



BIOMONITORING OF TOXICITY REDUCTION DURING IN SITU BIOREMEDIATION OF MONOAROMATIC COMPOUNDS IN GROUNDWATER

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Abstract—Biomonitoring of toxicity, using the *Ceriodaphnia dubia* acute toxicity test, was performed on a gasoline-contaminated aquifer in San Diego, Calif. undergoing *in situ* bioremediation. The bioremediation approach here featured nitrate enrichment of extracted groundwater (with subsequent reinfiltration back into the aquifer) to stimulate bacterial denitrification. Denitrification has been shown to support biodegradation of the monoaromatic hydrocarbons—benzene, toluene, ethylbenzene, and the isomers of xylene (BTEX). During the period between the start of bioremediation (October, 1989) and the end of nutrient enrichment (March, 1992), BTEX levels at the two most contaminated wells on site (MW1 and MW5) were reduced by about 81 and 99%, respectively. In acute bioassays of groundwater from MW1 during the first 6 months of bioremediation, concentrations of groundwater from about 3 to 10% produced 50% mortality of *Ceriodaphnia* after 24 h exposure, whereas groundwater concentrations of greater than 20% were required after remediation. When the 24-h LC50's for mortality were plotted as a function of BTEX concentration, the slope of the regression line was similar for both laboratory and field nitrate-enrichment studies. At MW5, where the BTEX level was dramatically reduced by 99% during bioremediation, the 24-h LC50 after bioremediation increased to 56% (from initial values of 12–15%), while uncontaminated groundwater from a well up-gradient of the gasoline leak, showed an LC50 value of 74%. These results demonstrate that even after bioremediation of an aquifer (with an associated BTEX reduction of 81–99%), the toxicity (as measured by *Ceriodaphnia*) of the groundwaters may not be reduced to precontamination levels.

Key words—*in situ* bioremediation, groundwater, biodegradation, toxicity testing, biomonitoring, *Ceriodaphnia dubia*, denitrification

INTRODUCTION

Bioremediation has received increasing attention for clean-up of gasoline-contaminated soils and groundwaters at leaking underground storage tank (LUST) sites. However, since microbiological processes may transform contaminants into unique intermediary metabolites, and gasoline is a complex mixture of over two hundred hydrocarbons, the identification and bioassay of every potentially toxic compound formed as intermediates or endproducts of the biodegradation process is expensive and difficult. In this regard, biomonitoring may be used as an efficient tool to determine the overall toxicity changes associated with bioremediation of the complex chemical mixture that exists at such hazardous waste sites (Marty *et al.*, 1991).

Denitrification is an anaerobic respiratory process, whereby nitrate is used as an alternate electron acceptor to oxygen for biodegradation. During denitrification, nitrate is reduced to either nitrous oxide or dinitrogen gas, while organic compounds are oxidized to the final endproducts carbon dioxide and

water. Denitrification has been shown to be effective in the biodegradation of the monoaromatic hydrocarbons—benzene, toluene, ethylbenzene, and the isomers of xylene (BTEX) in both laboratory (Trizinsky and Bouwer, 1990; Hutchins and Wilson, 1991; Mikesell *et al.*, 1991) and field-scale studies (Berry-Spark *et al.*, 1986; Sheehan *et al.*, 1988; Hutchins and Wilson, 1991). However, the degree of toxicity reduction in groundwaters associated with bioremediation under denitrifying conditions in an aquifer has never been assessed.

The purpose of this study was to use biomonitoring (employing the 24-h *Ceriodaphnia dubia* acute toxicity test) to follow the reduction of toxicity in groundwaters during *in situ* bioremediation of a gasoline-contaminated aquifer in San Diego, Calif. The particular bioremediation approach here featured both nitrate and nutrient (ammonium polyphosphate) enrichment of extracted groundwater with subsequent re-infiltration of this enriched groundwater back into the aquifer in a closed-loop system. Additionally, we performed a toxicity identification evaluation (TIE) of the groundwater to identify the

fraction of overall toxicity associated with volatile and non-polar organic compounds.

