Report for 2002AZ9G: Agricultural Chemicals as a Major Non-Point Source of Arsenic: Microbial Transformation of Organic Arsenicals

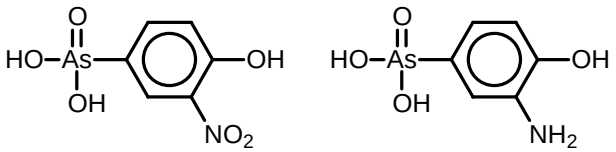
There are no reported publications resulting from this project.

Report Follows

Overview of Third Year's Activities

During the last year, research has focused on the biodegradation and toxicity of two nitrogen-substituted phenylarsenates. One of the compounds studied was roxarsone and the other compound was 4-hydroxy-3-aminophenylarsonic acid (HAPA), a biotransformation product of roxarsone. The structure of the two compounds are illustrated in Figure 1. Roxarsone is used extensively in the poultry industry and finds it way into the environment through land application of poultry manure. Additionally, the long term aerobic biodegradability of the herbicide, cacodylic acid (dimethylarsinic acid, DMA(V)), was evaluated in incubations with activated sludge.

Figure 1. Chemical structure of nitrogen substituted phenylarsenates: roxarsone and 4-hydroxy-3-aminophenylarsonic acid (HAPA).



roxarsone HAPA

Biodegradation of Nitrogen-Substituted Phenylarsenates.

Biotransformation of roxarsone to HAPA in anaerobic sludge. We previously reported the rapid reductive biotransformation of roxarsone to HAPA in microcosms inoculated with anaerobic sludge (2nd Annual Report). This year the research explored the environmental and physiological conditions for rapid bioconversion. Since the reaction requires the input of electrons, the role of different electron donating substrates on the rate of the bioconversion was examined as shown in Figure 2. Hydrogen, glucose and lactate supported the first, second and third fastest rates of biotransformation; respectively. Biotransformation also occurred at a modest rate in treatments not receiving any electron-donating substrate. Most likely due to the slow hydrolysis and degradation of biomass in the sludge, referred to as endogenous substrate. Acetate was a poor electron donating substrate, since it did not significantly increase the endogenous rate of roxarsone reduction. HAPA was shown to be formed stoichiometrically from the reduction of roxarsone. In the killed sludge control, a small fraction of roxarsone was converted to HAPA, most likely due to chemical reduction by reducing agents in the heat-killed sludge.

Reductive biotransformation reactions are known to be accelerated by extracellular redox shuttles. The impact of two extracellular shuttles, riboflavin and anthraquinone-2,6-disulfonate (AQDS) was evaluated. The electron shuttles were supplied at 0.05 mM to stimulate the reduction of 1 mM roxarsone by anaerobic sludge with hydrogen gas as the electron donor. The results of the experiment (Figure 3), demonstrate that AQDS and riboflavin significantly increase rates of roxarsone reduction. AQDS is often used as a model of quinone moieties in humus. Thus the results implicate humus as a possible electron shuttle that could dramatically increase the rates of roxarsone biotransformation.

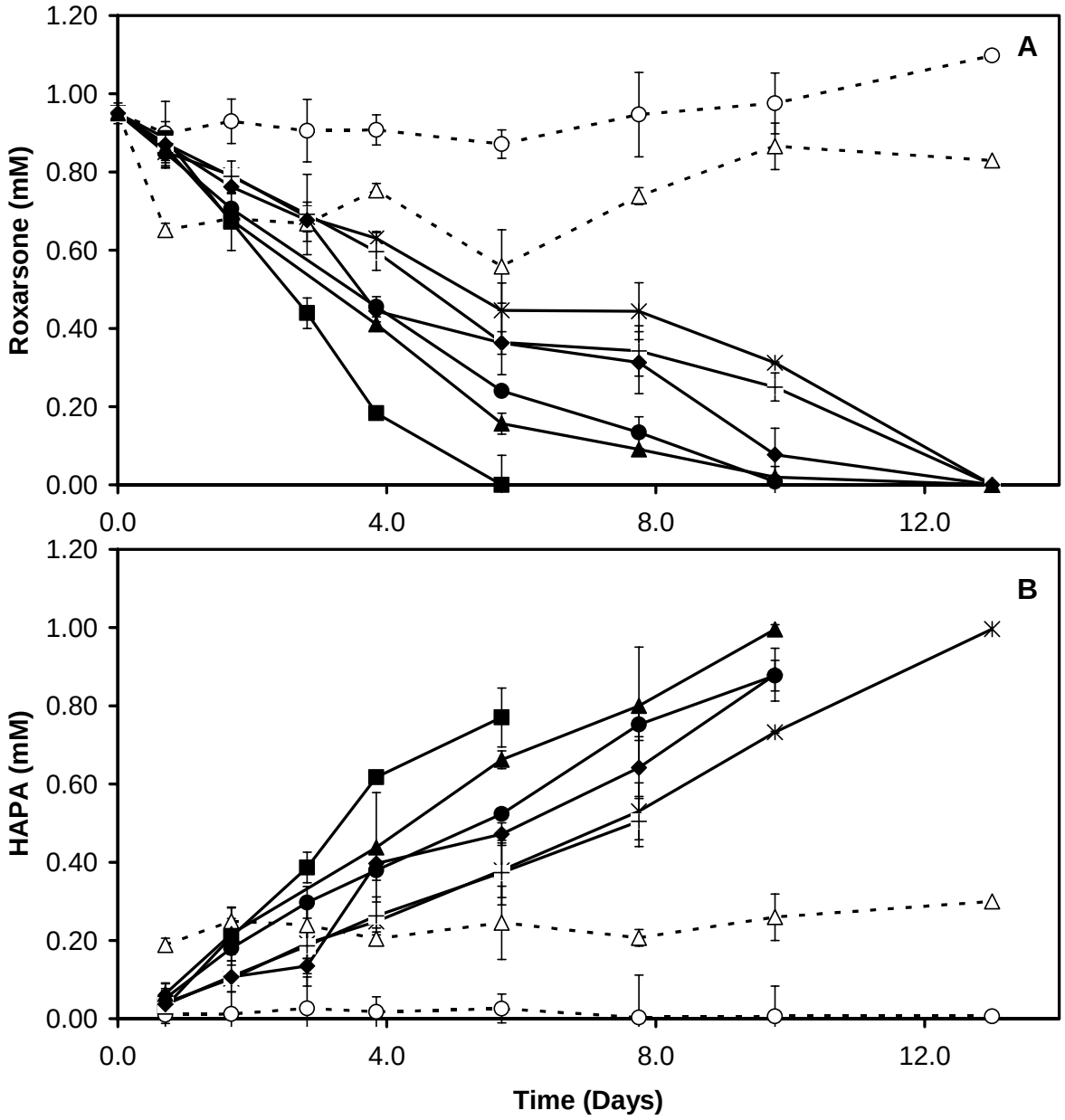


Figure 2. The role of electron-donating substrates on the time course of roxarsone biotransformation to HAPA in anaerobic sludge. The roxarsone and HAPA concentrations are shown in panels A and B; respectively. Legend: medium only control, open circles; killed sludge control, open triangles; no added electron donor, asterisk; acetate, plus sign; mixture of volatile fatty acids, closed diamonds; lactate, closed circles; glucose, closed triangles; hydrogen gas, closed squares

