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Sodium hypochlorite perturbation of a graywater treatment system

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A novel graywater treatment system consisting of an aerated batch reactor and biomass-retaining ultrafiltration unit was evaluated for treatment of shipboard wastes. The focus of this study was to determine the resilience of the biomass recycle reactor to perturbations of sodium hypochlorite, the major component of bleach. A bench-scale reactor was perturbed with 50, 190, and 1000 mg L^{-1} sodium hypochlorite and monitored for changes in respiration, substrate utilization, viable plate counts, fatty acid methyl ester profiles, and Biolog-GN patterns. Following the addition of hypochlorite, respiration and substrate utilization were not detected, and viable biomass decreased. Recovery times following perturbations were longer with higher concentrations of sodium hypochlorite. Community composition (determined by fatty acid methyl ester analysis) changed during the recovery from hypochlorite perturbations. However, more significant differences in community composition were noted between different perturbations and were a function of time. Irrespective of initial community composition, the reactor communities recovered from hypochlorite perturbations. Biolog patterns showed no notable change in the overall metabolic capacity of the community. The biomass recycle reactor's resistance to sodium hypochlorite perturbations contributes to its usefulness in treatment of shipboard wastes. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 191–197.

Keywords: biomass recycle reactor; wastewater treatment; phospholipid fatty acid analysis; sodium hypochlorite

Introduction

Graywater is a term used to describe any wastewater, except toilet wastes, in closed systems such as sea-faring passenger ships [18]. Our previous efforts have shown typical graywater to contain organics consisting mainly of polysaccharides and protein, less than 1% anionic surfactant, and low levels of inorganic elements [22]. As environmental regulations governing discharge to coastal seawater become more stringent, providing a method to reduce levels of graywater organics rapidly before discharge is important for environmental stewardship.

The ideal shipboard graywater treatment systems must reduce biodegradable organics in a large volume of water within a short hydraulic residence time (HRT). The waste separation and concentration techniques in common use do not provide a sufficiently rapid treatment when confined to fit the small spaces typical of marine vessels. Recently a laboratory-scale bioreactor with 100% biomass recycle has been tested for application as a graywater treatment system [20].

The biomass recycle reactor system consists of a reaction vessel where wastewater is biologically treated. Contents of the reactor are passed across an ultrafiltration membrane, which retains biomass in the reactor while allowing clean water to be discharged. Because the biomass in a cell recycle reactor reaches levels far grater than those in a continuous culture, the rate of substrate addition will support only a very slow net growth rate [6,21]. At this point, substrate may be used primarily for maintenance rather than biomass production [26]. As growth rates fall, biomass accumulation becomes a linear rather than an exponential function with time and cells become less reactive [20].

Although our previous work showed that biomass recycle reactors treating sterile simulated graywater were stable for up to 74 days, the system's resistance to perturbations is unknown [20]. This is of concern since, in addition to organic substrate in the graywater, pulses of chemicals used in shipboard operations, such as detergents and disinfectants, may enter the treatment system. It is not known how such toxic chemicals will affect the efficiency of the biomass recycle reactor. This investigation focused on the effects of sodium hypochlorite (bleach) on the resiliency of a biomass recycle reactor having low cell reactivity, treating simulated graywater.

Materials and methods

A biomass recycle reactor was operated as previously described by Konopka *et al* [20]. Briefly, the biomass recycle culture was maintained at 28°C in a glass reactor (working volume 580 ml; Kontes, Vineland, NJ, USA) with a medium feed rate of 120 ml h^{-1}. The reactor feed (Medium 3) contained 9.3 mM NH_4Cl, 3 mM NaCl, 2 mM $NaHCO_3$, 0.5 mM KH_2PO_4, 0.25 mM $MgSO_4$, 5 μM $CaCl_2$, 14 μM $FeCl_3$, 23 μM disodium EDTA, 26 μM sodium citrate, 87 mg L^{-1} laundry detergent, 400 mg L^{-1} starch, 150 mg L^{-1} gelatin, 17 mg L^{-1} linear alkylbenzene sulfonate, and 1 ml L^{-1} SL7 trace element solution [1]. The contents of the reactor were aerated and agitated with humidified air at a rate of 12 L min^{-1}. A polysulfone ultrafiltration membrane with a 100 000 nominal molecular weight cut

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off (Millipore Corp, Bedford, MA, USA) was used to retain the biomass in the reactor. Cells in the reactor were suspended with very little flocculation or attached growth. Neither microscopic observation nor phospholipid fatty acid signatures indicated the presence of protozoa in the biomass recycle reactor.

