Arch. Environ. Contam. Toxicol. 52, 410–417 (2007)
DOI: 10.1007/s00244-005-8218-8

ARCHIVES OF
Environmental
Contamination
and Toxicology

Bioremediation of Tetrachloroethylene-Contaminated Groundwater in a Model Aquifer: Effects on Green Frogs (*Rana clamitans*) and *Xenopus laevis* as Potential Wetland Receptors

Tana V. McDaniel,[1] Nathalie Ross,[2] Pamela A. Martin,[1] Helena Steer,[2] Ann-Marie Irwin Abbey,[2] Suzanne Lesage[2]

[1] Canadian Wildlife Service, Environment Canada, 867 Lakeshore Road, Burlington, Ontario, L7R 4A6 Canada
[2] National Water Research Institute, Environment Canada, 867 Lakeshore Road, Burlington, Ontario, L7R 4A6 Canada

Received: 28 August 2005 /Accepted: 16 September 2006

Abstract. Recent regulations require that the ecological effects of microorganisms introduced into the environment, such as for groundwater bioremediation, be assessed prior to their utilization. A native anuran (*Rana clamitans*) and a model anuran (*Xenopus laevis*) were used as potential wetland receptors of tetrachloroethylene (PCE)-contaminated groundwater, undergoing three bioremediation treatments: natural attenuation (NA), biostimulation (ST), and bioaugmentation (AU). Eggs of both species were exposed acutely (96 h) to remediated effluents. *Xenopus* tadpoles were chronically exposed to the effluents for 100 days and were screened for the presence of bacterial pathogens. There was no impact on the survivorship of the frogs exposed either acutely or chronically to the NA, ST, or AU effluents; nor was there any evidence of bacterial infection found, with the exception of control individuals. The results of these exposures suggest that bioremediation with KB-1™ culture poses a minimal threat to anuran development and survivorship.

Chemical contamination of groundwater is a serious issue affecting the availability and quality of drinking water in North America. Efficient technologies to deal with these concerns include *in situ* enhancement of microbial degradation of contaminants to less harmful substances. While these technologies have proven effective in the breakdown of contaminants, concern regarding the environmental impacts of introduced microorganisms into contaminated aquifers has prompted the regulation of their use (Environment Canada 1997; US EPA 1997). In the current paper, we present the results of an exposure study of effluents from a model aquifer undergoing bioremediation of chloroethylene contaminants on anuran larvae.

Correspondence to: Pamela A. Martin; *email:* pamela.martin@ec.gc.ca

To comply with recent federal regulations, more research is needed on the environmental impact of introducing novel microorganisms into the environment for groundwater bioaugmentation (AU). Groundwater AU consists of injecting microorganisms, isolated or cultured, that can degrade specific contaminants in an aquifer (Devinny and Chang 2000). Pure cultures of organisms or mixed consortia may be used to provide missing species, avoid lag phases, provide organisms in large numbers, ensure dispersal, remove contaminants at low concentrations, or provide secondary factors such as surfactant-producers. Although AU has been compared to other bioremediation approaches for its efficacy to biotransform chemical products (Lendvay *et al*. 2003), little information exists on its ecological effects.

Other bioremediation approaches include biostimulation (ST) and natural attenuation (NA). The ST approach promotes the biodegradative activity of groundwater indigenous species after ensuring adequate nutrients and electron acceptors are provided (Devinny and Chang 2000), and the NA approach refers to all naturally occurring processes that reduce contaminant concentrations or toxicity (Lorah and Olsen 1999; Sun *et al*. 2000). Although these approaches are exempted from notification regulations since no microbial additions are involved (Environment Canada 1997), it has been shown that changing environmental conditions, such as injected nutrients, may cause ecological effects (Ross *et al*. 1998; Iwamoto *et al*. 2000). All of these bioremediation approaches, applied to groundwater, might impact wetlands through groundwater resurgence (Tobias *et al*. 2001, Négrel *et al*. 2003).