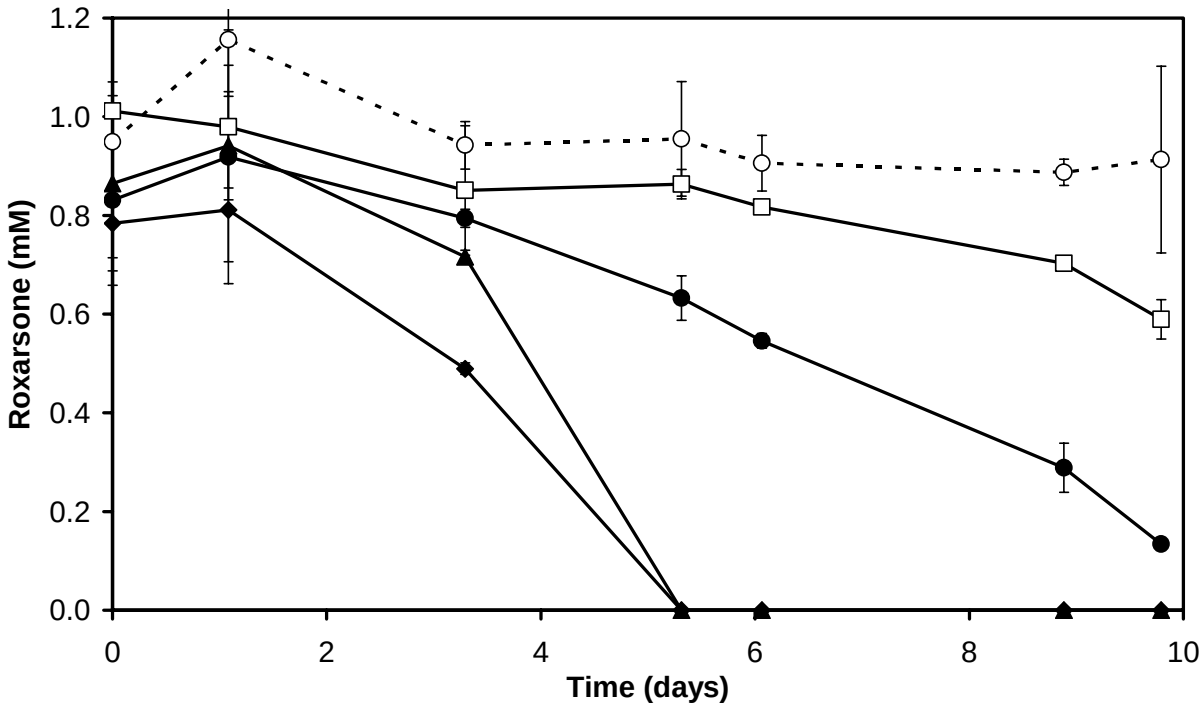


Figure 3. Effect of extracellular electron shuttles on the rate of roxarsone reduction in anaerobic sludge. The shuttles, riboflavin or anthraquinone-2,6-disulfonate (AQDS) were each supplied at 0.05 mM. Legend: medium only, open circles; no electron donor added control, open squares; hydrogen, closed circles; hydrogen and riboflavin, closed triangles; hydrogen and AQDS, closed diamonds.

Biotransformation of roxarsone to HAPA in aerobic sludge. The biodegradation of roxarsone was also evaluated under various redox conditions with aerobic activated sludge, obtained from a secondary treatment unit at Ina Road municipal wastewater treatment plant, Tucson. Biodegradation was compared under aerobic (O_2 added), denitrifying conditions (NO_3^- added) sulfate reducing (SO_4^{2-} added) and under methanogenic (no electron acceptor added) conditions as well as under methanogenic conditions with added lactate (as an electron donating substrate). The results (Figure 4) indicate that there was no bioconversion of roxarsone under aerobic and denitrifying conditions. Biotransformation was only evident under methanogenic and sulfate reducing conditions, with the highest level of biotransformation occurring in the methanogenic treatment with added lactate. HAPA was observed again as a biotransformation product, but it was not recovered stoichiometrically as was observed in the experiments with anaerobic sludge. Instead, HAPA only accounted for about 37 to 56% of the roxarsone eliminated. The lower than stoichiometric yield is not due to adsorption of arsenic on sludge or volatilization because total non-speciated arsenic concentrations were constant at 1 mM (which indicates that all arsenic was still present in solution). The missing arsenic was due to unidentified species (note that arsenate, arsenite and methylated species were monitored and not found). The results taken as a whole indicate that degradation does not occur readily under aerobic or denitrifying conditions. However an aerobic inoculum, can cause the reductive biotransformation of roxarsone to HAPA and other unidentified products when incubated anaerobically.

