The Effect of Klozur®CR on Sulfate-Reducing Bacteria (SRB) in Sediments from the Kalamazoo River

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Purpose

Three doses of Klozur®CR were added to slurry reactors containing sediment from the Kalamazoo River Superfund site contaminated with PAHs, PCBs, and mercury. The purpose of these studies was to investigate the affect of Klozur®CR on sulfate-reducing bacteria (SRB) indigenous to the sediments. The specific goals of this work were to quantify the affect of each dose on:

1) chemical oxidation of PCBs and PAHs,
2) numbers of culturable SRB,
3) relative abundance of SRB in the sediments relative to total bacteria, measured using molecular (i.e., 16S rRNA) probes.

The data in this report were collected over an 8-week period, which began with oxidant addition on October 3, 2008, and continued until the 8-week sample was taken on November 28th.

Materials and Methods

Reactors and Sediment Slurry

The slurry reactors consisted of 14 L closed, glass vessels. The lid had a central port housing a propeller-type mechanical mixer set at 200 rpm, and 3 additional ports. Four batch reactors were maintained; a control and three test reactors which received different doses of Klozur®CR. The slurry volume in each reactor was 10 L, with a solids content of 50% (w/v), meaning that each reactor contained 5 kg (dry weight) of sediment. Assuming a density of the sediment solids of 2.65 g/cm^3, each reactor contained approximately 2 L of dry solids and 8 L of water. Tap water was used as make up water. The sediment used in these studies came from the Kalamazoo River Superfund Site near Allegan, Michigan. The sediment was first homogenized in a cement mixer before being split into 4 aliquots, to ensure that the slurry in each reactor was similar. Table 1 shows the selected properties measured in the homogenized sediment. Soil analyses were conducted according to standard methods (ASA, 1994).

Three different doses of Klozur®CR were added per reactor 20 g, 50 g, and 100 g. The equivalent doses in terms of g of oxidant per g of dry sediment were; 4 g Klozur®CR /kg, 10 gKlozur®CR/kg, and 20 g Klozur®CR /kg (see Table 3). The reactors were maintained for 8 weeks, and were kept in an anaerobic chamber (N_2 gas), at 15°C. The pH was measured daily by inserting a pH probe into the slurry.

Analyses of PCBs PAHs and Persulfate

PCBs were extracted and analyzed according to the methods described by Cassidy et al. (2002). PAHs were extracted and quantified according to the procedures described by Cassidy and Hudak (2001). The persulfate anion was also quantified in filtrate (0.45 μm) samples using the iodometric titration method (Kolthoff and Stenger, 1947).

Table 1. Selected properties of the sediment slurry used in the studies.

Characteristic	Value
pH	6.8
Native organic carbon content (%)	4.5
Sand (%)	11
Silt (%)	43
Clay (%)	46
Culturable SRB (log MPN/g)	4.88 ± (0.41)[a]
PCB concentration (mg/kg)	7720
PAH concentration (mg/kg)	9063
Hg concentration (mg/kg)	175

[a] arithmetic mean ± (standard deviation).

Extraction and Hybridization of rRNA

RNA was extracted from the sediments using the bead-beating technique described by Devereux et al. (1992), and nucleic acids were further purified with Sephadex G25 spin columns according to Moran et al. (1993). RNA was denatured by adding 3 volumes of 2% (vol/vol) glutaraldehyde in 50 mM sodium phosphate (pH 7.0) to 1 volume of RNA extract and incubating at room temperature for 10 min (55). Denatured RNA was then diluted with sterile distilled H_2O containing 0.0002% (wt/vol) bromophenol blue and 1 μg of poly(A) ml^{-1}. Using a slot blot device (Minifold II; Schleicher and Schuell, Inc., Keene, N.H.) under slight vacuum, the various dilutions of sample and standard RNAs (in a volume of 100 ml) were applied to Immobilon-N membranes (Millipore Corp., Bedford, Mass.) that had been pre-wetted in 95% (vol/vol) ethanol and rinsed in distilled H_2O. Membranes were then dried at room temperature and baked at 80°C for 1 h prior to pre-hybridization and subsequent hybridization according to Stahl et al. (1988).