Household bleach (Blue Ribbon Bleach, Patterson Laboratories, Detroit, MI, USA) was added to a bioreactor in a pulse to achieve concentrations of 50, 190, or 1000 mg sodium hypochlorite L^{-1} (bleach contains 10 000 mg sodium hypochlorite L^{-1}) in one of three separate perturbation experiments. The 190 mg L^{-1} perturbation was performed 20 days after the 50 mg L^{-1} experiment within the same bioreactor. The 1000 mg L^{-1} perturbation was performed on a different run of the biomass recycle reactor, however operating conditions were the same in all three experiments. Ten milliliters of Antifoam B silicone emulsion (Baker, Philipsburg, NJ, USA) were also added to the culture treated with 1000 mg L^{-1} sodium hypochlorite. Samples were taken prior to and at various times following the addition of sodium hypochlorite. All of the following analyses were performed in triplicate.

Chemical analysis

Total chlorine in the reactor eluate was measured by placing one DPD (*N*, *N*-diethyl-*p*-phenylenediamine) Total Chlorine Reagent Powder Pillow (Hach Company, Loveland, CO, USA) in 5 ml of eluate immediately following sample collection. Absorbance at 530 nm in a 1-cm cell was measured immediately on a Gilford 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH, USA). Standards, made by diluting a chlorine stock solution (Hach Company), were treated in the same manner as the samples.

The main carbon source in the medium, starch, was monitored as anthrone reactive carbohydrate [17]. Samples for carbohydrate determination were taken from the eluate (liquid that had passed through the microfiltration unit).

Biomass measurements

Optical density (OD) at 600 nm was measured to give estimates of total cell density (an OD of $1 \approx 250$ mg cell protein L^{-1}, unpublished data). Samples with OD_{600} above 0.4 were diluted by a factor that placed turbidity in the range of 0.1–0.4. Samples for particulate protein were prepared by centrifuging the culture, decanting the supernatant and freezing the pellet until the analysis was performed. The pellets were thawed and placed in 1 N NaOH. Measurements were performed according to the Lowry *et al* method [25] using a bovine serum albumin standard (Sigma, St Louis, MO, USA). Viable cell numbers were estimated using viable plate counts performed on agar plates composed of Medium 3 excluding the linear alkylbenzene sulfonate (Medium 3.1). OD_{600} and viable plate counts were performed within a few minutes of sample collection to reduce the effects of residual chlorine on biomass measurements.

Viable biomass was also monitored as ATP in a separate biomass recycle reactor perturbed with 200 mg L^{-1} sodium hypochlorite, a level which would give a moderate decrease in biomass [12]. Culture samples were centrifuged immediately following sampling to remove sodium hypochlorite prior to analysis. ATP was measured to provide more insight to changes in viable cells following a perturbation than can be seen by viable plate counts. ATP was extracted and analyzed as in Cook *et al* [7].

Metabolic activity

The oxygen consumption rate of the culture was measured immediately following sample collection with a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) as described by Konopka *et al* [20].

Community and metabolic diversity

Fatty acid methyl ester (FAME) analyses of microbial lipids were made for the 50 and 190 mg L^{-1} sodium hypochlorite cultures. Ten milliliters of culture were diluted 1:1 with sterile medium and centrifuged for 10–12 min at $7500 \times g$ immediately following sample collection. The supernatant was decanted and the pellet was washed, lyophilized and frozen until the extraction was performed. Approximately 10 mg of lyophilized culture were suspended in 10 ml water stored over chloroform. One milliliter of this suspension was extracted using a chloroform-methanol extraction and total lipids were fractionated using column chromatography [10,28]. The phospholipid fraction was esterified by mild alkaline methanolysis [15]. FAMEs were analyzed by gas chromatography (Hewlett Packard 5890 GC with FID; Wilmington, DE, USA) [29]. Data were collected with Varian Chem-Star Workstation software (Sugar Land, TX, USA). Fatty acids were identified using external standards (Matreya, Pleasant Gap, PA, USA; Supelco, Bellefonte, PA, USA) and quantified by an internal standard (nonadecanoic acid methyl ester).