Tetrachloroethylene (PCE), a volatile chlorinated solvent used extensively in the dry-cleaning and degreasing industries, is a common contaminant of groundwater. In a US EPA national survey of volatile chemicals in groundwater supplies used for drinking water, PCE and its major metabolites were among the ten most commonly encountered chemicals (Westrick *et al*. 1984). Concentrations of PCE as high as 75 mg/L have been measured in Canadian aquifers (Environment Canada 1994). Although concentrations of PCE as high as 190 μg/L have been recorded in surface waters, concentrations are typically in the high ng/L to low μg/L range. The bacterial culture KB-1™ Dechlorinator has been introduced in several

sites successfully promoting PCE dechlorination to ethene (Duhamel *et al*. 2002; Major *et al*. 2002). PCE can be degraded *in situ* via anaerobic microorganisms in a series of reductive dechlorinations to produce trichloroethylene (TCE), dichloroethylene (DCE), vinyl chloride (VC), ethene, and ethane. The propensity of the chloroethylenes for microbial degradation has provided the impetus for the assessment of fully utilizing this process for bioremediation of contaminated sites. The KB-1™ culture is an enriched anaerobic bacterial consortium isolated from soil and groundwater impacted by TCE. The two main species in KB-1 responsible for the dechlorination of PCE are Geobacter and Dehalococcoides (DHC), which use dehalorespiration. Other species include *Sulfurospirillum deleyianum, Sporomusa* sp., *Spirochaeta* sp., *Methanosarcina* sp., *Methanomethylovarans* sp., and *Acetobacterium* (Duhamel *et al*. 2004). It is believed that KB-1™ consists predominantly of microorganisms that inhabit subsurface environments and is exempt from known human pathogens. Additional testing is required for assessing its potential ecological effects.

One primary concern to environmental regulators is the potential toxicity and pathogenicity of introduced microorganisms towards non-target organisms (Environment Canada 1997). Amphibians are an ideal model species because of their use in standardized toxicity assays, their susceptibility to pathogenic bacteria, and their potential for exposure. As a key component of wetland ecosystems, amphibians may be exposed to remediated effluents through groundwater recharges of wetlands (Todd 1980). Standardized toxicity assays, such as FETAX (ASTM 1998), allow for the testing of complex effluents for both acute toxicity and teratogenicity. Bacterial pathogens of amphibians are relatively well documented. Amphibians are susceptible to infection from a wide taxonomic range of bacterial pathogens (Anver and Pond 1984).

The goal of the present study was to assess the safety of three approaches of bioremediation of PCE using amphibian embryos and larvae as model wetland receptor species. Of primary interest was the introduction of a bacterial consortium, KB-1™, to enhance the degradation of chloroethylenes and its potential impact on amphibian survivorship and development. To assess the short-term impact on hatching success and early life stage development, green frog (*Rana clamitans*) and African clawed frog (*Xenopus laevis*) embryos were exposed acutely to effluent from each of the three bioremediation treatments, AU, ST, and NA, produced in a model aquifer. *Xenopus* larvae were exposed chronically to aquifer effluents for 100 days to assess long-term impacts on growth, development, and survivorship. To specifically address the potential for pathogenicity of the microbial consortium, *Xenopus* froglets from the chronic exposure were screened for incidences of bacterial infection.

Materials and Methods

Experimental Setup and Effluent Composition

A model aquifer was constructed at the Canada Centre for Inland Waters (CCIW, Burlington, ON) to compare the biosafety of three bioremediation approaches; NA, ST, and AU. The design and the characterization of the model aquifer are described in detail by Ross *et al*. (2002), but will be briefly outlined below. The model aquifer consisted of a stainless-steel tank (6.0 m long, 2.4 m wide, and 1.8 m deep) divided into three 0.8-m-wide parallel lanes and filled with clean, medium to fine grain sand. Water was pumped into the head tank of each lane, and the flow was maintained gravimetrically at 80 ml per min. The contamination source in each lane of the model aquifer consisted of PCE in silicone oil (10% w/w) mixed with coarse sand introduced in a 30-cm, 200-µm meshed sock inserted at 1 m depth. The chloroethylene concentration around the model aquifer injection point varied between 1 and 5 ppm and decreased to values between 0.89 to 0.10 ppm in the effluents (Figure 1).