Molecular Probing

Background work on the test sediments had previously identified *Desulfovibrio*, *Desulfobacteriaceae,* and *Desulfobulbus* as the three predominant genera of SRB. These genera and the molecular probes used to identify them are listed in Table 2.

Oligonucleotide probes were end labeled with ^{32}P, purified with Nensorb 20 cartridges (Dupont Corp., Wilmington, Del.), and hybridized at 40°C overnight (Devereux et al, 1992). After the membranes were washed, they were briefly air dried and the amount of probe was quantified with a gas proportional radioisotope detection system (Ambis, Inc., San Diego, California).

Two sets of hybridization membranes were used for slot blot analysis. One membrane was hybridized with a probe specific for a particular bacterial group or genus, while the other utilized a general probe designed to hybridize with 16S rRNA of practically all species in the domain *Bacteria* (EUB338) (Stahl et al., 1988) (Table 2). The specific probes utilized were SRB probes 687 (for the family *Desulfovibrionaceae*), 660 (for *Desulfobulbus* spp.), and 804 (for most members of the family *Desulfobacteriaceae*). Samples were added to membranes at three concentrations, with 50 to 200 ng per slot used for membranes assayed with the *Bacteria* probe and 600 to 1,800 ng per slot used on membranes for specific probes. Membranes assayed with the specific probes received reference rRNA extracted from pure cultures (Table 2) at a range of approxiatemly 3 to 25 ng per slot to generate a standard curve. Membranes hybridized with the general *Bacteria* probe (EUB338) received approximately 5 to 200 ng per blot of the same reference rRNA material that was used for the specific probes.

Table 2. The 16S rRNA oligonucleotide probes and target groups.

Target	Probe	Probe Sequence	Target Site[a]	Reference RNA
Bacteria Domain	804[b]	CAACGTTTACTGCGTGGA	6.6-6.9	
Desulfovibrio spp.	687[b]	TACGCATTTCACTTCCT	9.2-9.7	*Desulfovibrio piger* *Desulfovibrio vulgaris*
Desulfobacteriaceae spp.	660[b]	GAATTCCACTTTCCCCTCTG	10.5-10.9	*Desulfobotulus sapovorans* *Desulfobacterium vacuolatum*
Desulfobulbus spp.	338[c]	GCTGCCTCCCTAGGAGT	11.9-12.4	*Desulfobulbus elongatus* *Desulfobulbus propionicus*

[a] numbering relative to *Escherichia Coli*.
[b] Devereux et al. (1992).
[c] Stahl et al. (1988).

Relative Abundance (RA)

The relative abundance (RA) of the specific probe targets as a function of total *Bacteria* rRNA were determined by first quantifying radioactive signal per slot and correcting for background. The following equation was used to calculate RA: RA (%) = $[(m_{ss} \times m_{sr})/(m_{es} \times m_{er})] \times 100$, where m_{ss} is the slope of specific probe signal per unit of sample rRNA, m_{sr} is the slope of the specific probe signal per unit of reference rRNA, m_{es} is the slope of the *Bacteria* probe signal per unit of sample rRNA, and m_{er} is the slope of the

Bacteria probe signal per unit of reference rRNA. Samples having a non-linear slope of probe signal per unit of rRNA (i.e., $r^2 < 0.90$) were omitted from analyses.

Most Probable Number (MPN)

SRB in sediment slurry samples were enumerated by the most probable number (MPN) technique. A 10 mL sediment sample was transferred to a sterile blender. The medium of Widdel and Pfennig (64), without Na_2SO_4 or an electron donor, was used to rinse residual sample into the blender and to prepare a 1-in-10 dilution (weight/volume) of the sediment. This dilution blended for 5 min. to homogenize, and the homogenate was aseptically transferred to a sterile serum bottle, sealed, and reduced. This dilution was the inoculum for a quadruplicate MPN dilution series (10^{-2} to 10^{-9}) in tubes sealed with serum stoppers. The medium of Widdel and Pfennig (1981) was also used for MPN determination. To ensure a suitable electron donor for any SRB, the medium contained: 10 mM acetate; 10 mM ethanol, 5 mM benzoate; 10 mM butyrate, 10 mM malate, and 10 mM propionate. Incubations were at 20°C. Growth was determined as the increase in optical density at a wavelength of 600 nm. Sulfate removal was confirmed by ion chromatography (IC). Tubes showing both growth and $SO4^{2-}$ consumption were considered positive and were used to calculate the relative abundance of SRB per gram of sediment in the sample.