For each sampling time, the data (averages of three replicates) were converted to % of the total fatty acid and principal component analysis (PCA) was used to compare fatty acid profiles between samples. A correlation matrix was constructed from the fatty acid profile data, eigenvalues were developed, and then eigenvectors determined. Multiple analyses were conducted in order to load factor 1 with the highest level of variance. Following these analyses, an approach using no rotation of data was selected. Loading scores for each sample point were also determined. The loading scores indicate which of the fatty acid molecules account for the majority of distribution of the treatment along the axis of the PCA graph. Analysis was conducted using Systat v.8 (Chicago, IL, USA).

Carbon source utilization patterns were determined using Biolog-GN microtiter plates (Biolog, Hayward, CA, USA). Samples for Biolog plate inoculation were taken before each perturbation and at four different times following the additions of sodium hypochlorite. A culture from the bioreactor was diluted to an OD between 0.15 and 0.25 with sterile 0.85% saline. Wells on the Biolog plates were filled with 140 μl of the cell suspension and incubated at 32°C for 24 h. Any wells which had the same color as the control were scored as negative, whereas wells with a purple color darker than the control were scored as positive.

Results and discussion

Bleach disinfection efficiency

The efficiency with which sodium hypochlorite killed bacteria from the biomass recycle reactor was estimated by adding varying amounts of sodium hypochlorite to 1 ml of culture removed from the biomass recycle reactor, and performing viable plate counts on agar Medium 3.1 immediately following a reaction time of 1 min. Sodium hypochlorite concentrations of approximately 2.5, 5, 25, and 50 mg L^{-1} killed 29%, 47%, 98%, and 99% of cells removed from the bioreactor, respectively. This information was used to determine the concentration of sodium hypochlorite that could cause a significant amount of stress when added to the biomass recycle reactor. The minimum concentration of sodium hypochlorite which caused a 99% reduction of viable cells was 50 mg L^{-1}. This concentration, along with 190 mg L^{-1} and 1000 mg L^{-1}, were used in the bioreactor perturbation experiments.

Biomass recycle reactor perturbations

Viable biomass: Following additions to the biomass recycle reactor of 50–1000 mg L^{-1} sodium hypochlorite, viable plate counts dropped by over 99% (Figure 1a). The decrease in plate counts was greater with increasing hypochlorite concentration. The ATP measurements from a separate 200 mg L^{-1} hypochlorite perturbation also indicate an initial drop in viable biomass in the first hour following the addition of bleach (Figure 2). ATP levels continued to decline from 1 h to 5 h, but at a slower rate. This slow decrease in ATP may be caused by a slower death rate resulting from the loss of chlorine from the system, or continued degradation of extracellular ATP that was released when bleach was added to the reactor.

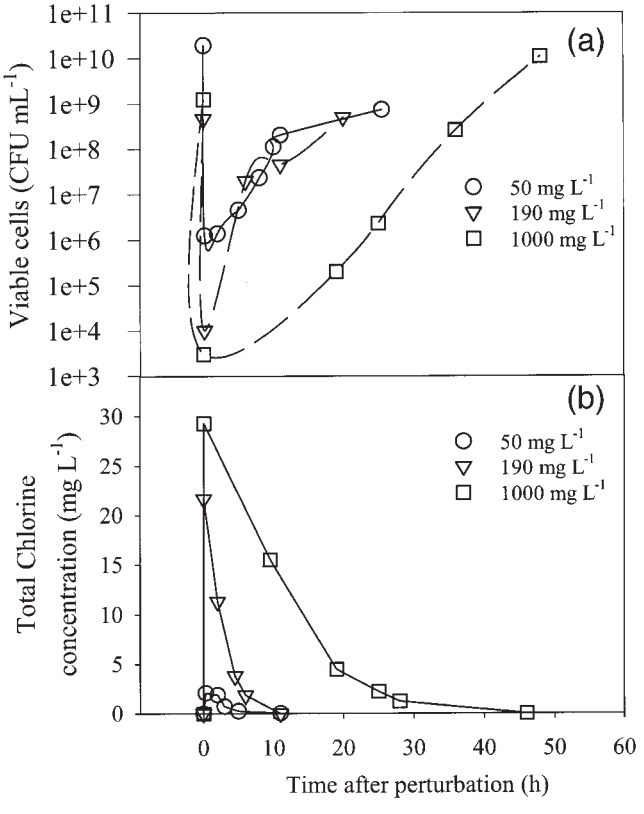

Figure 1 (a) Changes in viable cells measured as colony forming units (CFU) ml^{-1} for a biomass recycle reactor culture following perturbations with 50, 190, and 1000 mg L^{-1} sodium hypochlorite. (b) Concentrations of total chlorine (mg L^{-1}) measured in the ultrafiltration eluate of biomass recycle reactors perturbed with 50, 190, and 1000 mg L^{-1} sodium hypochlorite.