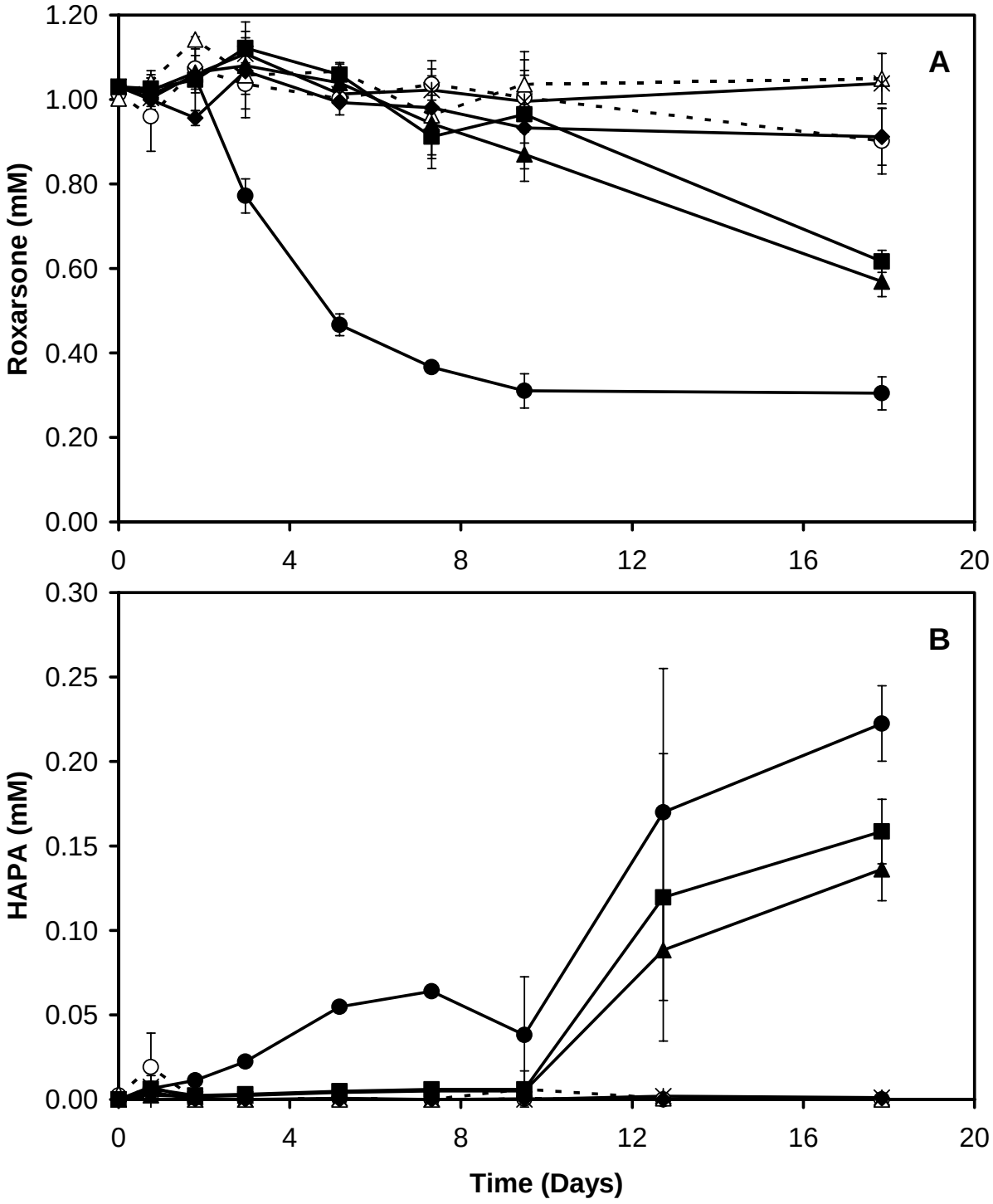


Figure 4. Biotransformation of roxarsone in aerobic activated sludge incubated under variable redox conditions. Roxarsone and HAPA concentrations are given in panels A and B; respectively. Legend: medium only control, open circles; killed sludge control, open triangles; aerobic, astericks; denitrifying, closed diamonds; sulfate reducing, closed triangle; methanogenic, closed square; methanogenic+lactate, closed circle.

Long-term incubation of roxarsone with anaerobic sludge. In various experiments with anaerobic sludge, roxarsone was rapidly converted to HAPA in stoichiometric amounts. However after long incubation periods (*eg* 120 days), HAPA that had previously accumulated was found to be largely eliminated (by 95.3%) as is shown in Figure 5. Two inorganic arsenicals, arsenate and arsenite were found to be formed. The combined recovery of arsenate and arsenite accounted for about 20.5% of the arsenic added initially to the system as roxarsone. The lower than stoichiometric yield was not due to adsorption of arsenic on the sludge or volatilization because total non-speciated arsenic concentrations were constant at 1 mM (which indicates that all arsenic was still present in solution). The remaining arsenic is therefore attributed to unidentified arsenic species in solution.

Long-term incubation of HAPA with anaerobic sludge. A similar experiment was conducted as that described in the paragraph above, except that HAPA was incubated for a long incubation period (of 113 days). The results of this experiment are shown in Figure 6 for incubations of 29 and 83 days, and in Figure 7 for the incubation of 113 days. After 29 days, HAPA was largely recovered. In the biologically active methanogenic and sulfate reducing treatments small eliminations were evident but these were of questionable significance. On days 83 and 113, HAPA levels dropped in all treatments and controls, but the decreases were significantly greater in the biologically active methanogenic and sulfate reducing treatments. The HAPA levels in the denitrifying treatment were similar to the medium only and killed sludge control on day 83, whereas the values declined a little more than the controls on day 113. Inorganic arsenicals (arsenate and arsenite) were shown to accumulate as a result of the decline in HAPA concentration. The increases were significantly greater in the biologically active treatments compared to the medium only and killed sludge control. However, as was indicated above, the recovery of inorganic arsenicals was only partial. The best recovery of inorganic arsenicals was 16.9% on day 83 and 24.7% on day 113 compared to the initial HAPA used at the beginning of the experiment. As was noted before, the remaining arsenic can be attributed to unidentified arsenic species in solution based on total non-speciated arsenic measurements. The loss of HAPA in the medium only and killed sludge controls would suggest that chemical mechanisms of HAPA transformation were occurring. The bottles were wrapped with aluminium foil, so photolysis reactions can be excluded. Nonetheless, a greater removal of HAPA and greater release of inorganic arsenicals in the biologically active treatments would indicate the involvement of biologically catalyzed mechanisms as well.

