline concentration S_0 = 7.5, ●, and 7.0 mg C liter^{-1} ○, respectively. (b) (d_{14} to d_{20}) with X_0 adjusted to 6.2 $\times$ 10^5 cells ml^{-1}; S_0 = 6.8 mg C liter^{-1}.

It is frequently suggested that the presence of a known, easily biodegraded cosubstrate may help to sustain the survival or/and growth of a bacterial inoculum (Schmidt and Alexander, 1985) and improve xenobiotic degradation. Sodium benzoate was used in this study as a supplementary substrate in tests with aniline in shake flask assays. Sodium benzoate (Fig. 5a) was removed within 1 day (*i.e.*, about 6 days before aniline), but had no significant effect on the degradation rate of aniline when compared to experiments with aniline as the only source of carbon (Fig. 5b). Since the cosubstrate added in this assay ($\approx$3.7 mg C liter^{-1}) accounts for less organic matter than that which occurs in the inoculum itself, adding a cosubstrate served no beneficial function in these biodegradation tests. This exogenous source of carbon would also increase oxygen consumption and could aid in the selection of a nonspecific bacterial population.

Biofilm Chemostat Reactors

The ability of an attached marine biomass to degrade aniline in BCR was tested under different operating conditions. One advantage of such reactors is that the biofilm may be pregrown on glass beads inside the BCR before starting the biodegradation test. Controlling the concentration of the nutrient medium (yeast extract in synthetic seawater) that was continuously fed into the reactor will set the biofilm density, similar to an initial

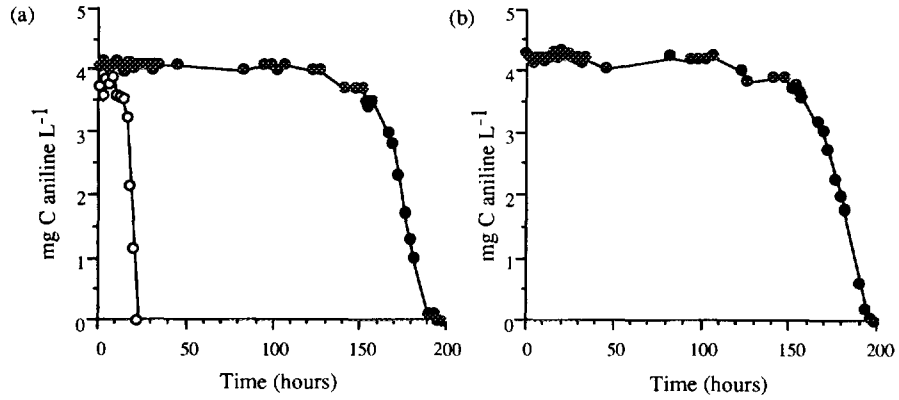

FIG. 5. Biodegradation of aniline in shake flask test with (a) mixed substrate, sodium benzoate, ○ (S_0 = 3.7 mg C liter^{-1}) + aniline, ● (S_0 = 4.2 mg C liter^{-1}) and (b) aniline alone, ● (S_0 = 4.3 mg C liter^{-1}).

density of inoculum in a shake flask test. A yeast extract surface flux of 0.13 to 0.58 μg C day^{-1} cm^{-2} resulted in a number of attached bacteria in the reactor at steady state (generally obtained within 5–8 days at 20°C) of 5.0 × 10^6 to 1.1 × 10^7 cells cm^{-2} (Table 1). A fraction of this biomass was viable (13–20% CFU), and respired dissolved oxygen became a limiting factor for the reactor receiving high concentrations of biodegradable organic matter (dissolved O_2 in reactor BCR3 was always below 0.1 mg liter^{-1}).

At steady state, the biofilm reactors were gently rinsed and fed with synthetic seawater supplemented only with aniline. The kinetics of aniline biodegradation (Fig. 6) had different patterns, with a significant lag phase (5 days) for BCR3, which contained more fixed biomass but operated with little to no dissolved oxygen. In this case, the expected positive effect of a high biomass concentration was counterbalanced by the absence of terminal electron acceptor (oxygen). Addition of 2 g liter^{-1} of mercuric chloride (10% of the total interstitial volume) in BCR1 at Day 8 killed viable bacteria and completely inhibited the aniline biodegradation (results not provided).

The relationship between the HRT and the aniline removal was studied in the same biofilm reactor for five HRTs by changing them from 32 to 2 hr. The biofilm reactor was inoculated with marine bacterial inoculum as described previously and continuously fed with 5.0 mg C aniline liter^{-1}. Steady state was obtained for each HRT tested after 5.6 ± 0.9 HRT on average. Total number of attached CFU measured at steady state for each HRT was approximatively the same [(4.9 × 10^7) ± (0.7 × 10^7) CFU cm^{-2}] throughout the test. Decreasing the HRT from 32 to 2 hr increased the residual aniline concentration at steady state (Fig. 7) from 0 mg C aniline liter^{-1} (HRT = 32 hr) to 3.5 mg C aniline liter^{-1} (HRT = 2hr), which is in agreement with the observations of Sanchez *et al.* (1992) and the theory of reactors (Tempest, 1970; Harder *et al.*, 1977).