As chlorine concentration in the eluate decreased, cells resumed growth as seen by increasing viable counts and ATP. Growth rates calculated from plate counts from the 50, 190, and 1000 mg L^{-1} perturbations were 0.565, 0.236, and 0.325 h^{-1}, respectively during the recovery period. There appeared to be a biphasic increase in ATP (first order rate constants = 0.05 h^{-1} from 4.5–7 h after perturbation, and 0.24 h^{-1} from 7–12 h after perturbation) before the concentration stabilized near the original level. A similar growth pattern was seen for biomass recycle reactor cells in nutrient shift-up experiments, although the mechanism responsible is not clear [20].

Chlorine was not detected at 11 h for both the 50 mg L^{-1} and 190 mg L^{-1} treatments, and at 48 h for the 1000 mg L^{-1} (Figure 1b). Following a period of growth, plate counts returned to near original levels once the chlorine level became undetectable. Recovery times for plate counts increased with higher sodium hypochlorite concentrations. It should be noted that the viable plate count taken at the end of the 1000 mg L^{-1} treatment exceeded the plate count taken before the perturbation. The bioreactor cells may have been more culturable following recovery from the perturbation than they were before due to a change in physiological state. Before the perturbation, the cells were in a state of low reactivity and slow growth rate [20]. After the perturbation killed much of the biomass, more substrate was available per viable cell and growth was rapid during recovery. These cells may have been better able to grow on agar medium than the cells with low reactivity.

Microbial activity: The hypochlorite additions, which killed >99% of the bacteria, caused a decrease in microbial

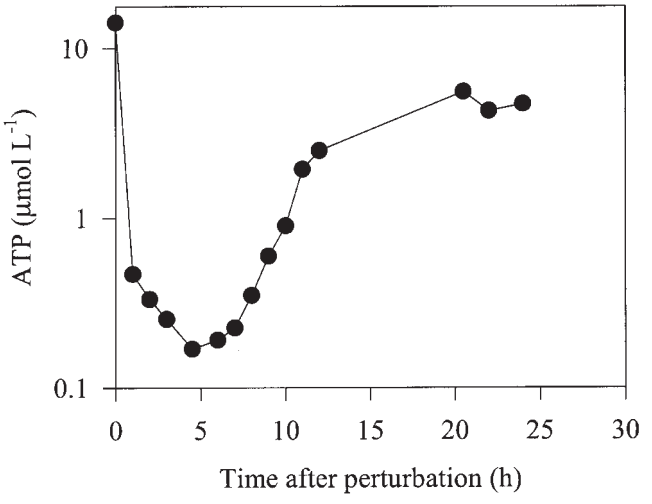

Figure 2 Concentrations of ATP (μmol L^{-1}) in samples taken from a biomass recycle reactor perturbed with 200 mg L^{-1} sodium hypochlorite.

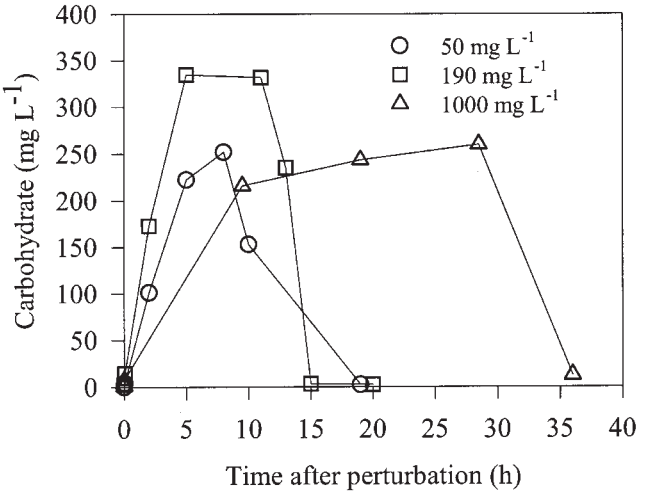

Figure 3 Changes in total carbohydrate in eluate collected from a biomass recycle reactor perturbed with 50, 190, and 1000 mg L^{-1} sodium hypochlorite.