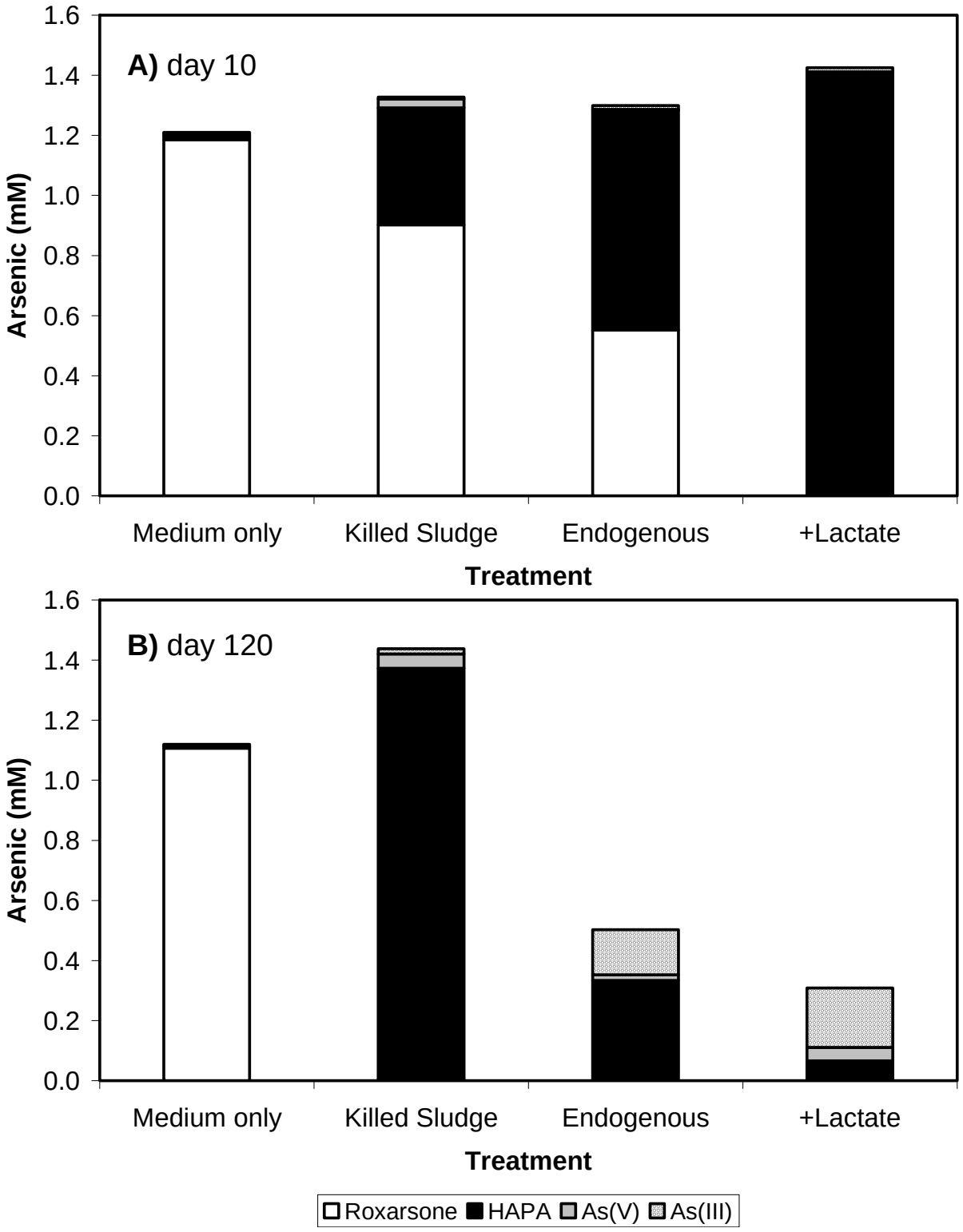


Figure 5. Long term incubation of roxarsone and recovery of products in anaerobic sludge under methanogenic conditions. The incubation was either supplemented with electron donating substrate (+lactate) or not (endogenous). The products recovered are shown after 10 and 120 days of incubation in panel A and B; respectively.

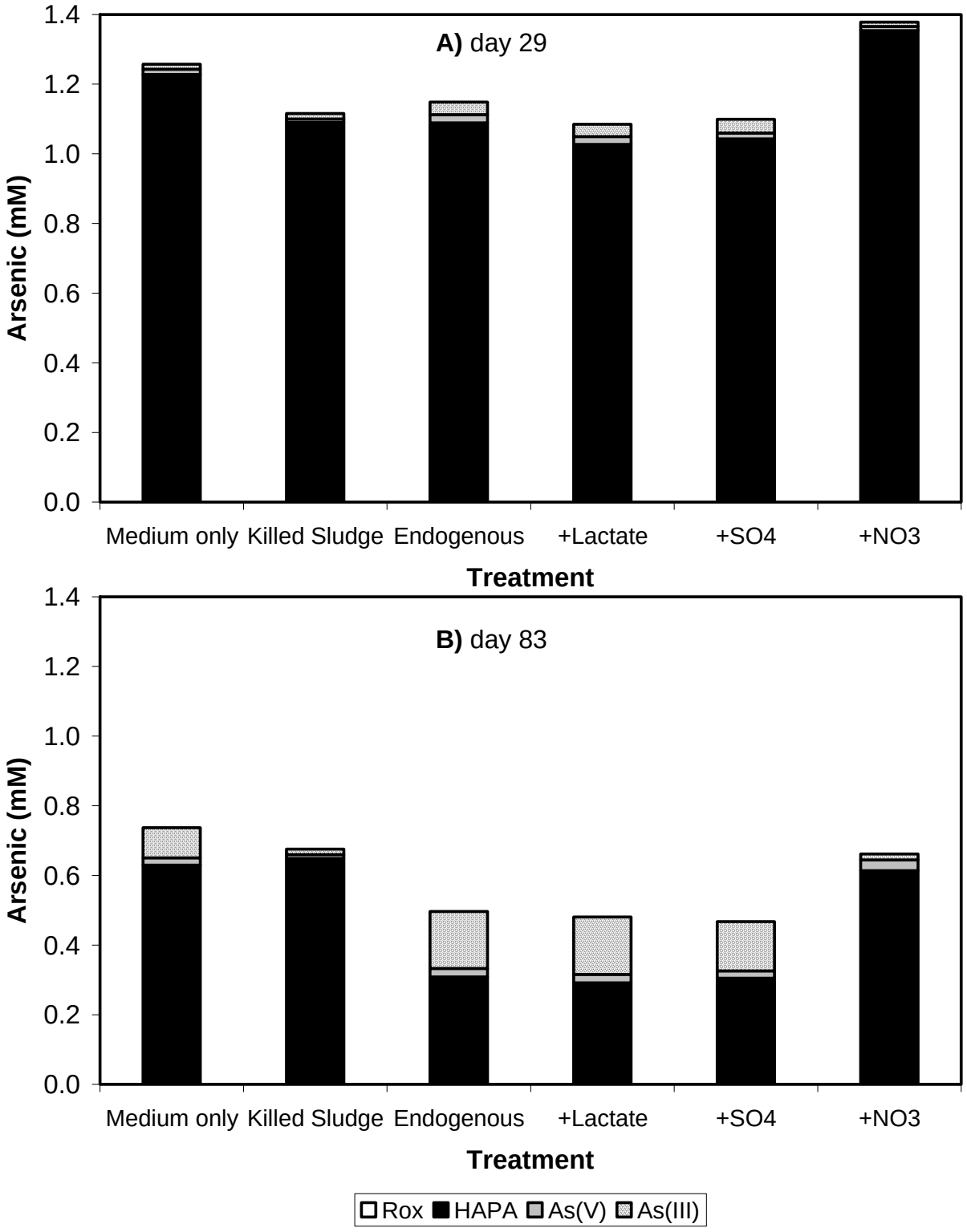


Figure 6. Long term incubation of HAPA and recovery of products in anaerobic sludge under methanogenic conditions, sulfate reducing and denitrifying conditions. The methanogenic incubation was either supplemented with electron donating substrate (+lactate) or not (endogenous). The products recovered are shown after 29 and 83 days of incubation in panel A and B; respectively.