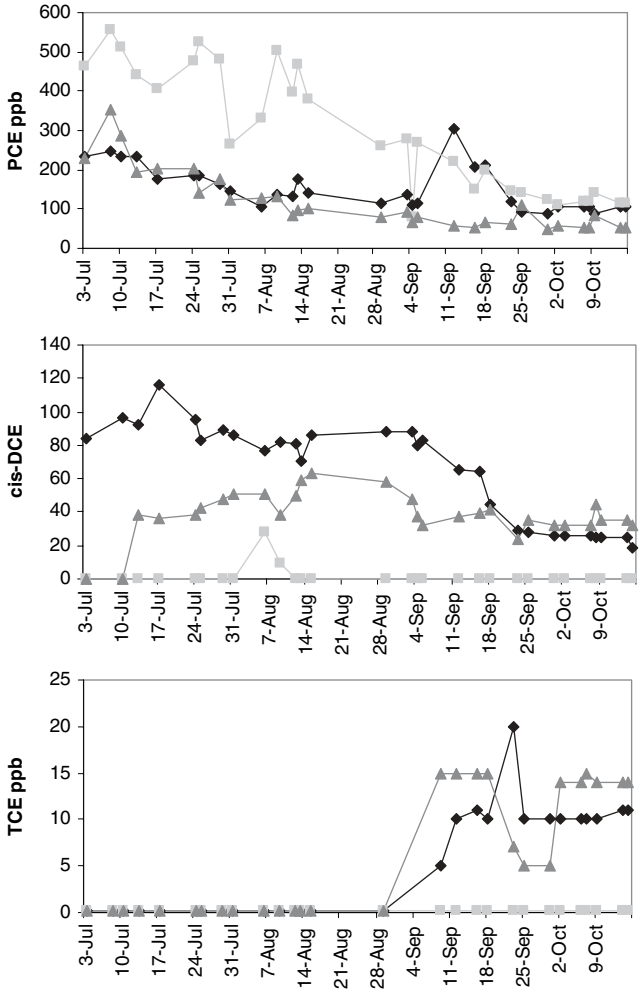

Fig. 1. Measured concentrations of perchloroethylene (PCE), trichloroethylene (TCE), and *cis*-dichloroethylene (*cis*-DCE) in the aquifer effluents from the three bioremediation lanes [(♦) Bioaugmentation (BA), (▲) Biostimulation (ST), and (■) Natural Attenuation (NA)] during the four-month period for which frogs were exposed

The NA lane was left unaltered. The ST lane received injections of nutrients (methanol and lactic acid) twice weekly, and the AU lane received the same regime of nutrient injection as the ST lane plus a single injection of KB-1™ culture (Major *et al*. 2002). The effluents were sampled at the exit of the model aquifer, located approximately 4.3 m from the contamination source and 4.0 m from the nutrient injection/AU injection zone.

The source of water for the model aquifer was groundwater from a research well located on the grounds of the CCIW. Carbon-filtered, dechlorinated tap water was used to dilute the aquifer effluent

during amphibian testing. Concentrations of chloroethylenes in the aquifer effluents (AU, ST, NA), as well as control groundwater and dechlorinated tap water, were measured biweekly using purge and trap (US EPA method 503 0B) GC/MS (US EPA method 82 60B; US EPA 1996) as described in Ross *et al*. (2002). The minimum detection limit for this technique was 0.1 ppb; however, the practical detection limit, or limit of quantification, was closer to 1.0 ppb for most samples. In addition to the chemical analyses, the effluents and KB-1 culture were tested for the presence of potential pathogens: *Cryptosporidium parvum, Campylobacter* sp., *Shigella* sp., *Staphylococcus aureus, Clostridium perfringins, Escherichia coli, Pseudomonas aeruginosa*, and *Yersinia* sp. (Bergey *et al*. 1984).

Effects of Acute Exposures to Effluents

Egg masses of green frogs *(R. clamitans)* and *X. laevis* were collected when less than 24 h old, before gastrulation was completed. Green frog egg masses were collected from a site in south central Ontario, Canada, where chloroethylenes were not detected in the surface water. *Xenopus* egg masses were obtained from a breeding colony at the University of Guelph (Guelph, ON). Three egg masses were used for each species with each egg mass representing a replicate. An egg mass represents the clutch of an individual female frog. Each egg mass was divided into lots of approximately 30 embryos and immersed in 300 ml of exposure solution contained in a sealed 1-L Mason® jar. The jars were held in a circulating water bath to maintain a constant temperature of 22°C ± 1°C. All exposures were conducted under an exhaust hood. A 14L/10D photoperiod, using full spectrum lamps (Interlectric Corporation F32T8 True Lite fluorescent tubes), was used to mimic natural light conditions.