MATERIALS AND METHODS

Field scale remediation project

The project was carried out at the Main Street Facility (owned by San Diego Gas and Electric Company), located in the City of San Diego. This property was used as a fueling depot for fleet vehicles. The site is located on a coastal plain near the San Diego Bay. The shallow groundwater (water table approximately 32 ft below land surface) has a relatively high total dissolved solids, and has been designated by the Regional Water Quality Control Board as non-beneficial use.

Figure 1 is a diagram of the locations of the wells and the infiltration gallery at the Main Street site. Details on the nature and extent of contamination of the site have been previously reported (Gersberg *et al.*, 1993). The *in-situ* bioremediation system consisted of three major components (Fig. 1):

- down-gradient groundwater extraction system (from wells MW5 and EW6);
- nutrient feed system (potassium nitrate plus ammonium polyphosphate);
- up-gradient groundwater infiltration system (at the tank excavation pit).

Hydraulic gradient measurements at the site determined that groundwater flow is in a southerly direction. Pumping groundwater from two down-gradient extraction wells (EW6 and MW5) to the infiltration gallery created a mostly closed loop to recycle the groundwater enriched with added nutrients and electron acceptor (nitrate). Nutrient enrichment began on 12 October 1989, and until 4 March 1992 (the last day of nutrient enrichment), the groundwater was recycled by infiltration into the contaminated soils (at the

site of the tank pit excavation) at a rate of 800–900 gallons per day.

The nutrient solution was added to the recycled groundwaters by flow proportional peristaltic pump. The nitrate (as KNO_3) was added to the final concentration of 177 mg l^{-1} $\text{NO}_3^- \text{--N}$ until March, 1990, when this level was reduced to about 100 mg l^{-1} to reflect the decreasing BTEX levels in the aquifer. A long-chain polyphosphate compound (as ammonium polyphosphate) was also added to facilitate phosphorous distribution into the aquifer (to a final concentration of about 58 mg l^{-1}) as well as supply ammonia-N (at about 18 mg l^{-1}).

Chemical analyses and toxicity testing

The disappearance of monoaromatic hydrocarbons was followed by peak attenuation on a gas chromatograph equipped with a purge-and trap concentrator (Carroquino *et al.*, 1992). All dissolved nutrients in the groundwater were analyzed by colorimetry according to *Standard Methods* (APHA, 1985). *Ceriodaphnia* toxicity testing was performed following guidelines of the U.S. EPA (1985). Standard methods were modified as necessary for testing volatile compounds, i.e. 40 ml vials were filled with no headspace and sealed with caps fitted with teflon septa to prevent loss of BTEX by volatilization. Dissolved oxygen, hardness and total alkalinity were measured using the Hach Kit (Hach Company, Loveland, Colo.) titration methods (Carroquino *et al.*, 1992).

For the toxicity identification evaluations we performed 7-day chronic toxicity testing following the guidelines established by the U.S. EPA (1991a, b). Plastic 23 ml cups were used to hold the test organisms, with one organism per cup. Neonates used were less than 24 h old. Test cups were filled with no headspace, and capped with clear plastic lids to prevent volatilization of contaminants. Tests were conducted with 6 or 7 different dilutions of groundwater (including the control) and five replicates per concentration. Manipulations performed were aeration, filtration, and