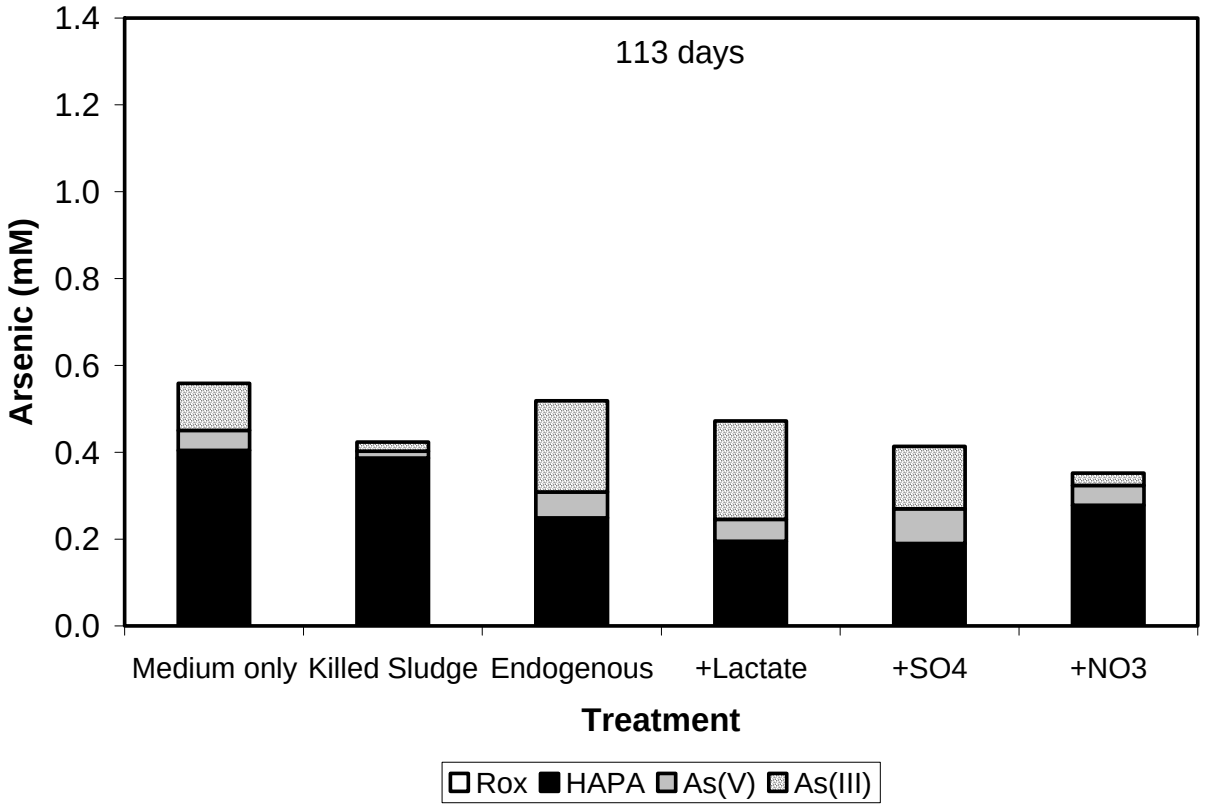


Figure 7. Long term incubation of HAPA and recovery of products in anaerobic sludge under methanogenic conditions, sulfate reducing and denitrifying conditions. The methanogenic incubation was either supplemented with electron donating substrate (+lactate) or not (endogenous). The products recovered are shown after 113days of incubation.

Toxicity of oxidized HAPA products.

During the research of this project, we have noticed that HAPA is very susceptible to autooxidation in air. The products of HAPA oxidation were observed to be toxic to microbial activities in the anaerobic sludge. The phenomenon was first noted when studying the toxicity of HAPA to methanogens. If the HAPA stock solution was prepared without the antioxidant (ascorbic acid), HAPA was observed to be significantly more toxic compared to HAPA prepared with ascorbic acid. To evaluate the toxicity of oxidized HAPA, stock solutions of HAPA were adjusted to pH 9 and aerated for different periods of time: 4 minutes, 1 hour, 16 h, 4 days and 16 days. These oxidized HAPA solutions were incubated at different dilutions to determine the 50% inhibiting concentrations (expressed in terms of the original HAPA concentration in the stock solution). Toxicity was assayed by measuring the methane production in anaerobic sludge with fed with acetate as substrate. To illustrate the protocol, the graph in Figure 8 shows the time course of methane production in assay bottles exposed to different levels of 16h oxidized HAPA. The slope of each line (the activity) is compared to the slope of the uninhibited control (0 mM HAPA) and the relative activity is plotted as a function of the HAPA concentration. The activity versus HAPA concentration graph is shown in Figure 9. The concentration that corresponds to the point where the relative activity line crosses 50% is referred to as the 50% inhibiting concentration (50%IC). The assignment of a 50%IC of 1.16 mM and 0.15 mM for HAPA oxidized for 4 minutes and 16 hours, respectively, is shown in Figure 9. This procedure was repeated for each of the oxidation times. The net result is a graph, which illustrates the change in the 50%IC of HAPA versus oxidation time as shown in Figure 10. The first few minutes of the oxidation dramatically increases the toxicity of HAPA (lowers the 50%IC). As the oxidation continues for the next 16 h, the toxicity continues to increase reaching a maximum around 16 h. Therafter, continued oxidation up to 16 days results in only a small decline in the toxicity of the oxidized HAPA solutions. Preliminary measurements with a mass spectrometer indicate the formation of oligomers as oxidation products of HAPA. These oxidation products, apparently exhibit a high level of microbial toxicity.

To test whether the toxicity is universal and not just restricted to prokaryotes, a mitochondrial toxicity test (MTT) was conducted. The MTT test revealed that HAPA incubated without ascorbic acid was significantly more toxic than HAPA incubated with ascorbic acid (Figure 11). Clearly, the difference can be attributed to the formation of oxidized products from HAPA during the aerobic assay. In addition, 240 µM of HAPA previously oxidized for 16h was also more toxic than HAPA protected from oxidation by ascorbic acid. The early oxidation products formed from HAPA during the MTT were apparently more toxic than HAPA oxidized for 16 h prior to the test. Also it was observed that HAPA was more toxic to mitochondria compared to roxarsone. Roxarsone displayed the same weak toxicity in the presence and absence of the antioxidant. This observation is consistent with the fact that roxarsone is not susceptible to autooxidation.

The results taken as a whole indicate a rapid initial biotransformation of roxarsone to HAPA. The HAPA formed is unstable in air and is rapidly autooxidized to very toxic intermediates. Therefore land application of poultry manure may have serious ecological consequences due to the formation of toxic oxidized HAPA species. The sequence of events required for the toxic products to accumulate would be an initial anaerobic conversion followed by aerobic conversions. This sequence is consistent with initial composting of manure followed by land application.