Static renewal exposures (96 h) of aquifer effluent were conducted on green frogs and *Xenopus* using a modified FETAX protocol (ASTM 1998). Exposure solutions were composed of 12.5, 25.0, and 50.0% aquifer effluents. Controls consisted of groundwater from the aquifer head tank. The proportion of groundwater/effluent was made up to 50% consistently across all treatments by the addition of the appropriate amount of groundwater and all solutions were brought up to 100% with aerated tap water. Exposure solutions were renewed once every 24 h. Exposure solutions were not aerated during the 96-h exposure. During one 24-h renewal period, chloroethylene levels were measured at 0 and 24 h, to determine the range of chloroethylene levels in the jars.

Dissolved oxygen, pH, and temperature were monitored in all exposure jars daily to ensure that acceptable conditions for egg survival were maintained. At the end of the 96-h exposure period, all of the embryos had hatched and survivorship was assessed. Tadpoles were then euthanized using a 1% solution of tricaine methane sulfonate and were assessed for developmental malformations. Malformations were classified according to the *Atlas of Abnormalities* for *Xenopus* (Bantle *et al*. 1998).

Effects of Chronic Exposure to Effluents

Chronic exposures were initiated on *Xenopus* embryos less than 24 h old and continued for 100 days (d). Subsequent to this, tadpoles were raised in tap water until they completed metamorphosis, or until 156 d had passed. *Xenopus* tadpoles and embryos were exposed to effluents from each of three lanes mixed with aerated tap water to a dilution of 25% in order to simulate the mixing of groundwater into a freshwater wetland through groundwater recharge. Controls were exposed to clean groundwater at the same dilution rate. Three clutches from three individual females were used, and were divided up, each providing one replicate of each treatment. A total of 30 eggs were used in each replicate. *Xenopus* were raised in 1-L glass Mason® jars for 6 d until feeding began. At this point, tadpoles were transferred to 25-L glass aquaria containing 10 L of exposure solution. Tanks were suspended in a circulating water bath, with a constant temperature of 22°C ± 1°C. Tadpoles were fed Nasco Frog Brittle once per day; feces and leftover food were removed. The exposure solution was changed completely every 4 d; on the intervening days, 1.75 L of freshly collected aquifer effluent was added to each tank after removing an equal volume of water. For 4 h immediately following the addition of fresh effluent, tanks were not aerated. During one 24-h renewal period, chloroethylene levels were measured at 0, 4. and 24 h in the exposure tanks and at time 0 in the effluent and compared to expected nominal values to characterize the loss of chlorethylenes due to mixing and volatilization. Tanks were then aerated for 20 h; pretrials with the effluent indicated that aeration was necessary to maintain dissolved oxygen levels above 5 mg/L. When tadpoles reached metamorphosis, stage 66 (Nieuwkoop and Faber 1994), they were euthanized using a 1% solution of tricaine methane sulfonate, weighed, and the snout vent length (SVL) was measured. Frogs were assessed for malformations under a dissecting scope. Malformations were classified according to standard nomenclature (Meteyer *et al*. 2000; Ouellet 2000).