Results and Discussion

The three doses of Klozur®CR added per reactor, the equivalent doses per g of dry sediment, and the range in pH values over the 8 week test period are listed in Table 3. The reactors were maintained for 8 weeks, and were kept in an anaerobic chamber (N_2 gas), at 15°C. Table 2 lists the doses and the resulting pH values for each dose. The data in Table 3 show that the pH increased with increasing dose of Klozur®CR. This is expected, because the (50% by weight) calcium peroxide in Klozur®CR drives the pH higher as the dose increases. The high pH caused by the calcium peroxide is what activates the sodium persulfate to form the sulfate radical.

Table 3. The three doses of Klozur®CR used, and the resulting range in pH.

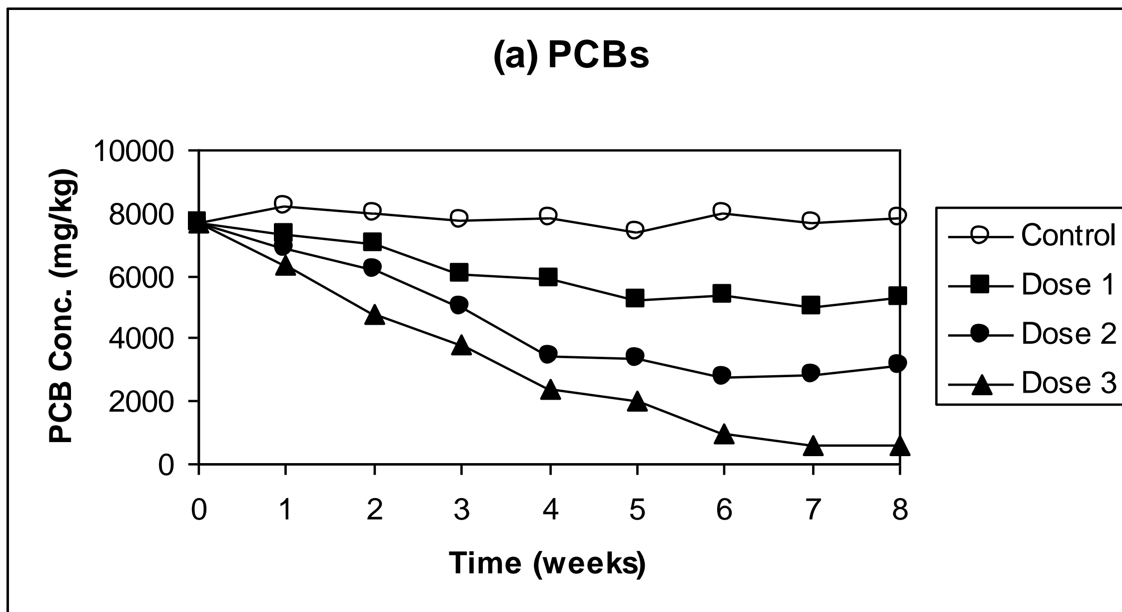
Dose	Klozur®CR added per reactor (g)	Klozur®CR added per mass of sediment (g/kg)	pH
Control	0	0	6.6-6.9
Dose 1	20	4	9.2-9.7
Dose 2	50	10	10.5-10.9
Dose 3	100	20	11.9-12.4