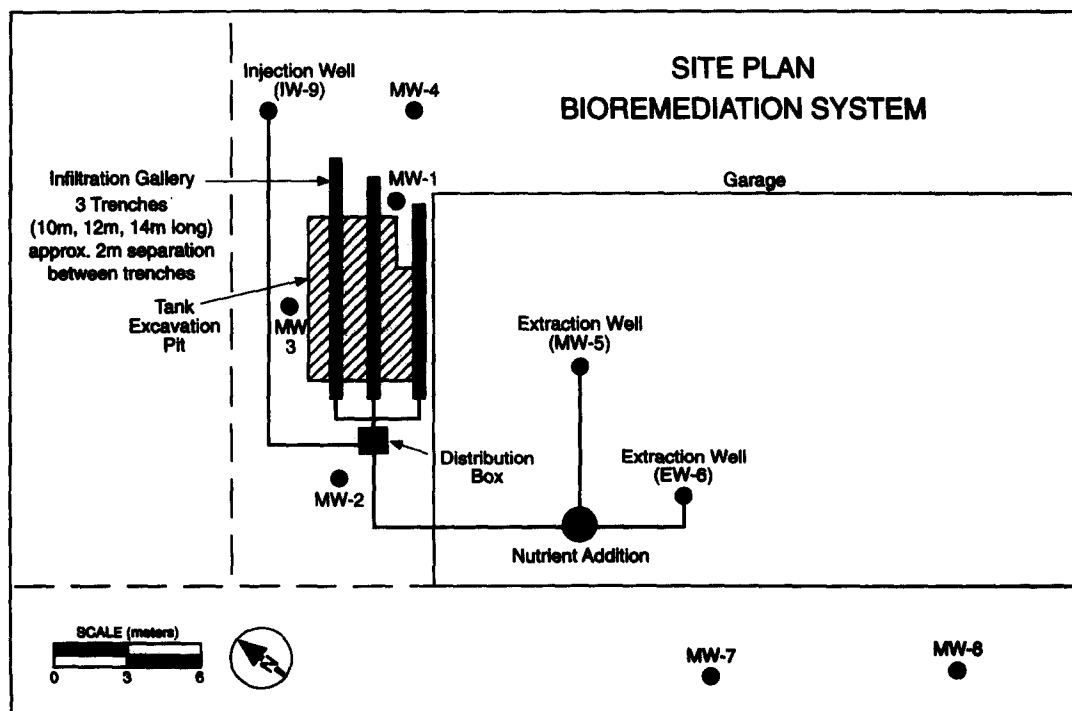


Fig. 1. Site plan of the *in situ* bioremediation project.