Bacterial Screening on Xenopus *Chronically Exposed to Effluents*

Upon euthanizing, frogs were initially examined macroscopically for any visible lesions. To determine if harmful bacterial infection would result from exposure to the elevated bacterial levels associated with the bioremediation treatments, swabs were taken from a subsample of frogs from each treatment group. Euthanized frogs were stored at −80°C and then thawed 20 min prior to swabbing. Swabs were conducted in sterile conditions under a laminar flow hood. Frogs were first swabbed externally with ethanol and their dorsal surface exposed to UV light for 20 min to sterilize the skin and prevent contamination while opening the abdominal cavity. Both aerobic and anaerobic swabs were taken from the abdominal cavity and from the lower intestinal tract to provide a fecal sample. BD BBL™ Port-A-Cul swabs were used in the collection and transport of aerobes and BD BBL™ Culture Swab Plus with charcoal were used for anaerobes. Three frogs from each treatment (one frog from each replicate tank) were swabbed; swabs were pooled so that each microbial assessment contained material from each of the three frogs. This may have led to higher than normal counts. Swabs were immediately transported to the Animal Health Laboratory at the University of Guelph for routine bacteriology including enterobacterial: *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Aeromonas* spp.; Gram-positive bacteria, and a subset of anaerobes including *Fusobacterium* spp. and bacteriodes. In addition, screening was conducted for the presence of specific pathogens: *Yersinia* spp. *Salmonella* spp., and *Campylobacter* spp. Anaerobic fecal samples were screened for *Clostridium perfringens*. If bacteria were detected in any of the abdominal samples, the analysis was redone with three new frogs to ensure results were replicable. The relative density of bacterial colonies was expressed as categories: 0 representing no colonies, 1+ occasional colonies, 2+ few colonies, 3+ moderate number of colonies, and 4+ representing a large number of colonies.

Statistics

All analyses were performed using STATISTICA 6.1 (StatSoft 2003). For the acute exposures, differences in survivorship and malformation rates among effluent concentrations were analyzed using a one-way

analysis of variance (ANOVA) with each jar considered a replicate ($n = 3$). Each effluent type was compared independently with the control group. For the chronic exposures, differences in survivorship, time to metamorphosis, and malformation rates among effluent groups (i.e., AU, ST, NA, Control) were analyzed using a one-way ANOVA with three replicate tanks per effluent type. Percentage data were normalized by using the arc-sign transformation (Sokal and Rohlf 1999). Clutch effects for survivorship, size, time to metamorphosis, and malformation rates were analyzed using an ANOVA with clutch as the categorical predictor variable across effluent type. An ANCOVA was conducted to test for differences in body size using cumulative tadpole density in each tank as a continuous predictor variable. Post hoc tests for differences among groups were Tukey's honestly significant difference test.

Results

Composition of Effluents

Neither PCE nor any of its breakdown products were detected in the groundwater used for control exposures. The parent contaminant, PCE, was the primary chloroethylene to be detected in effluent from the NA lane, with only low concentrations of *cis*-DCE occasionally detected (Figure 1). In the ST lane, biodegradation was promoted through reductive dechlorination; levels of PCE were lower than in the NA lane, and *cis*-DCE was measured at levels between 24 and 63 ppb. However, no further degradative products were observed in the ST lane throughout the period of the study. In the AU lane, PCE was dechlorinated to ethene (0 to 0.3 ppb) with intermediate degradation products, such as VC (5 to 9 ppb), remaining. At the beginning of the chronic exposures, concentrations of PCE in the aquifer effluents ranged from 440–550 ppb (NA), 200–350 ppb (ST), and 190–250 ppb (AU) with 70–100 ppb of *cis*-DCE in the AU lane.

Concentrations of chloroethylenes were measured in the effluent only, on a regular basis. After mixing and dilution of the effluent, concentrations of chloroethylenes in the tadpole exposure solutions were considerably lower. The nominal concentrations in the amphibian exposure tanks were expected to be 25% of the measured concentration of the effluent immediately after mixing with aerated water. In all likelihood, the actual concentrations were substantially less than the nominal concentration for most of the exposure period. Measurements of chloroethylenes in tank water 4 hours after mixing with the effluent indicated that PCE levels were 17% to 25% of nominal concentrations, while concentrations of *cis*-DCE were 27% to 40% of nominal concentrations. After aeration for 20 hours, concentrations of both compounds dropped to trace levels, at which point more effluent was added. This would bring the range of expected PCE concentrations in the tadpole tanks at between 0 and 100 ppb over the 4 months of exposure, and the range of *cis*-DCE concentrations between 0 and 8 ppb.