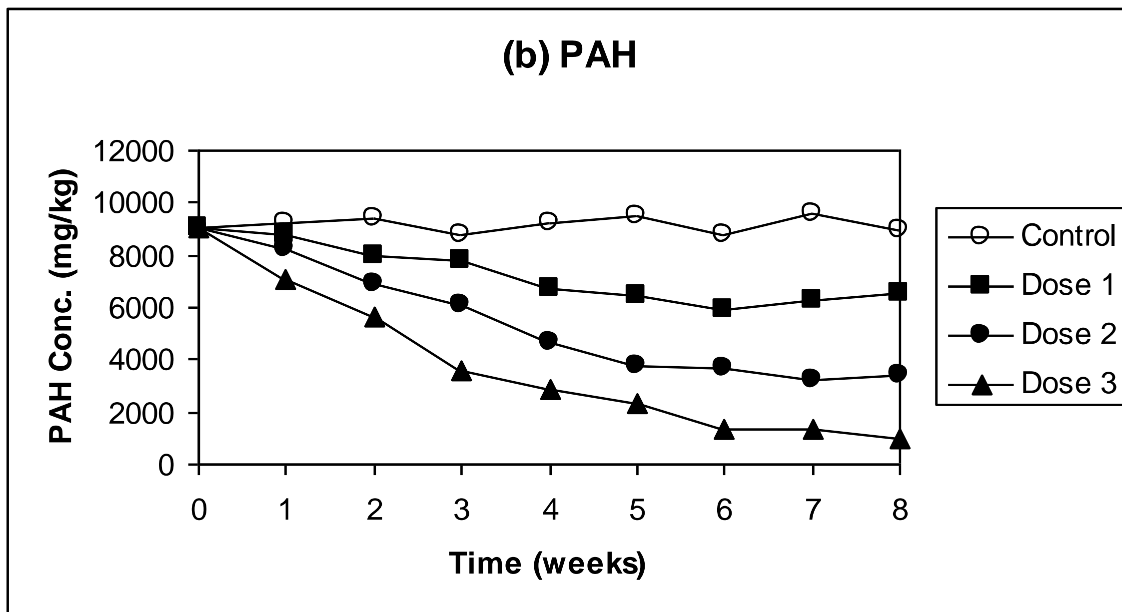
Removal of PCBs and PAHs

Figure 1 shows the concentrations of PCBs and PAHs with time for the control reactor and for the three doses of Klozur®CR. The control reactors showed no significant

reduction in PCBs or PAHs. This is not surprising, since neither PCBs nor PAHs would be expected to undergo significant anaerobic biodegradation in the slurries over an 8 week period. Increasing the dose of Klozur®CR resulted in increased removal of both PCBs and PAHs during the 8 week period. Both PCBs and PAHs have been previously shown to be chemically oxidized by persulfate. Increasing contaminant destruction with increasing oxidant dose is expected, and is also consistent with the higher pH values observed with increasing oxidant dose (Table 3), which enhances the alkaline activation of the persulfate in Klozur®CR.

Figure 1 Concentrations of (a) PCBs and (b) PAHs in the reactors.





Persulfate Concentrations
Persulfate concentrations in the three test reactors during the 8 week study period are shown in Figure 2. It is clear from Figure 2 that the rate of persulfate activation increased with increasing dose. For Dose 1, Dose 2, and Dose 3, the Persulfate concentrations were below detection after 4 weeks, 6 weeks, and 7 weeks, respectively.

Figure 2. Persulfate concentrations in the reactors.