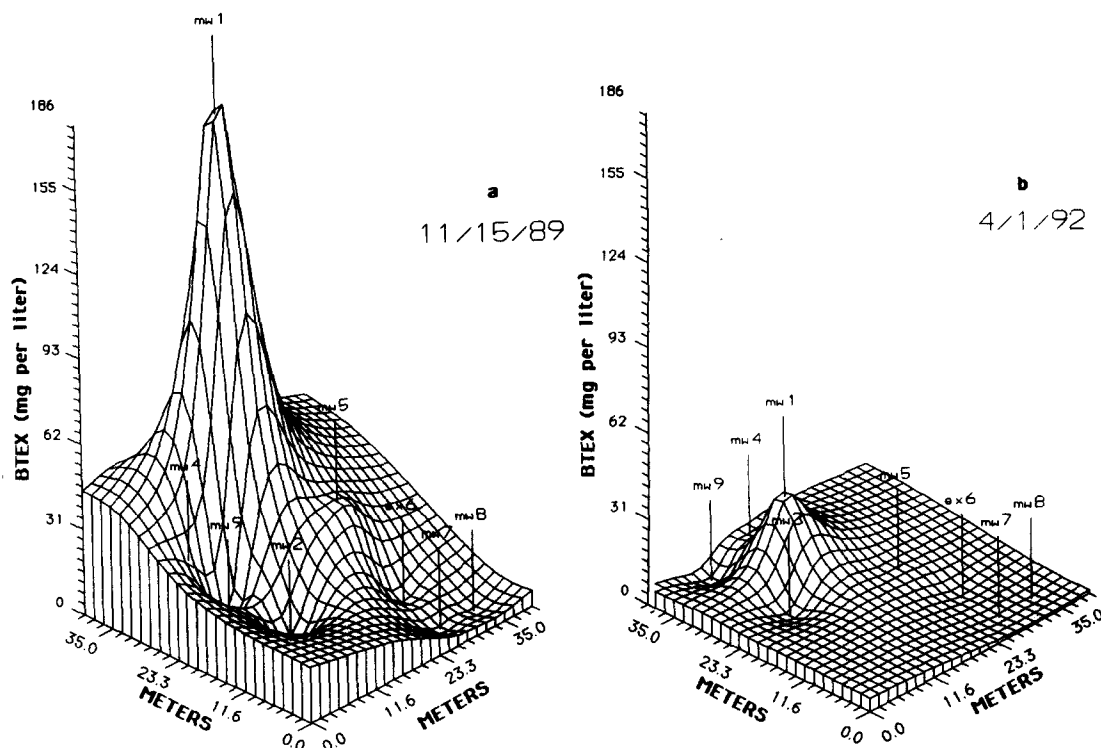


Fig. 2. Groundwater concentrations of BTEX (a) just after start of bioremediation project on 15 November 1989, and (b) at the end of nutrient enrichment on 1 April 1992.

treatment using a C_{18} solid phase extraction (SPE) resin. No pH adjustments were made during these testing procedures.

Data analysis

The 24-h LC50 for the acute toxicity data was calculated using the Moving Average-Angle Method (U.S. EPA, 1985). For the 7-day chronic mortality toxicity testing, the trimmed Spearman-Kärber method was used to estimate the LC50 and associated confidence interval (Hamilton *et al.*, 1977). Regression lines were determined for both the laboratory and field toxicity studies using BTEX level as the independent variable and LC50 as the dependent variable.

RESULTS AND DISCUSSION

Just before the start of bioremediation, BTEX levels in the two most contaminated wells (MW1 and MW5) were approximately 176 and 40 mg l^{-1} , respectively. By the end of the bioremediation project, BTEX levels in the groundwater at these same two wells declined by about 81 and 99%. This remediation of the aquifer is illustrated three-dimensionally in Fig. 2, with the spatial relationship of BTEX concentrations at the site shown just after the start of bioremediation (15 November 1989) and after 27 months of nutrient enhancement (4 March 1992).

The relationship between BTEX concentration and acute toxicity (as expressed by the 24 h LC50) of groundwater at the most contaminated well on-site (MW1) is shown in Fig. 3. During the first 6 months of bioremediation, concentrations of groundwater

from about 3 to 10% produced 50% mortality of the *Ceriodaphnia*, whereas groundwater concentrations of greater than 20% were required after bioremediation. Since survival of *Ceriodaphnia* was 100% for the highest groundwater dilution tested (ranging from 1 and 6%) in every bioassay except one (90% survival), the dilution water itself showed little or no daphnid toxicity. There is a possibility that some of the observed toxicity may have been due to low dissolved oxygen levels, and either ammonia (added directly to the aquifer), or nitrite generated via nitrate reduction in the aquifer. Dissolved oxygen was monitored in each of the toxicity tests, and (at the dilutions used) never fell below 5.2 mg l^{-1} , well above the adverse effect level for *Ceriodaphnia*. As for ammonia, even though it was added to the recycled groundwater at a final concentration of about 18 mg l^{-1} , adsorption of ammonia onto aquifer solids probably minimized breakthrough into the aquifer, so that ammonia-N levels in the groundwater at MW1 never exceeded 1.1 mg l^{-1} during any of our toxicity tests. Nitrite did begin to accumulate in the aquifer at MW1 within the first 4–8 weeks after the start of nutrient enrichment; however, subsequently it appeared that the microbial community was able to adapt to the influx of nitrate since nitrite-N was not detectable throughout the aquifer after this initial period of acclimation.