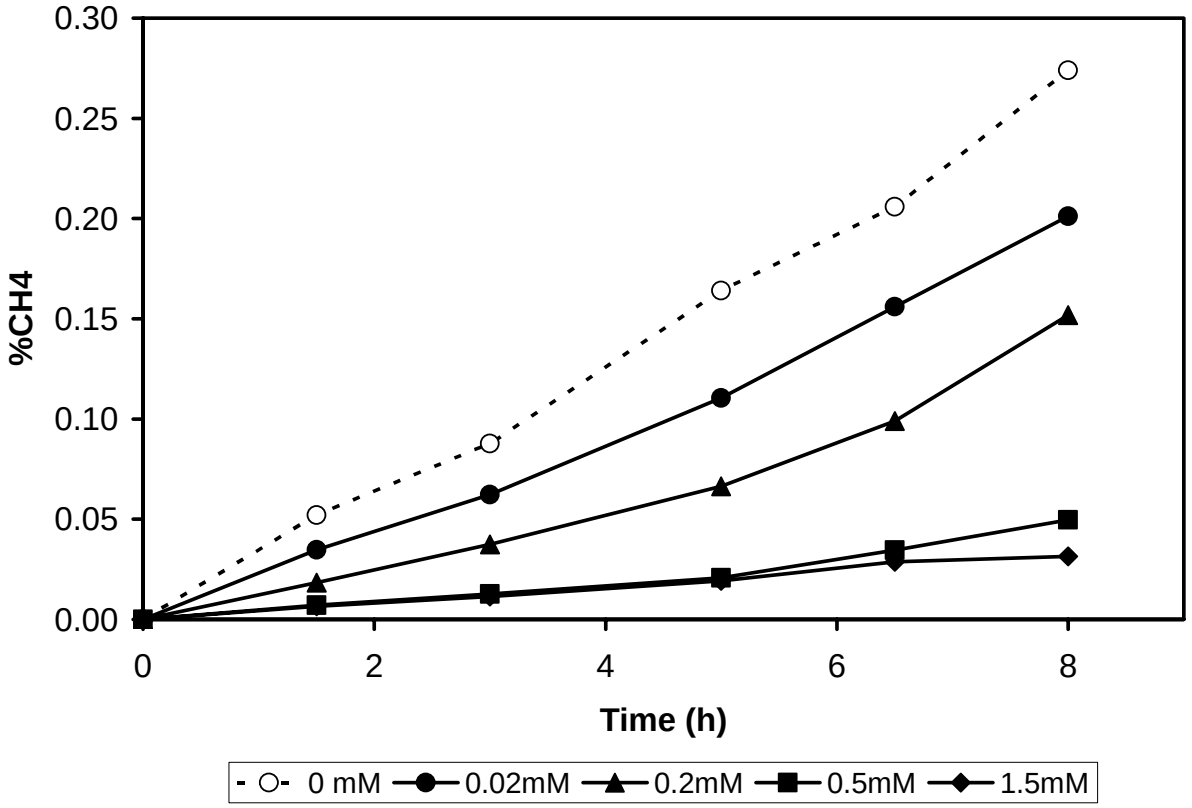


Figure 8. The time course of methane production during the acetoclastic methanogenic toxicity assay supplied with different concentrations of 16-h oxidized HAPA. The concentrations correspond to HAPA in the original unoxidized stock solution.

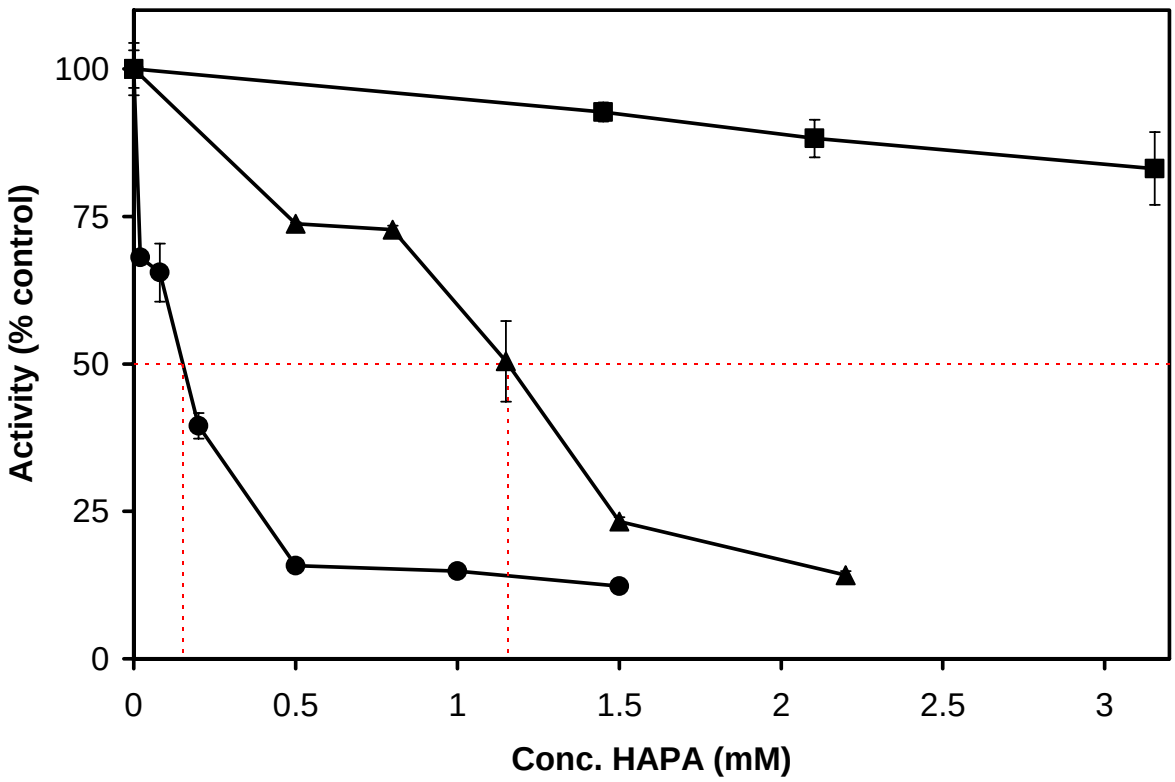


Figure 9. The relative activity of HAPA oxidized for 16 hours (closed circles), 4 minutes (closed triangles) and unoxidized HAPA (closed squares) as a function of the HAPA concentration. The concentrations correspond to HAPA in the original unoxidized stock solution.