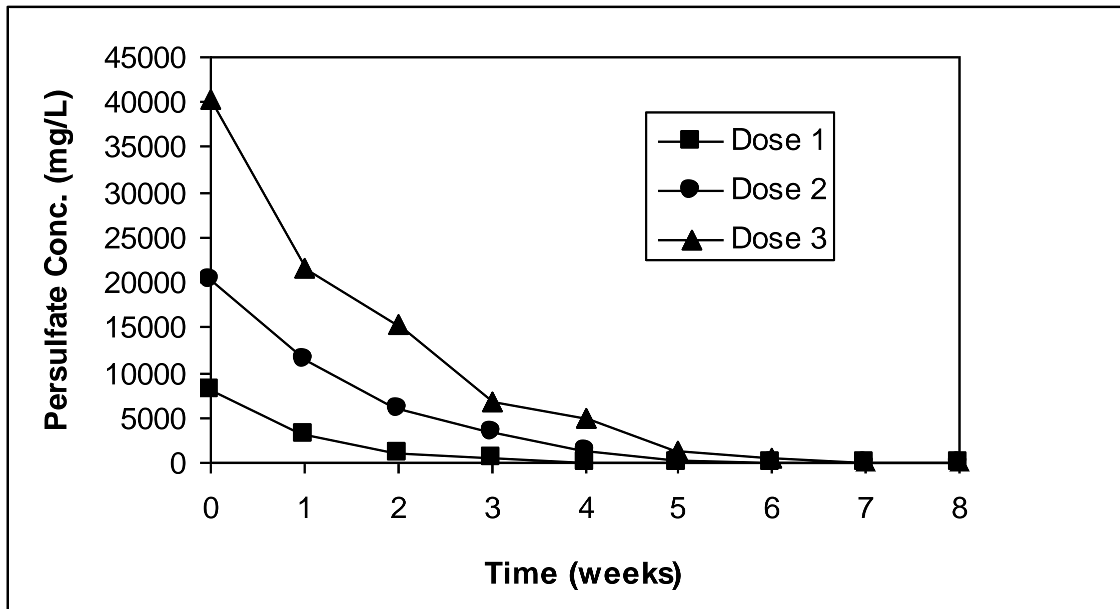


Table 4 lists the percent removal of PCBs and PAHs in the reactors. As shown in Figures 1 and 2, increasing dose of Klozur®CR increased the percent removal of the contaminants. Dose 3 resulted in the greatest extent of contaminant oxidation, 91% for PCBs and 88% for PAHs.

Table 4. Percent removal of PCBs and PAHs in the control and test reactors

Dose	**PCB removal (%)**	**PAH Removal (%)**
Control	0	0
Dose 1	31	27
Dose 2	59	61
Dose 3	91	88

Affect of Klozur®CR on SRB

Figure 3 shows the results for MPN analyses of sulfate-reducing bacteria (SRB) in the reactors, with error bars to show the standard deviation. In the control, no significant change in MPN of SRB were observed. However, adding Klozur®CR did result in significant changes in MPN of SRB in the sediment. In general, as the dose of Klozur®CR increased, the MPN of SRB decreased. This is not surprising, since the radicals produced during oxidation with Klozur®CR (including the sulfate and hydroxyl radicals) can inhibit and/or kill micrrorganisms. Moreover, SRB are strict anaerobes, and the addition of Klozur®CR would certainly result in oxidative stress to the bacteria present in the sediments, including the release of molecular oxygen from the calcium peroxide present in Klozur®CR. Doses 1 and 2 resulted in an initial decrease in MPN during the first 3 weeks, followed by an increase in MPN after week 4. Dose 1 actually resulted in a greater MPN after 8 weeks than observed in the control, which was anaerobic throughout the 8 week period. This increase in MPN after an initial decrease is probably due to the accumulation of sulfate, which is the residual product of the persulfate in Klozur®CR. Sulfate is the preferred electron acceptor of SRB, and SRB can utilize PAHs as electron donors. In addition, chemical oxidation of PCBs and PAHs can result in the accumulation of low molecular weight fatty acids and alcohols (e.g., acetate, oxalate, propionate, ethanol) (Cassidy et al., 2002; Ndjou'ou and Cassidy, 2006), which are ideal electron donors for SRB. Concentrations of sulfide, the product of reduction of sulfate by SRB, were not included in this study.

Figure 3. MPN of sulfate-reducing bacteria (SRB) in the reactors. The error bars show the standard deviation of MPN from the quadruplicate analyses.