activity. However, in each case the remaining microbes were able to reestablish the bioreactor system. Increases in carbohydrate coincided with the drop in viable plate count since there were not enough active cells left in the system able to degrade the substrate which entered the reactor (Figure 3). In addition, community respiration rates were low while carbohydrate levels were high (Figure 4). During this period, there were not enough viable cells remaining in the reactor to consume oxygen at a rate above the detection limit. Substrate oxidation inhibition [8] and oxygen consumption suppression [30] with exposure to chlorine have been noted previously. Both substrate consumption and community respiration returned to original levels as viable biomass recovered. While chlorine declined to levels below that which caused a 30% kill in the disinfection efficiency study, cells that survived the exposure to chlorine grew and degraded the protein and carbohydrate that had accumulated in the reactor. The higher biomass levels that resulted from this growth reconstituted a community respiration rate greater than or equal to that found before the perturbation.

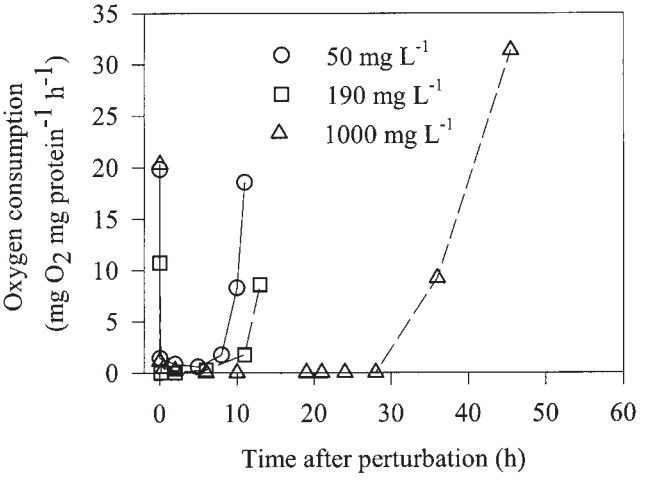

Figure 4 Oxygen consumption as a measure of respiratory activity of a culture in a biomass recycle reactor perturbed with 50, 190, and 1000 mg L^{-1} sodium hypochlorite.

Community diversity

There was concern that exposure to sodium hypochlorite might alter community structure by selecting for chlorine-resistant organisms, which may have different substrate utilization abilities. This selection might cause a subsequent change in graywater treatment efficiency. FAME profiles in conjunction with multivariate statistics can distinguish among microbial communities [16]. Disturbances to microbial communities may cause changes in biomass, community structure and physiology, all of which can be measured by FAME analysis [11]. The focus of our lipid analysis was to detect significant changes in community structure as a result of a bleach perturbation.

The majority of differences in fatty acid profiles of the bioreactor population taken for the 50 mg L^{-1} and 190 mg L^{-1} sodium hypochlorite perturbations can be described by the first two principle components (~60% of the total variance). PCA allows visualization of the variance in the data as expressed by best fitting a set of factor equations. A plot of factor 1 *vs* factor 2 shows separation between samples from the 50 mg L^{-1} sodium hypochlorite treatment and samples from the 190 mg L^{-1} treatment along the PC axis 1 (44% of the variance) (Figure 5). Loading along this axis is driven by differences in the amounts of 16:0, i17:0, cy17:0, and 14:0, and to a lesser degree by 18:2ω9,12, 16:1ω9c, and i14:0 fatty acids. These comprise higher percentages of the total fatty acids in the 190 mg L^{-1} sodium hypochlorite perturbation. Many of these fatty acids are typical of most eubacteria; the iso branched-chain saturated fatty acids are considered indicative of Gram-positive bacteria [5,9]. The presence of the polyunsaturated fatty acid suggests that eukaryotes may play a larger role in the population present during the 190 mg L^{-1} sodium hypochlorite perturbation [5].

