

## Enumeration and characterization of BTEX-degrading bacteria from hypoxic environments functional with mixed electron acceptors

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Our work developed out of an interest in the behaviour of bacteria resident in benzene-, toluene-, ethylbenzene- and xylene(s)-(*i.e.*, BTEX)-contaminated aquifers. We observed that such environments are oxygen-limited, yet continuous monitoring over extended periods suggested that BTEX contaminants were disappearing and the contaminant plume may be diminished. Such observations suggested that significant microbial populations were active *in situ*. Moreover, laboratory studies with microcosms suggested the ascendancy of bacterial populations under hypoxic (*i.e.*, oxygen-limited) conditions whose growth and BTEX-degrading activities were associated with the reduction of nitrate under conditions whereby oxygen concentrations were less than twenty percent of saturation.

Contamination of groundwater with petroleum products is a serious problem. Spills occurring on the surface, leaking underground storage tanks and leaking pipelines may lead to subsurface contaminant plumes containing significant amounts of BTEX compounds. This group of compounds ranks second only to trichloroethylene in occurrence as groundwater contaminants in the USA. Microorganisms which degrade these compounds, moreover, are ubiquitous. They abound in nature, and therefore their potential for bioremediation has been recognized for both *in situ* and *ex situ* treatment schemes. However, most groundwater contaminated with BTEX compounds is found below the surface where its reduced oxygen content imposes severe limitations on the rate and extent of biochemical transformations of these compounds to harmless derivatives. In view of the foregoing, our laboratory studies have been directed towards the following questions.

1. What are the ambient conditions in a BTEX-contaminated aquifer?

2. What is the behaviour of indigenous bacteria under these conditions?

3. What are the properties of a bacterial strain functional under hypoxic conditions?

4. How does this strain compare with other BTEX-degrading strains?

### Aquifer characteristics

For the past several years, we have been sampling three sites in which groundwater was contaminated by petroleum products including BTEX (benzene, toluene, ethylbenzene and xylenes) components. All three of these contaminated aquifers are characterized by their low dissolved oxygen levels in the contaminated regions (approximately 1-2 mg/l), in contrast to uncontaminated adjacent regions in the aquifer (approximately 3-4 mg/l). The occurrence of dissolved nitrate also showed a corresponding relationship when the contaminated and uncontaminated regions were compared. The nitrate concentrations were low or non-detectable in the contaminated regions, but higher in the uncontaminated regions. These relationships were observed for all three aquifers leading to the conclusion that oxygen and nitrate depletion characteristic of the contaminant plume regions were indicative of an active ongoing biological process (Mikesell *et al.*, 1991).

### Activity of indigenous microbial communities

Interesting correlations between the presence of denitrifying bacteria competent for growth on BTEX were observed when cores of aquifer material that had been sampled vertically were tested for microbial

activity as a function of depth and location within the contaminant plume region. Bacterial strains growing on BTEX aerobically were observed for all samples independent of depth and/or BTEX concentration. However, when such samples were incubated under hypoxic conditions (*i.e.*, less than 2 mg/l dissolved oxygen), bacterial growth and denitrification matched BTEX degradation observed for microcosm cultures inoculated with bacteria eluted from the soil core samples. Therefore, there were differences observed between bacteria that would degrade BTEX under hypoxic-nitrate reducing conditions and those which grow on BTEX under aerobic conditions.

#### Diversity of bacteria from hypoxic environments

Samples from the BTEX-contaminated aquifers were incubated in mineral salts buffer overnight to elute bacteria from particles. These eluates were then plated on solid mineral salts medium followed by incubation aerobically or under hypoxic conditions in the presence of BTEX vapors. Aerobic cultures were incubated 48 h and hypoxic cultures 1 week. Colonies were picked onto the same medium and incubated as before. These cultures, then, became the source for subsequent tests for the mineralization of individual BTEX components under both aerobic and hypoxic conditions. For this work, *p*-xylene was used as representative of xylenes. The colonies selected for such tests were picked on the basis of their distinctive morphologies and colony size to facilitate estimates of the diversity of the microbiota that might be isolated from BTEX-contaminated environments.

A subset of the above bacterial isolates that showed good growth on all four of the BTEX substrates when tested on them singly was analysed (Mikesell *et al.*, 1993). These isolates were also compared with respect to their growth on BTEX under either aerobic or hypoxic conditions as a further indication of the diversity of microorganisms resident in the samples. The results from such tests showed that not all bacteria isolated under one condition (*e.g.*, aerobiosis, carbon source) grow under dissimilar conditions. For example, of the eight isolates selected for growth on benzene under aerobic conditions, none grew on BTE or X under hypoxic conditions. In contrast, however, for seven bacteria selected for growth on ethylbenzene under aerobic conditions, five of the strains also grew on BTE or X under hypoxic conditions. These results show how the substrate used to select such strains and also the gas atmosphere may provide an estimate of the diversity which obtains in BTEX-contaminated aquifers. Moreover, it shows that pure cultures of bacteria can be isolated which perform well under hypoxic conditions (Kaphammer *et al.*, 1991; Mikesell and Olsen, 1992, 1993).