A regression line was calculated using BTEX values as the independent variable and toxicity values

(24-h LC50) as the dependent variable. The correlation coefficient between BTEX and toxicity levels was 0.73. The value for the slope of the regression line (-0.158) indicates that the LC50 decreased by 0.158% for each additional mg l^{-1} of BTEX in the groundwater. In order to evaluate whether the above equation could be used in a predictive fashion to estimate BTEX toxicity, we spiked dilution water with 70 mg l^{-1} of benzene, toluene, and ortho and para-xylene, in levels and proportion of each similar to that which existed *in situ*. The 24-h LC50 for this spiked water was 16.9%; while the regression equation for our field toxicity data would predict an LC50 of 13.6%. Although this difference between the measured and predicted values was not statistically significant, the fact that our field data predicts a higher toxicity than that observed for a pure mixture of the monoaromatics is not surprising, since the groundwater at MW1 also contained a significant concentration of total petroleum hydrocarbons (generally $100\text{--}200 \text{ mg l}^{-1}$ TPH) which may also exert toxicity to *Ceriodaphnia*. Such residual toxicity may also explain why the LC50 value for the y-intercept (toxicity level at 0 mg l^{-1} BTEX) in Fig. 3 is relatively low (24.6% dilution). Indeed, in groundwater at monitoring well MW5 where the TPH level was much lower (less than 10 mg l^{-1}), the 24-h LC50 value reached 56% when the BTEX decreased to concentrations below 1 mg l^{-1} (Table 1).

At MW5, which was down-gradient from MW1

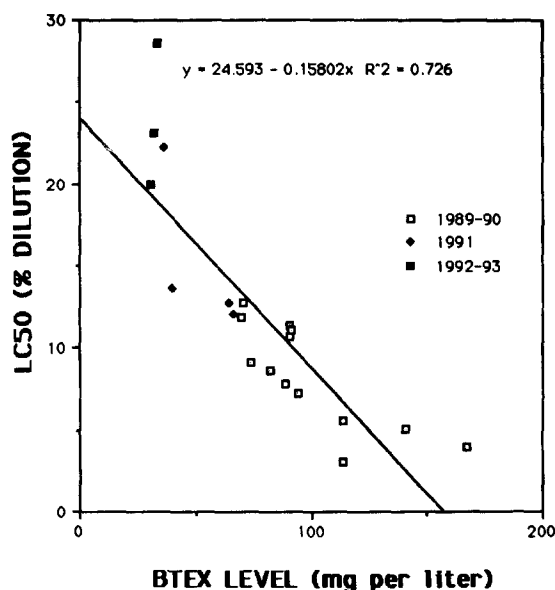


Fig. 3. Linear regression of toxicity (24-h LC50) as a function of BTEX level in groundwater obtained from biomonitoring during remediation at well MW1 (most contaminated well on-site). Data points represent LC50 values measured during biomonitoring in 1989–1990 (first year of nutrient enrichment), 1991 (second year of enrichment), or 1992–1993 (last year of nutrient enrichment or after project ended).

Table 1. Toxicity values (24-h LC50), BTEX concentration, and level of nitrate in groundwater at monitoring well MW5 before and after bioremediation

	Date		
	Before nutrient enrichment	After nutrient enrichment ^a	
	2/27/91	5/27/91	6/21/93
LC50 (% dilution)	14.6	11.9	56.10
Benzene (mg l^{-1})	5.8	4.0	0.26 ^b
Toluene (mg l^{-1})	16.9	9.4	0.01
Total xylenes (mg l^{-1})	19.6	10.5	0.13
Nitrate-N (mg l^{-1})	0.3	2.5	0.15

^aBiodegradation may have proceeded even after nutrient enrichment ended due to residual nitrate in groundwater.