We suggest that these differences along factor 1 reflect changes occurring as a function of time in the reactor, rather than as a result of the addition of bleach. That is, there is a succession of bacterial types in the reactor with time. This conclusion takes into consideration that the bioreactor was run continuously for 20 days between the two perturbations. This succession is also illustrated by changes in FAME profiles from a reactor run without perturbation (Figure 6). FAME profiles from days 13–18 of an unperturbed reactor run are different from earlier days (32% of the variance on PC axis 1). This variance, like that seen between the 50 mg L^{-1} and 190 mg L^{-1} sodium hypochlorite perturbations, is driven primarily by the smaller saturated fatty acids (branched and unbranched). The relative amounts of these fatty acids are higher than in the earlier days of biomass recycle operation.

Changes in fatty acid profiles resulting from the perturbations are expressed along factor 2. Differences in the levels of cy19:0, 18:0, 10Me18:0 and 18:1ω9+/11c are controlling separation. Generally, the fatty acids controlling the variance for factor 2 are at lower relative amounts at the end of the 50 mg L^{-1} and the beginning of the 190 mg L^{-1} perturbations. These changes are likely a result of bleach addition.

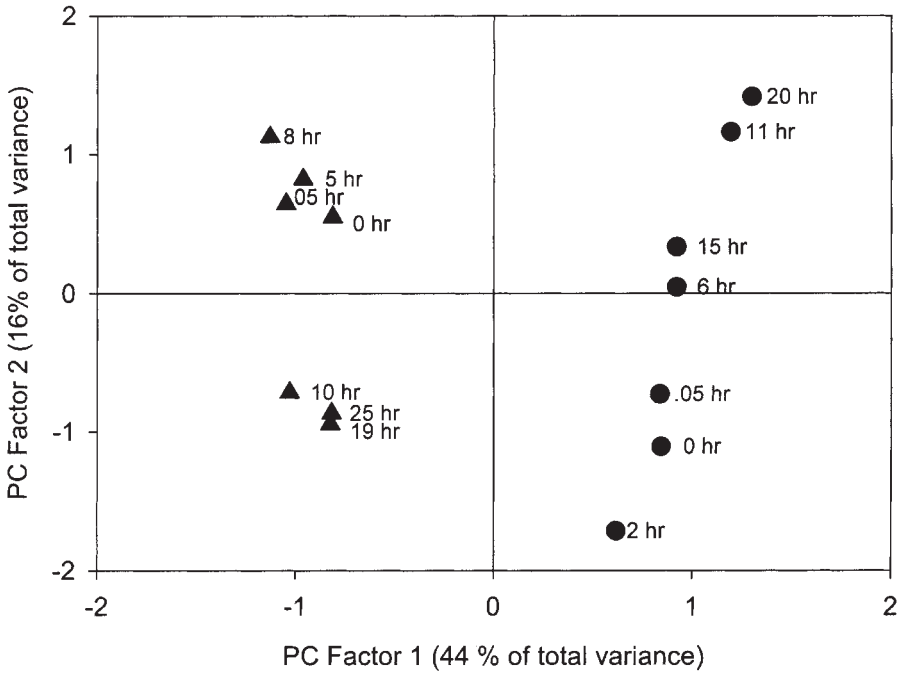

Figure 5 Principle component analysis (PCA) plot (factor 1 *vs* factor 2) of phospholipid fatty acid (PLFA) profiles for the 50 mg L^{-1} (▲) and 190 mg L^{-1} (●) sodium hypochlorite perturbations. Times denote sampling time (in hours) following addition of sodium hypochlorite.

