

Summary

(Effect of Ethanol on Hydrocarbon-degrading Bacteria in the Saturated Zone: Microbial Ecology Studies)

Research was conducted to better understand how ethanol could affect the composition of subsurface microbial communities, with an emphasis on indigenous bacterial populations capable of benzene, toluene, ethylbenzene, and xylene (BTEX) degradation. Prior to this study, there were no rapid methods available for assessing the populations of anaerobic aquifer bacteria that could carry out *in situ* BTEX biodegradation. In this study, a novel DNA-based method was developed for this purpose and was used to investigate how key factors could influence the populations of toluene-degrading bacteria in microcosms incubated anaerobically with BTEX under a variety of conditions. The factors investigated included: (1) the presence or absence of ethanol, (2) the presence of various electron acceptors typical of contaminated aquifers, and (3) the fuel exposure history of the aquifer sediments used to inoculate the microcosms. The molecular method involved RTQ-PCR (Real-Time, Quantitative Polymerase Chain Reaction) analysis of a recently-discovered gene, *bssA*, that is known to be associated with the first metabolic step of anaerobic toluene degradation. Thus, analyses based on the *bssA* gene provided an estimate of the populations of anaerobic, toluene-degrading bacteria. In addition, a companion method was used to estimate total eubacterial populations using 16S rDNA.

The microcosms under study were inoculated with aquifer sediments from four sites with different histories of exposure to fuel hydrocarbons: a leaking underground fuel tank (LUFT) site at Travis Air Force Base (AFB) in California, a LUFT site in Sacramento (California), an ethanol- and fuel-contaminated terminal in the Pacific Northwest ("Northwest Terminal"), and a background, uncontaminated site in Tracy (California).

- The RTQ-PCR method developed for this study was successful at determining population trends that were consistent with observed degradation activity. The most convincing results were for Travis AFB microcosms incubated under denitrifying conditions; these microcosms clearly had the most rapid toluene degradation and the highest *bssA* abundances (representative of anaerobic toluene-degrading bacterial populations) of all the sites and conditions studied.
- Ethanol had no clear effect on the abundance of *bssA* copies in any of the microcosms analyzed. However, under the conditions tested (i.e., with electron acceptors supplied in excess), a change in numbers of *bssA* copies would not be expected because ethanol often had no discernible effect on toluene degradation activity.
- Ethanol could promote relatively minor changes in microbial ecology that could result in major changes in hydrocarbon degradation activity. The one very clear case of an effect of ethanol on toluene degradation was in Northwest Terminal microcosms incubated under denitrifying conditions; ethanol promoted toluene degradation in these microcosms. In the presence of ethanol, toluene was mostly degraded within 15 days, whereas in the absence of ethanol, there was no apparent degradation in over 50 days. The most likely explanation is that ethanol degradation fortuitously increased the populations of toluene-degrading bacteria. However, numbers of *bssA* copies were not

higher after ethanol degradation, suggesting that, if such a population increase occurred, it was within the range of experimental error.

- Ethanol could decrease the relative populations of anaerobic hydrocarbon-degrading bacteria. In some cases, ethanol was associated with marked (e.g., nine-fold) increases in total eubacterial populations but no discernible changes in *bssA* abundances or in hydrocarbon degradation activity. This indicates that ethanol, which is generally more degradable than BTEX compounds under anaerobic conditions, can disproportionately support the growth of bacteria that aren't anaerobic hydrocarbon degraders.
- Of the anaerobic electron-accepting conditions tested, denitrifying conditions were clearly the most supportive of anaerobic hydrocarbon degradation. Travis AFB microcosms incubated under denitrifying conditions had the most rapid toluene degradation and the highest *bssA* abundances of all the sites and conditions studied. Over the first four days of incubation, during which time most of the toluene and ethanol (when present) had been consumed, numbers of *bssA* copies increased 100- to 1000-fold. For Travis AFB microcosms incubated under sulfate-reducing, ferric iron-reducing, and methanogenic conditions, toluene degradation was slower and no comparable increases in *bssA* copies were detected.
- Results of this study suggest that the newly-developed, DNA-based method could provide insights into the complexity of *in situ* microbial ecology of fuel-contaminated aquifers, but that further developments would be beneficial. For example, some of the results in this study are a reminder that genetic potential (represented by DNA) is not always realized and that genetic expression (represented by mRNA) is also important. To illustrate, Sacramento aquifer material (before incubation) had the highest absolute numbers of *bssA* copies of any aquifer material analyzed. However, the toluene degradation rates of Sacramento denitrifying microcosms were comparable to those of Tracy denitrifying microcosms, which had by far the lowest absolute numbers of *bssA* copies. The method developed in this study could be modified to quantify gene expression by using mRNA. Ideally, both DNA and mRNA approaches could be used to assess the populations and activities of hydrocarbon-degrading bacteria in the subsurface.
- To evaluate whether these laboratory microcosm results can be extrapolated to actual gasohol releases at LUFT sites, this molecular ecology approach should be used to characterize the spatial and temporal changes in subsurface microbial communities as a result of controlled gasohol release field experiments.
- If enhanced *in situ* bioremediation is considered at sites contaminated with gasohol, addition of nitrate as an electron acceptor could produce positive results. Denitrifying conditions were most favorable for anaerobic BTEX degradation for all sites tested, and, for Travis AFB microcosms, supported the rapid growth of toluene-degrading bacteria. These results are consistent with the findings of other BTEX biodegradation studies. Nitrate addition is easier to implement than oxygen addition because nitrate is much more soluble in water and is not a gas. Concerns regarding the use of nitrate include the following: (1) it is regulated in groundwater, (2) nitrogen gas formed by denitrification could create bubbles and disrupt groundwater flow, and (3) the degradation of benzene, the most toxic of the BTEX compounds, may not occur under denitrifying conditions at all sites. These concerns need not disqualify *in situ* nitrate addition, as added nitrate concentrations could be kept below regulatory guidelines and *in situ* benzene degradation cannot be guaranteed under any electron-accepting conditions.