Other compounds present in the aquifer effluents include methane and ethane, and propionate. Levels of methane ranged from 0 to 52 ppb in the AU effluent, 0 to 67 ppb in the ST effluent, and 0 to 0.7 ppb in the NA effluent. Propionate was also found in the effluents in low concentrations around 20 ppb in all effluents. Within the aquifer, densities of viable bacteria ranged from $1.47 \times E05$ to $3.42 \times E05$ bacteria per ml in water samples taken from wells just prior to where the effluent exits the aquifer, suggesting that not all bacteria had been filtered out by the tank. Measured concentrations of total heterotrophic bacterial densities in the aquifer effluents ranged from 840 to 2300 cfu per 100 ml as quantified by membrane filtration; however, this method did not allow us to identify the bacteria. All three aquifer effluents tested negative for the pathogens *Cryptosporidium parvum, Campylobacter* sp., *Shigella* sp., *Staphylococcus aureus, Clostridium perfringins, Escherichia coli, Pseudomonas aeruginosa*, and *Yersinia* sp. However, the source water for the aquifer did contain large numbers of *Pseudomonas aeruginosa*, indicating that control individuals may have been exposed to this bacterium.

Effects of Acute Exposures of Effluents

Acute exposure to aquifer effluents (NA, ST, and AU) did not lead to a decrease in survivorship of either green frogs or *Xenopus* embryos (Table 1). Survivorship of green frog embryos did not fall below 95% in any of the treatment or control groups, whereas survivorship of *Xenopus* embryos was significantly lower in the control group than in the AU 25% effluent exposure group (ANOVA $F = 4.3$, $p = 0.044$). There was a slight nonsignificant increase in malformation rates in *Xenopus* embryos exposed to the highest concentration of the ST effluent and AU effluent relative to controls. The majority of these malformations involved dorsal flexure of the tail and notochord.

Effects of Chronic Exposure of Effluents

At 68 d prior to metamorphosis, the control group appeared to have higher survival than those exposed to the remediated effluents, although this was not significant (ANOVA $F = 2.0$, $p = 0.19$, Table 2). By day 100, any earlier differences were no longer apparent ($F = 0.35$ $p = 0.79$, Table 2). There was no significant clutch effect for survivorship. Of the surviving individuals, time to metamorphic climax did not vary among the groups. There was a significant clutch effect ($F = 3.1$ $p = 0.049$), with individuals from clutch three reaching metamorphic climax four to five days earlier than the other two clutches. There was no increase in the incidence of malformations in metamorphosed frogs exposed to any of the aquifer effluents as compared to the control group ($F = 1.44$, $p = 0.3$).

Size of frogs at metamorphic climax varied significantly among treatments (Table 2). There was a significant difference in weight, with frogs from the control and ST groups being 16% smaller on average than those from the AU and NA group. Snout-vent length was also significantly lower in control frogs as compared to the NA or AU group. However, size was significantly correlated with the number of frogs per tank (weight $R^2 = 0.25$, length $R^2 = 0.24$). When density was considered in the model as a covariate, there was no significant effect of treatment for either weight ($F = 0.9$, $p = 0.4$) or snout vent length ($F = 1.1$, $p = 0.35$).

Table 1. Survivorship and malformation rates in green frog and *Xenopus* embryos exposed to aquifer effluent from three bioremediation scenarios: Natural Attenuation (NA), Biostimulation (ST), and Bioaugmentation (AU) and control groundwater for 96 h

			Green Frogs				*Xenopus*			
			Survivorship (%)		Malformations (%)		Survivorship (%)		Malformations (%)	
Treatment	*cis*-DCE (ppb)	PCE (ppb)	mean	stdev	mean	stdev	mean	stdev	mean	stdev
Control	0	0	98.9	1.9	5.6	5.1	75.6*	8.4	7.8	5.1
NA 12.5	0	8 – 20	95.5	1.9	6.7	3.3	82.2	10.7	8.9	5.1
25	0	14 – 33	98.9	1.9	5.5	1.8	88.9	10.7	3.3	0
50	0	20 – 59	93.5	3.4	6.4	5.5	95.6	3.8	8.8	1.8
ST 12.5	0	5 – 8	95.7	1.7	2.2	1.9	88.9	7.7	6.7	3.3
25	0	7 – 13	95.6	3.8	2.3	4.0	93.3	6.7	5.6	3.9
50	0	16 – 28	97.8	3.9	5.3	4.7	82.2	16.4	14.4	1.9
AU 12.5	4 – 8	4 – 17	95.6	3.9	7.7	6.9	85.6	3.9	13.3	6.7
25	7 – 15	7 – 36	97.8	3.9	8.9	5.2	91.1	3.9	4.4	1.9
50	17 – 18	15 – 37	97.8	3.9	5.5	3.9	85.6	5.1	14.4	3.9

The range over the 24-h renewal period of measured concentrations of the two main contaminants in the effluent, *cis*-DCE and PCE., are given for each effluent type.
stdev = standard deviation.
* Significant at the $p < 0.05$ level.