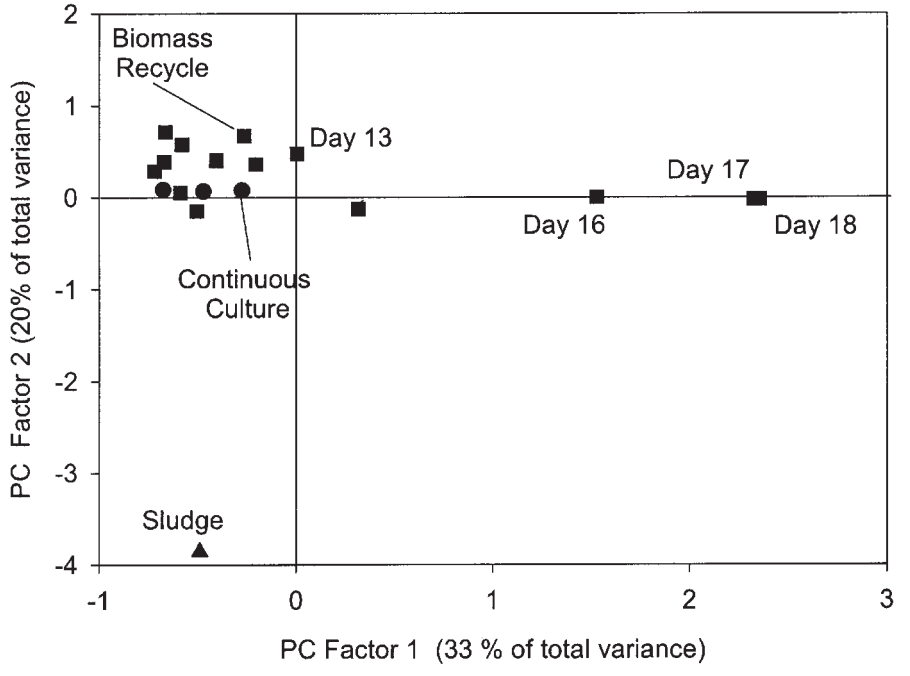

Figure 6 PCA plot (factor 1 *vs* factor 2) of PLFA profiles from a bioreactor run as a continuous culture (●), days 1–3, followed by a switch to biomass recycle operation (■) for days 4–18. A PLFA profile from the sludge (▲) used to inoculate the bioreactor is also plotted.

From our data it is clear that the bioreactor populations change over time and, as a result, different fatty acid profiles are present at the start of each perturbation. This reflects the dynamic nature of the biomass recycle unit. In addition, our data indicate that over the 20–25 h perturbation experiments, bleach caused a less significant shift in the fatty acid profiles. These community changes, likely a result of selection for more chlorine-resistant strains, are not as important to population dynamics as the changes seen over time. The shifts in community composition come without any consequential change in microbial ability as the biochemical process rates following the perturbations were at or above the rate found prior to the perturbation.

Environmental stresses that alter the metabolic status of bacteria, such as desiccation and starvation, can trigger changes in the ratios of *trans* to *cis* isomers of fatty acids

[14]. Changes in the ratio of 16:1ω9t to 16:1ω9c were considered as a possible indicator of stress during the perturbation experiments. *Trans*/*cis* ratios did not vary much for the 50 mg L^{-1} sodium hypochlorite perturbation, but changes were noted for the 190 mg L^{-1} treatment (Figure 7). The *trans*/*cis* ratio in the 190 mg L^{-1} hypochlorite perturbation was high before the addition of bleach (time = 0) and gradually decreased over time (the sharp drop in *trans*/*cis* shown at 5 min following the perturbation is likely an experimental artifact).

Other studies found that higher *trans*/*cis* ratios correspond to periods of stress [14], therefore we expected to see ratios increase following the addition of bleach. This was not the case, however. We believe that the sodium hypochlorite addition relieved some of the nutrient deprivation stress placed on the cells by the biomass recycle conditions. This 'stress relief' concept may also be supported by our results that showed an increase in culturability of cells following the 1000 mg L^{-1} perturbation. It is reasonable to assume that bacteria cultured in biomass recycle for an extended period are under more stress than those present at the onset of biomass recycle conditions when cell density is lower. We know from previous work that a biomass recycle reactor population's reactivity decreases with longer bacterial residence times and higher biomass [21]. The addition of bleach killed a large portion of the population, resulted in higher amounts of substrate available per cell and the *trans*/*cis* ratios began to decline. Ratios continued to decline throughout the period of exponential growth when substrate availability was high. The ratios did not begin to increase again until the biomass had recovered to the original level (20 h).

Carbon source utilization

Biolog-GN patterns were used to determine if exposure to bleach would cause a shift in the ability of the community to use a diverse number of substrates, thus resulting in lower graywater treatment efficiency. Approximately 80% of the Biolog-GN substrates were utilized by the biomass in every sample taken from each perturbation. The remaining substrates were utilized to varying degrees. When determining differences in metabolic capacities based on Biolog patterns, it is important to note which individual substrates produced positive responses and how each affects the overall interpretation of metabolic diversity of the community [19]. Although responses for a few of the substrate wells varied from plate to plate, 77 of the same substrates present in the Biolog-GN plates were utilized in all of the samples. This indicates stability in the metabolic capabilities of the bioreactor community since bacteria were still able to use a wide range of carbohydrates, amino acids, polymers, and carboxylic acids and recover from the bleach perturbations.