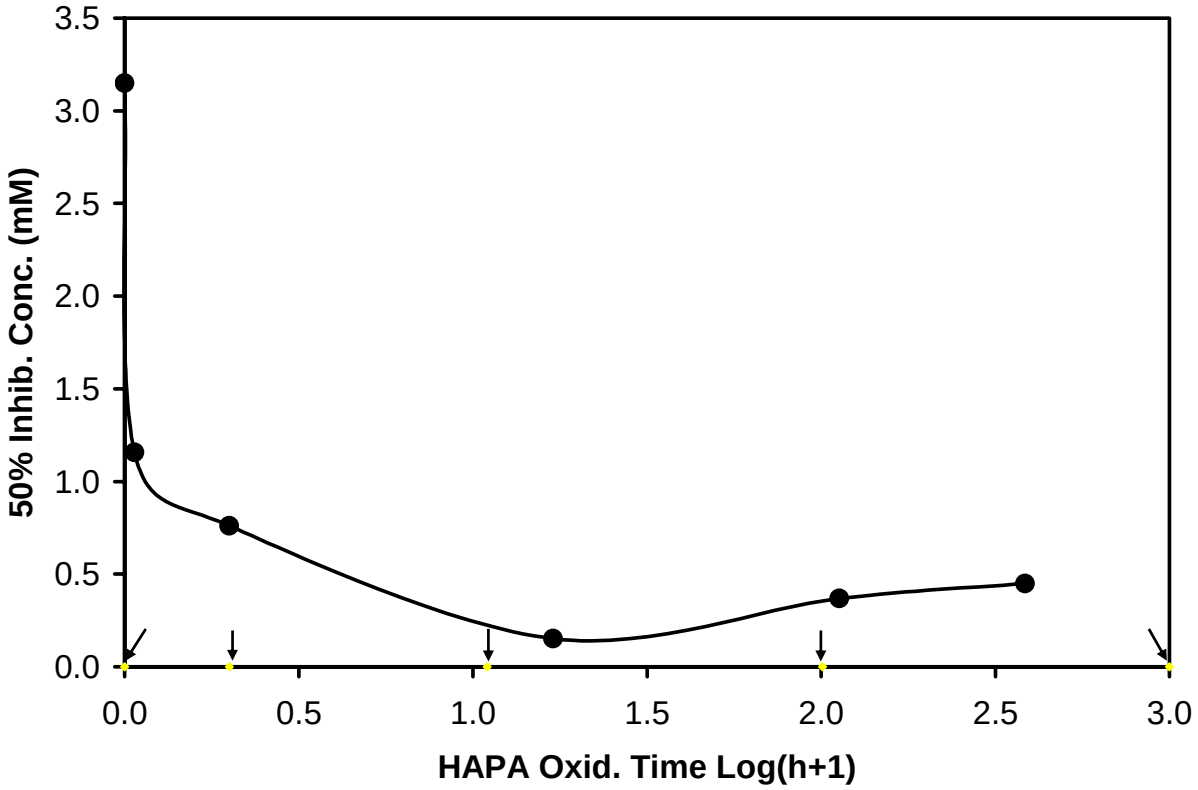


Figure 10. The 50% inhibiting concentration (50%IC) of HAPA to acetoclastic methanogenesis as a function of HAPA oxidation time. The graph is plotted on a logarithmic scale. From left to right the arrows indicate oxidation times corresponding to 0, 1, 10, 100 and 1000 h, respectively. The inhibitory concentration plotted at time 0 corresponds to the highest concentration tested of unoxidized HAPA, which correspond to 17% inhibition.

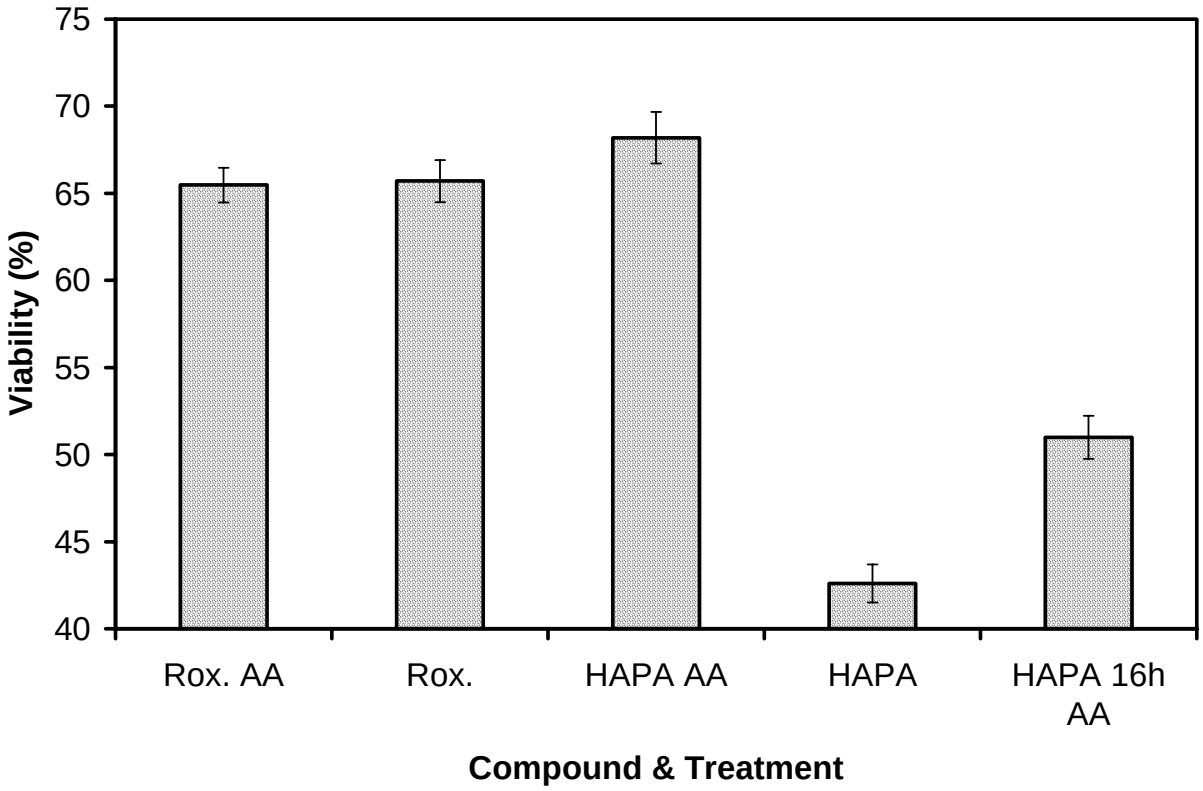


Figure 11. The toxicity of roxarsone and HAPA to the mitochondrial toxicity text (MTT) when supplied at a final assay concentration of 240 µM. Several assays conducted with ascorbic acid (AA) to prevent further oxidation of the compounds. The viability data of compounds incubated with AA is expressed as a percent of control viability in an assay the same level of AA.

Long-Term Aerobic Biodegradability of Dimethylarsinic acid.

The long term aerobic biodegradability of dimethylarsinic acid, DMA(V), was evaluated in incubations with activated sludge. During the first 50 days no degradation was evident. However on the next sampling at 121 days, evidence for aerobic biodegradation was apparent. The evidence is three-fold. DMA(V) concentrations decreased 1.01 mM more in inoculated treatments compared to uninoculated controls (Figure 12A) and arsenate, As(V), increased to 0.4 mM as a mineralized product of DMA(V) conversion. (Figure 12B). Respiratory evidence is shown in Figure 13. The complete treatment containing sludge and DMA(V) consumed 4.72% more O_2 compared to the endogenous substrate control (sludge only). The theoretical oxygen demand of the 1.01 mM DMA(V) consumed was equivalent to 72.7% of the extra O_2 consumption due to the presence of DM(V) (that which is beyond the endogenous respiration). This O_2 balance is very satisfactory when considering experimental error in the measurements.