#### Pure culture studies

Studies with single bacterial strains were also performed as for the microcosm cultures described above. For this work we used three strains designated W31, CFS215 and PKO1 which we isolated from BTEX-contaminated soil and compared their behaviour with other BTEX-degrading strains described by others previously. These studies showed that the ability to degrade BTEX was not simply a matter of denitrifying ability since not all denitrifiers degraded BTEX under hypoxic conditions. Moreover, these studies suggested fundamental enzymological differences occurring between the two groups of microorganisms as shown in figure 1. In this regard, all the strains used had in common the production of catechols from toluene or benzene. Moreover, such catechols were metabolized to ring fission products with the obligatory requirement for one molecule of oxygen. The ring fission enzyme, catechol dioxygenase, accordingly offered a good subject for comparing the two groups of organisms depicted above with regard to an oxygen-requiring enzyme's kinetic characteristics both for oxygen and substrate. When Michaelis constants ( $K_m$ ) were determined for oxygen, they were approximately one order of magnitude lower for two of the bacterial strains studied which were active under hypoxic-denitrifying conditions (CFS215, PKO1) than the other *Pseudomonas* strains depicted in figure 1. Thus such organisms functional under oxygen-limiting conditions may be comprised to more efficiently utilize low ambient concentrations of dissolved or atmospheric oxygen necessary for critical oxygen-requiring steps.

Our work to date suggests that a group of microorganisms has evolved which is for the most part indistinguishable from closely related species, but which has adapted to growth and metabolism in

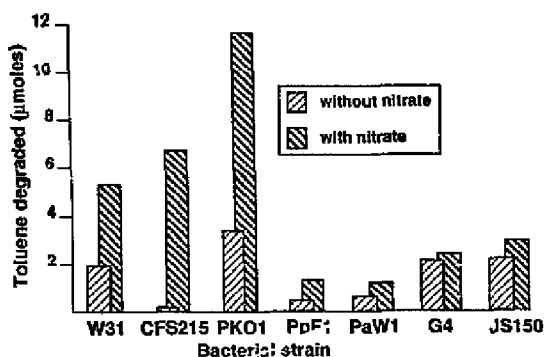


Fig. 1. Toluene degradation with nitrate under hypoxic conditions ( $< 2 \text{ mg } \text{O}_2/\text{l}$ ).

low oxygen environments. Such microorganisms, then, may facilitate the natural bioremediation of toxic xenobiotic molecules *in situ*, thus reducing the impact of such substances on human health and the environment.

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## Disposal of slop oil and sludges by biodegradation

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Absorbing various crude oils into an oleophilic peat matrix prior to biodegradation apparently focuses enhanced bacterial action on the surface of the absorbent. This observation has been exploited to accelerate and otherwise improve landfarming operations and has been the basis for a novel bioreactor suitable for use on-site by operating personnel at refineries and petrochemical complexes. This innovation provides a solution to the problem of disposing of high hydrocarbon content oily wastes such as sludges and slop oils generated on-site.

**Introduction**

Considerable effort is required to convert a scientific discovery into practical technology. In order to proceed, sufficient benefits must be identified with a specific need or opportunity to warrant the costs and risks associated with the development process. Success depends on the degree to which the development process can meet the requirements and constraints associated with the target application.

This article describes the development of a bioremediation technology for the treatment and disposal of high hydrocarbon content oily wastes known as "slop oil" or "sludges". These arise in oilfield and refinery operations from the imperfect separation of oil water emulsions or the accumulation of waste oily materials in tank bottoms and the like. Volumes are usually modest. At a given location, tens to hundreds of cubic meters of such oily waste can be generated each year with a hydrocarbon content of 10 to 80 %. Significant solids can be entrained (up to 40 %). There are few ways to handle these wastes effectively and economically. The volumes are too large to treat casually and too small to support expensive facilities or processes at a given site.

Increasing environmental awareness has eroded traditional disposal options. To date, these wastes have been used on nearby gravel roads as a dust control agent, sent directly to local landfills or transferred to local oil reclaimers specializing in the breaking of persistent emulsions. With tightening environmental regulation, these options are disappearing. A need exists for a simple on-site disposal process with low operating and capital costs and minimal long-term liability.