Very long HRTs of 15 or even 22 days, as used by Nyholm and Kristensen (1992) in suspended bacteria chemostats, improved the probability of biodegradation, but such extended

TABLE 1

Influence of Organic Carbon Surface Flux on the Number of Attached Cells, Proportion of Viable Bacteria, and Residual Dissolved Oxygen during Aniline Biodegradation Tests in Three Biofilm Chemostat Reactors

Parameters measured	BCR_1	BCR_2	BCR_3
Yeast extract at the inlet of the reactor (mg C liter^{-1})	1.94	4.64	8.89
Surface flux (μg C day^{-1} cm^{-2})	0.13	0.30	0.58
Number of attached cells after 14 days of continuous feed (cells cm^{-2})	5.0 × 10^6	6.9 × 10^6	1.1 × 10^7
% of viable attached bacteria per reactor (CFU counts) after 14 days of continuous feed	19	20	13
Dissolved O_2 in the reactor during aniline biodegradation (mg liter^{-1})	2.7 to 4.2	1.1 to 2.7	0 to 0.1

Note. Hydraulic residence time, 32 hr; temperature, 20 ± 2°C.

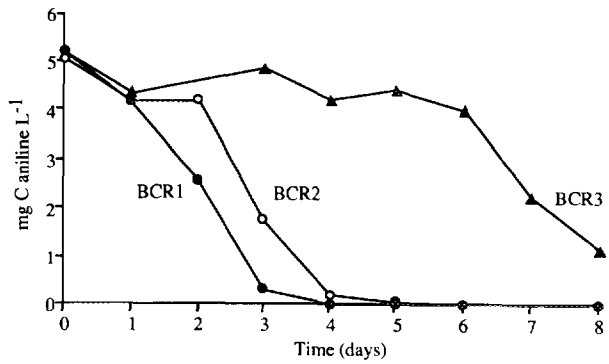

FIG. 6. Biodegradation of aniline (5.15 mg C liter^{-1}) in three biofilm chemostat reactors (BCR1, BCR2, and BCR3) with pregrown biofilms (for operating conditions, see Table 1).

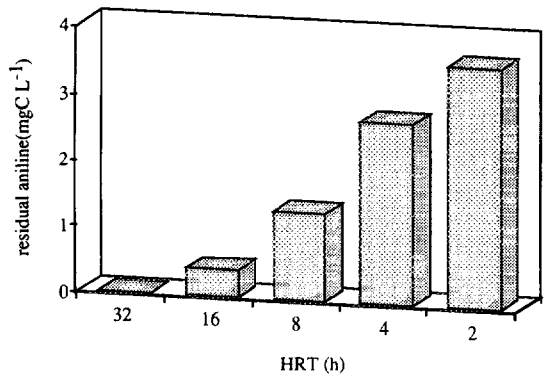

FIG. 7. Biodegradation of aniline in biofilm chemostat reactor (BCR) and influence of the hydraulic residence time (HRT = 32, 16, 8, 4, and 2 hr) on the residual of aniline at steady state. (Influent aniline concentration, S_0 = 5.0 ± 0.2 mg C liter^{-1} throughout the test; temperature, 20 ± 2°C.)

HRTs are not practical. A HRT in a biofilm reactor between 16 and 32 hr appears more feasible.

The cometabolic biodegradation of aniline and sodium benzoate was measured with two BCRs running in parallel: one was continuously fed with aniline (2.2 mg C liter^{-1}) plus sodium benzoate (2.9 mg C liter^{-1}) and the other with aniline alone (4.4 mg C liter^{-1}). There was a significant increase (4 days) in the time required for complete aniline biodegradation in the aniline plus sodium benzoate reactor compared to the test without cosubstrate (Fig. 8). As in the shake flask tests, there appears to be little advantage in continuously feeding cosubstrate in this specific system.

CONCLUSIONS

The two main objectives of biodegradation tests are to mimic as closely as possible the real environment and to provide information on potential for specific chemicals to be biodegraded by a representative ecosystem sample under standardized conditions.

This study demonstrates that seawater biodegradation tests can be readily carried out using bacterial inocula from local marine aquaria, synthetic seawater (which reduces the geographic and seasonal variations in quality), and a glass bead fixed-bed reactor which provides a large adhesive surface area for marine bacteria (around 10^7 cells cm^{-2}). Such reactors may be used with pregrown biofilm or directly filled with a mixture of inoculum and synthetic seawater plus the chemical to be tested. The cosubstrate (easily biodegradable compounds) used in this study provided no better conditions for chemical testing. These continuously fed biofilm systems provide a great opportunity for testing different environmental conditions (by changing the flux for example). However, the HRT appears to be a key parameter controlling rates of biodegradation. A 32-hr HRT did provide complete aniline removal in 3 days and a 16-hr HRT led to 75% removal after 8 days. Experiments are under way to optimize removal while minimizing HRT (e.g., >16 h but <32 h). As these reported conditions were defined for aniline as the reference compound, other recalcitrant or toxic chemicals would require separate testing to investigate the usefulness of biofilm ecosystems for biodegradation testing.

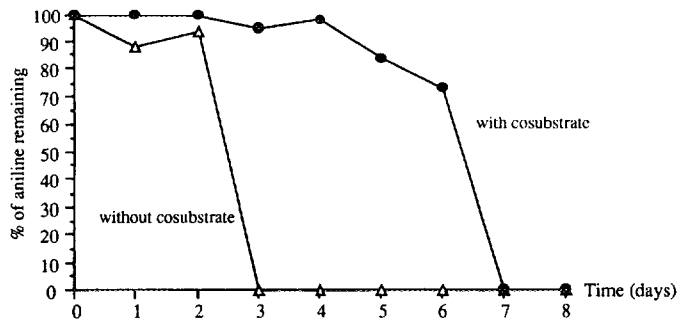

FIG. 8. Biodegradation of aniline in biofilm chemostat reactors (BCR) continuously fed with aniline (with or without cosubstrate). Cosubstrate was removed within 2 days (not shown). HRT, 32 hr; temperature, 20 ± 2°C.

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