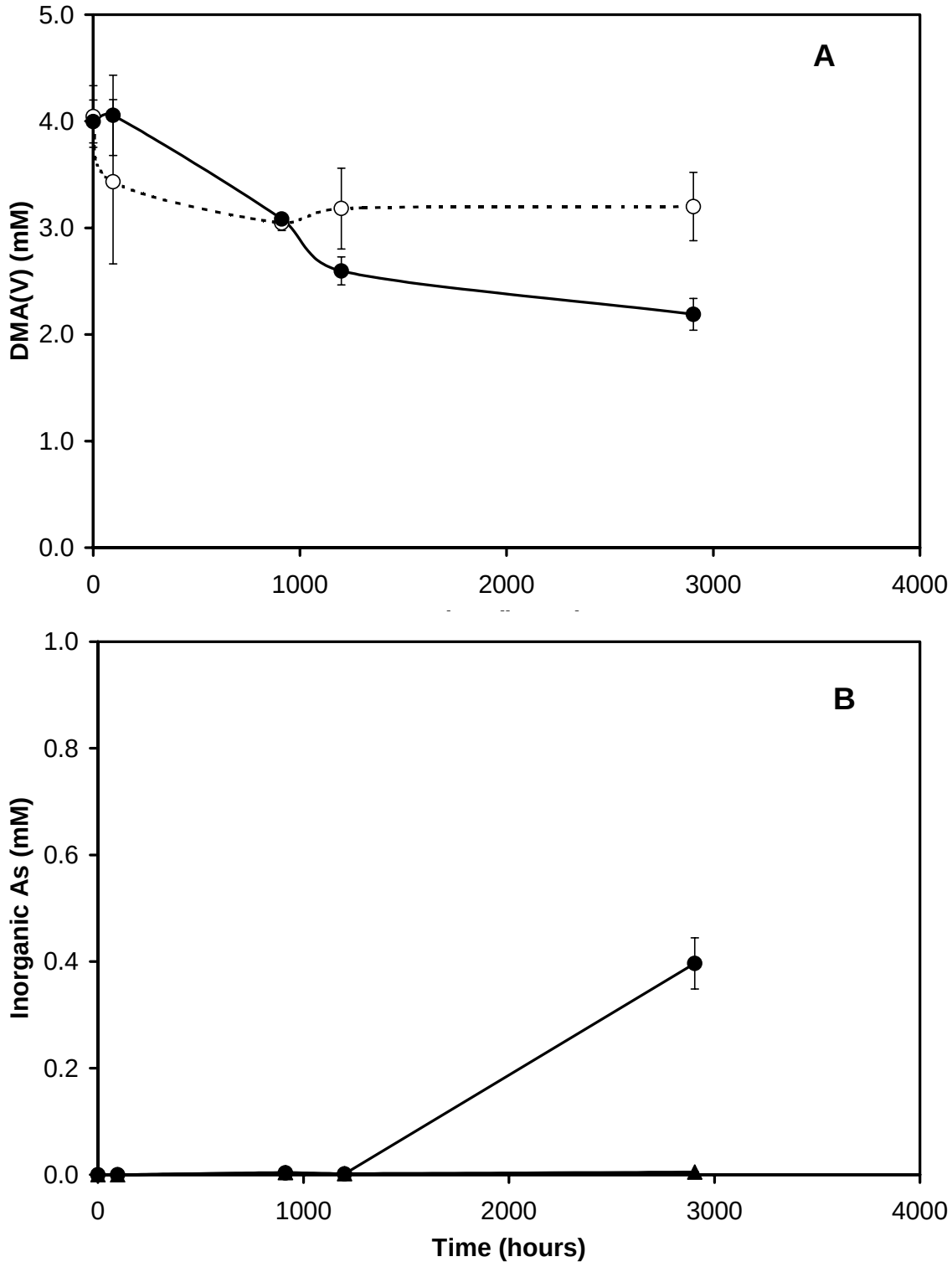


Figure 12. Aerobic biodegradability of DMA(V) by aerobic activated sludge. Panel A shows the consumption of DMA(V). Legend: DMA(V) in uninoculated control, open circles; DMA(V) incubated with active sludge, closed circles. Panel B shows the release of inorganic arsenicals. Legend: formation of As(V), closed circles; formation As(III), closed triangles.

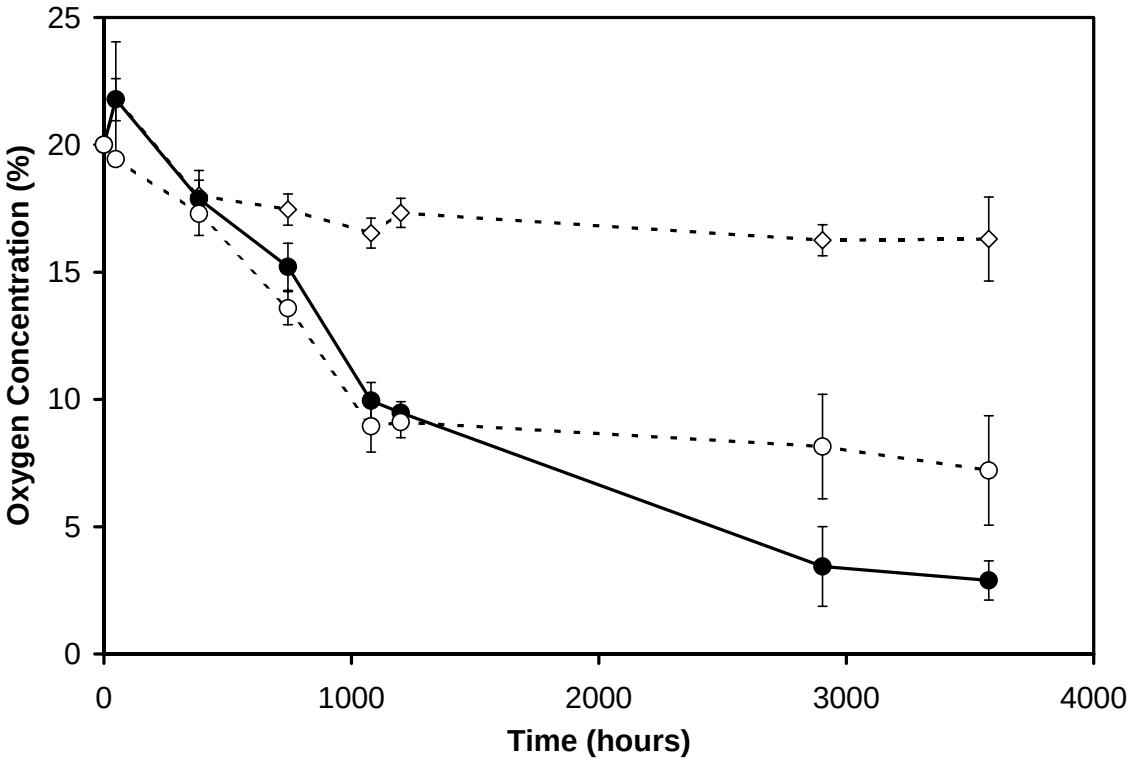


Figure 13. O_2-consumption during the experiment evaluating the aerobic biodegradability of DMA(V) with aerobic activated sludge. Legend: O_2 concentration in head space with blank medium, open diamonds; O_2 concentration in head space with medium and activated sludge, open circles; O_2 concentration in head space with medium, activated sludge and DMA(V), closed circles.