^bBTEX and nitrate values represent means for groundwater analyses on 16 April 1993 and 7 July 1993.

and the infiltration gallery (Fig. 1), nitrate did not appear at significant levels until approximately 24 months after nutrient enrichment began. This delay in nitrate appearance at MW5 was most probably due to the low rate of groundwater extraction/infiltration and the relatively low hydraulic gradient at the site (0.004). At the same time that nitrate appeared in July 1991, benzene, toluene and total xylene levels decreased (from an initial total BTEX level of about 30 mg l^{-1}) by 83, 95, and 63%, respectively. Since then, BTEX levels at MW5 have remained very low, with values ranging from 0.15 to 1.10 l^{-1} for all the sampling dates in 1993. Biomonitoring data for groundwater at MW5 showed pre-remediation (before nitrate appearance) 24-h LC50 values of 14.6% on 27 February 1991 and 11.9% on 27 May 1991 (Table 1). On 21 June 1993 (post-bioremediation), groundwater from MW5 was sampled and tested for acute toxicity. The 24-h LC50 was 56.1% (Table 1). Since the post-remediation BTEX concentration at MW5 was about 1 mg l^{-1} or less, and the TPH level was also low (less than 10 mg l^{-1}), we may then consider the 24-h LC50 value for groundwater at well MW5 to approximate the final toxicity value attainable by bioremediation.

Although nitrate was not the sole electron acceptor available in the aquifer (dissolved oxygen level was about 1 mg l^{-1} or less), the contemporaneous appearance of nitrate and disappearance of BTEX in the anoxic aquifer at MW5, suggest that denitrification played a major role in the toxicity reduction observed. Carroquino *et al.* (1992) showed that when uncontaminated groundwater taken from a well up-gradient of the LUST excavation pit (Fig. 1) was tested for toxicity to assess the background toxicity of these waters to *Ceriodaphnia*, the 24-h LC50 was only 74.1%. This latter value suggests that chemicals in the groundwater up-gradient of our contaminated site exerted toxicity to *Ceriodaphnia*, and since this region of the aquifer underlies an old industrialized area, this possibility cannot be ruled out. It is therefore illustrative to normalize the post-remediation toxicity value at MW5 (24-h LC50 of 56.1%) by dividing it by the background groundwater toxicity value of 74.1%, to

obtain a less biased measure of the efficacy of bioremediation. The resultant normalized toxicity value of 75.7% points to the fact that nitrate-enhanced bioremediation may not completely restore these groundwaters to the condition that existed before contamination, at least in the time frame of this study. However, since the nature of the chemicals that exert residual toxicity (to *Ceriodaphnia*) at MW5 after such bioremediation remains unknown, our toxicity test data needs to be interpreted with caution, as the results cannot necessarily be quantitatively extended to predictions of human toxicity.

There have been few reports in the literature documenting toxicity reduction associated with bioremediation in either laboratory or field settings. Marty *et al.* (1991) demonstrated changes in toxicity of aqueous soil extracts at a pesticide disposal site before and after bioremediation. Most pesticides decreased in experimental plots during the 63-day treatment; most notable were a decrease in endosulfan I level from an initial value of 1353 mg kg⁻¹ to a final level of 192 mg kg⁻¹, and a decrease in chlorothanoniol concentration from an initial level of 869 mg kg⁻¹ to a final level of 84 mg kg⁻¹. In static short-term bioassays using Medaka (*Oryzias latipes*) larvae, before bioremediation concentrations from 2.2 to 2.9% produced 50% mortality, whereas extract concentrations of 18–34% were required after bioremediation.