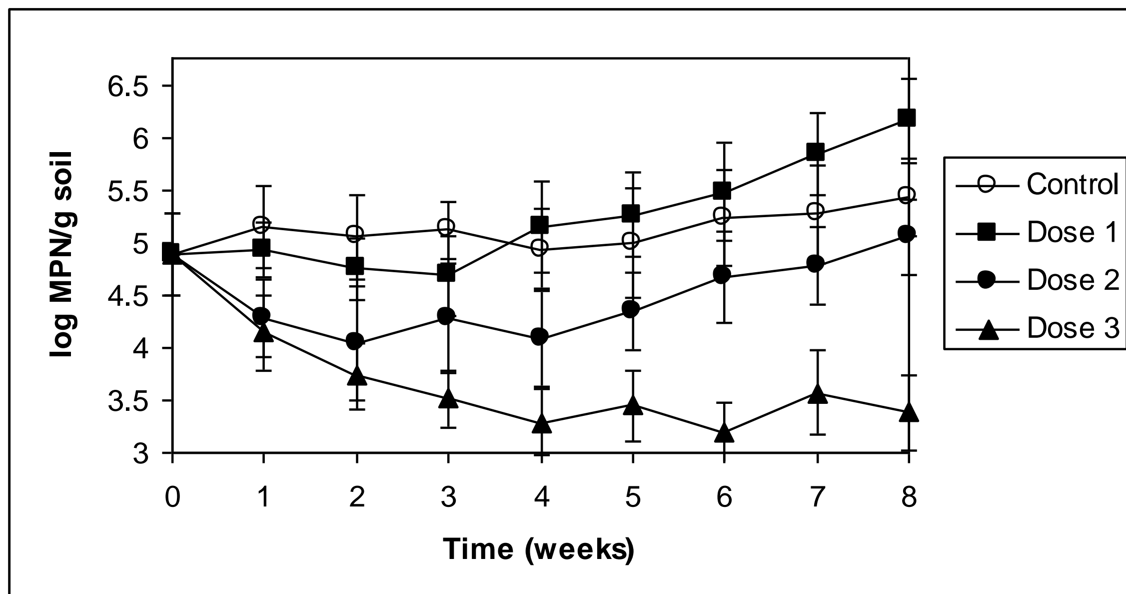
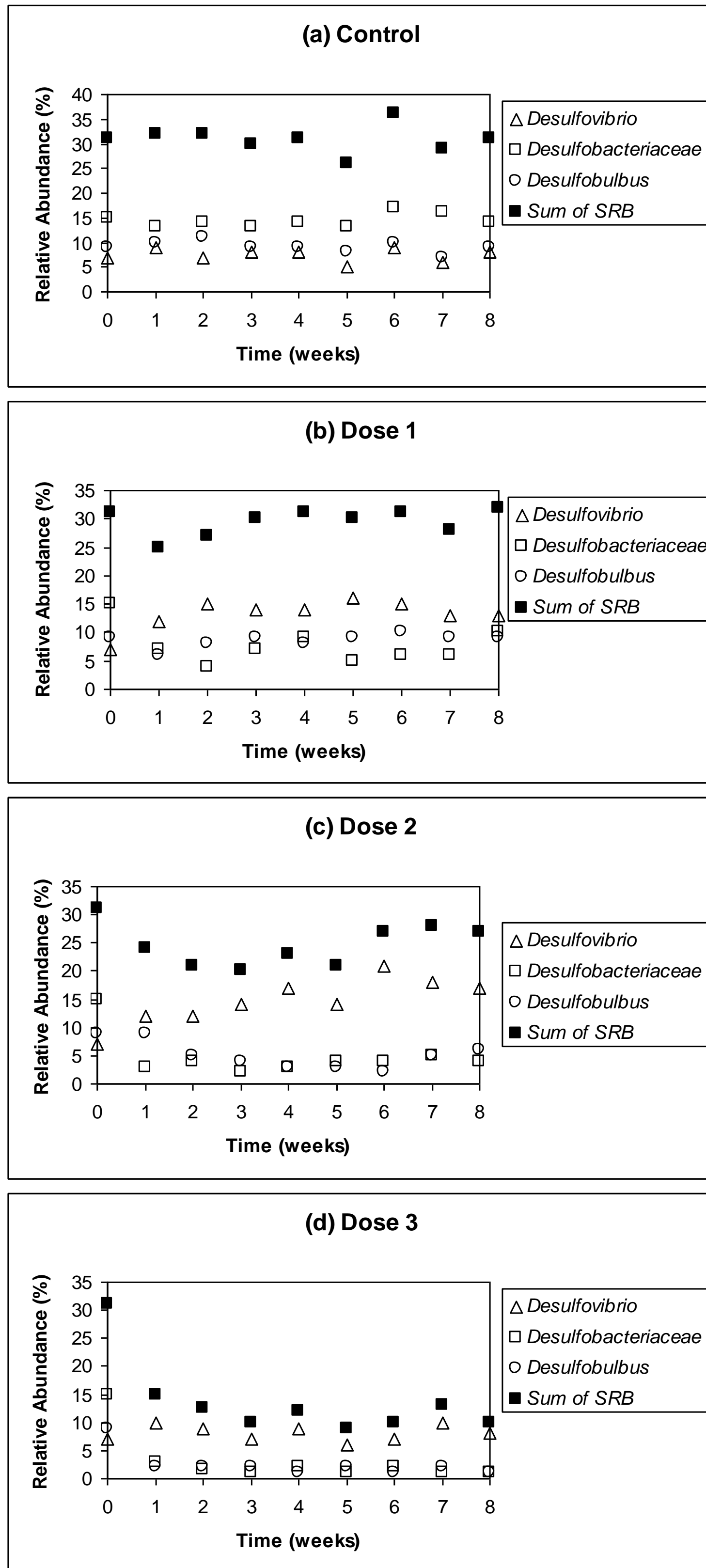