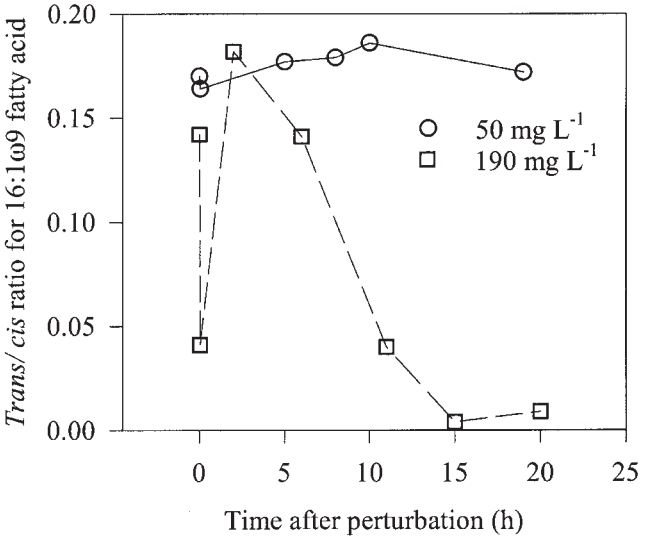

Figure 7 Changes in *trans/cis* isomer ratios for 16:1ω9 fatty acid used to monitor changes in nutritional status after perturbation of biomass recycle reactor with 50 or 190 mg L^{-1} sodium hypochlorite.

Conclusions

Despite decreases in activity and biomass, as well as some changes in community composition, the biomass recycle reactor regained operating efficiency after each perturbation. The low growth rate imposed by the carbon substrate-limited conditions in the biomass recycle reactor may have contributed to the resilience of the culture. Although the mechanism for resistance in the biomass recycle reactor is unclear, starved cells are similarly resistant to stress [27]. *E. coli* O157:H7 becomes more resistant to chlorine disinfection with longer starvation periods [24]. Nystrom *et al* [27] found that certain proteins are produced in response to starvation and may contribute to resistance to heat, UV and cadmium chloride in a marine bacterium. The physiological state of cells in the biomass recycle reactor closely resembles that of cells found in natural and treated waters, which display increased biocide resistance compared to cells grown in nutrient-rich culture. Kuchta *et al* [23] found that *Legionella pneumophilia* grown in substrate-limited tap water were more resistant to chlorine than those grown on nutrient rich media. Biocide resistance by growth-limited organisms has also been seen for *Pseudomonas* sp [3] and mycobacteria [4]. The mechanisms for survival may be similar in such cells and ones from the biomass recycle reactor.

The low growth rate imposed by nutrient-limited conditions may bring about a physiological change that results in resistance. These physiological alterations most likely involve the cell envelope, which can provide a barrier between the bactericide and intracellular components [2,23]. Gilbert and Brown [13] showed that the envelope composition changed in response to nutrient availability and affected its barrier qualities. Lipopolysaccharides, outer membrane proteins, extracellular polysaccharides, and phospholipid-fatty acids found on the outer portion of the cells may contribute to biocide resistance [2]. Lisle *et al* [24] suggested that chlorine resistance in starved *E. coli* is biphasic, with the components of the cellular envelope interacting first with the biocide and reducing the concentration that enters the cells. Intracellular components are then able to repair damage caused by the sublethal concentration of chlorine. Determination of the biochemical mechanisms contributing to resistance of individual cells in the biomass recycle system is beyond the scope of this study.

It was established that the biomass recycle reactor

employed in this experiment is useful as a graywater treatment system for starch-based waste streams such as found on passenger ships [20]. Short HRT, quick response to substrate pulses and low sludge production are among the advantages of biomass recycle reactors. This investigation further demonstrates the usefulness of biomass recycle systems for waste water treatment by showing that it can rapidly recover from a perturbation which kills over 99% of its biomass. These findings suggest that the biomass recycle reactor is particularly well suited to situations where continuous monitoring of the effluent is not practical as the system will resume normal function within a short period of time and before any problems may be detected.

Acknowledgements

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