Aquatic toxicity biomonitoring using the cladoceran *Ceriodaphnia dubia* offers certain advantages as compared to fish-based biomonitoring, due to the short lifespan, ease of culturing, and relatively high sensitivity of *Ceriodaphnia* to toxicants. Carroquino *et al.* (1992) using laboratory microcosms of BTEX-contaminated groundwater from the same region of aquifer as in the present investigation, compared toxicity reduction (measured by the acute *Ceriodaphnia* toxicity test) after nitrate (and phosphate) enrichment, with that after enrichment with nitrate (and phosphate) as well as hydrogen peroxide (H₂O₂). In this case the H₂O₂ acts as a source of oxygen (upon decomposition) which both inhibits denitrification and stimulates aerobic respiration. After a statistical comparison of treatments, these authors found that the addition of nitrate plus hydrogen peroxide was no more effective (in reducing toxicity) than the addition of nitrate alone. When LC50's from toxicity tests on nitrate-enriched groundwater microcosms (Carroquino *et al.*, 1992) are plotted as a function of BTEX concentration (Fig. 4), the slope of the regression line for these laboratory microcosm enrichments (0.187) is very similar to the value (0.158) obtained from biomonitoring in our field-scale bioremediation study (Fig. 3). This implies that such laboratory studies may yield useful information on the toxicity response to be expected in the field.

In order to identify the general nature of the toxicity present in gasoline-contaminated aquifer, we conducted several toxicity identification evaluations

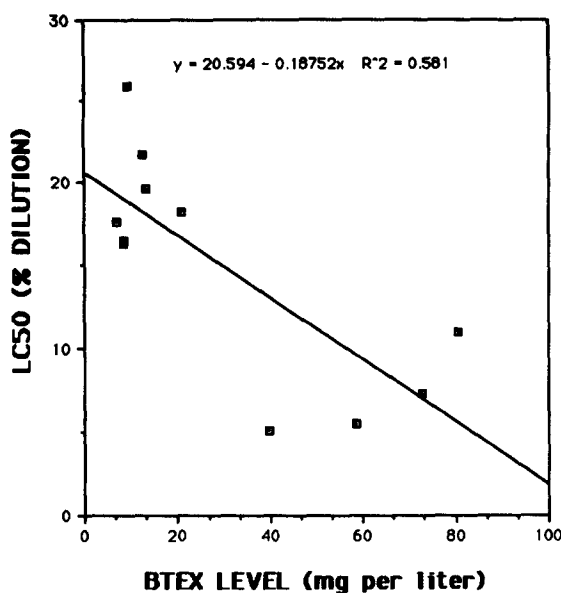


Fig. 4. Linear regression of toxicity (24-h LC50) as a function of BTEX level in groundwater from monitoring well MW1 enriched with nitrate-N (50 mg l⁻¹) and phosphate-P (10 mg l⁻¹) and incubated in laboratory microcosms with no headspace. Acute toxicity tests were performed on subsamples from these microcosms as biodegradation proceeded.

(TIE) on groundwater from monitoring well MW1 which contained approximately 30 mg l⁻¹ BTEX. A TIE consisted of a baseline 7-day chronic mortality-toxicity test, followed by this same toxicity test after aeration, filtration, passage through a C₁₈ SPE column (to remove non-polar organic hydrocarbons), and subsequent to the combination of all of these treatments. The baseline toxicity tests yielded LC50 values ranging from 14.08 to 15.19%. Results after aeration were equivocal, with one test showing no toxicity reduction upon aeration, while a second test showed significant toxicity reduction to an LC50 value of 30.78%. Surprisingly, the combination of aeration, filtration and SPE treatment resulted in an LC50 of only 34.0%. Even though these 7-day chronic mortality results were not directly comparable to the 24-h acute mortality results of our field biomonitoring, they do suggest that even after extensive physico-chemical treatment of the groundwater (which is aimed at removing both volatile compounds and non-polar organics such as alkanes) considerable residual toxicity still remained, most probably due to polar organic compounds. Taken together with our results at monitoring well MW5, where significant toxicity remained even after nearly complete bioremediation of BTEX, these findings suggest that (at least in the time frame of our study) it may be difficult for remediation strategies such as *in situ* bioremediation, or pump-and-treat with air-stripping, to reduce the toxicity as measured using

Ceriodaphnia, of gasoline-contaminated groundwaters to the pre-contaminated condition.

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