Figure 4 shows the relative abundance (RA) of each genus of SRB and their sum in the sediment during the 8 week study period for; (a) the control, (b) Dose 1, (c) Dose 2, and (d) Dose 3. Before treatment in the test reactors, and throughout the 8 week study period in the control, *Desulfobacteriaceae* was the most abundant genus of SRB at approximately 15%, whereas *Desulfovibrionaceae* and *Desulfobulbus* each comprised less than 10% of the total bacteria present in the sediment. The sum of the three genera comprised approximately 30% of the total baterial population in the sediment. This is typical of sediments, which have a much higher concentration of SRB than most soils. Dose 1 reduced the sum of SRB to between 25-30%, Dose 2 reduced the sum of SRB to between 20-25%, and Dose 3 reduced the sum of SRB to between 12-15%. These results are consistent with the MPN results (Figure 3) and can be explained by increasing death of SRB with increasing dose of Klozur®CR. A population shift within the SRB community in the sediments was also observed with increasing doses of Klozur®CR (Figure 4). *Desulfovibrionaceae*, which was the genus of SRB with the lowest RA in the sediments before treatment, became the predominant genus for all three doses of Klozur®CR. These data indicate that species of the genus *Desulfovibrionaceae* have a greater ability to survive, and even thrive during periods of oxidative stress than *Desulfobacteriaceae* and *Desulfobulbus* species.

The results from the measurements of most probable number (MPN) (Figure 3) and the relative abundance (RA) of oligonucleotide probes of SRB in the sediments (Figure 4) clearly show that SRB were not completely killed or inhibited by addition of Klozur®CR. In fact, after an initial decrease in MPN, the lowest dose of Klozur®CR resulted in a higher MPN of SRB relative to the control (Figure 3). These observations are consistent with recent review articles on SRB, which report that SRB, and particularly *Desulfovibrio* species, posses a variety of enzymes that act together to achieve an efficient defense against oxidative stresses (Dolla et al., 2006).

Figure 4. Relative abundance (RA) of each genus of SRB and their sum in the sediment with time for; (a) the Control, (b) Dose 1, (c) Dose 2, and (d) Dose 3.



Conclusions

Klozur®CR was effective at chemical oxidation of the contaminants in the sediments tested, removing 91% of PCBs and 88% of PAHs at the highest dose tested (20 g/kg sediment). The results from the measurements of most probable number (MPN) and the relative abundance (RA) of oligonucleotide probes of SRB in the sediments clearly show that SRB were not completely killed or inhibited by addition of Klozur®CR. In fact, after an initial decrease in MPN, the lowest dose of Klozur®CR resulted in a higher MPN of SRB relative to the control (Figure 3). *Desulfovibrio* species showed the greatest tolerance to Klozur®CR, but *Desulfobacteriaceae* and *Desulfobulbus* species also survived the highest dose of Klozur®CR.

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