Table 2. Survivorship (day 68 [stage 52–64] and day 100 [stage 60–66]), time to transformation, and size at metamorphosis of *Xenopus* tadpoles exposed to aquifer effluent from three bioremediation scenarios (Natural Attenuation (NA), Biostimulation (ST), and Bioaugmentation (AU)), and control for 100 days

	Survivorship day 68 (%)		Survivorship day 100 (%)		Time to transform (d)		Weight (g)		Snout-vent length (mm)	
Treatment	mean	stdev	mean	stdev	mean	SE	mean	SE	mean	SE
Control	88.3	1.4	70.2	11.4	104.3	2.2	0.36^{B}	0.01	15.3^{C}	0.2
NA	63.3	17.4	58.9	18.4	104.8	2.6	0.42^{A}	0.02	15.9^{AB}	0.2
ST	68.7	27.8	65.4	25.0	103.1	1.8	0.37^{B}	0.02	15.5^{BC}	0.2
AU	60.6	5.8	58.4	4.3	100.9	2.3	0.45^{A}	0.02	16.4^{A}	0.2

Letters indicate means that are significantly different at the $p < 0.05$ level. stdev = standard deviation; SE = standard error.

Bacterial Screening on Xenopus *Chronically Exposed to Effluents*

Only in the control frogs were any bacteria isolated from the abdominal cavity (Table 3). To ensure these large numbers were not due to contamination of the samples, the assay was repeated on three separate frogs from the control treatment with similar results. Frogs from NA, ST, and AU effluents, swabbed and cultured at the same time, did not show bacterial growth from abdominal swabs, eliminating the hypothesis of contamination of samples. No frogs, including controls, tested positive for *Yersinia*, *Salmonella*, or *Campylobacter*. Fecal swabs indicated a number of aerobic organisms in all treatment groups but contained no *Clostridium perfringens.*

Discussion

Few impacts on survivorship or malformation rates were seen as a result of acute embryonic exposure to effluents of an aquifer contaminated with low levels of PCE and treated with three bioremediation approaches. This suggests that bioremediation through the addition of a microbial consortia known to degrade chlorinated solvents (AU with KB-1™), or the enhancement of the native microbial community (ST), poses a minimal threat to early life stages of amphibians. There was no impact on the hatching success of either green frogs or *Xenopus* as compared to the control group. Hatching success was high with the exception of the control group for the *Xenopus* exposure. Mortality induced by chloroethylene toxicity was not expected given the low levels of contamination in the effluents. Concentrations of PCE and its metabolites were well below levels known to be toxic to fish by at least an order of magnitude (Call *et al*. 1983). Previous exposures indicated that levels as high as 20 mg/L PCE, 60 mg/L of TCE, and 100 mg/L of DCE did not induce significant mortality in American toad (*Bufo americanus*), green frog (*Rana clamitans*), wood frog (*Rana sylvatica*), or spotted salamander (*Ambystoma maculatum*) embryos (McDaniel *et al*. 2004).

Exposure to aquifer effluents did not result in an increase of malformation rates in green frog embryos or *Xenopus*. Levels of PCE, TCE, and *cis*- and *trans*-DCE in the aquifer effluents were considerably below those found to cause malformations in exposures to amphibian embryos (Fort *et al*. 1993). McDaniel *et al*. (2004) determined that the 96-h EC_{50} for malformations

Table 3. Counts of bacterial swabs of metamorphosed *Xenopus* froglet*s* raised in aquifer effluents from the three bioremediation scenarios and control groundwater

Treatment	Swab type	Bacteria	Count[a]
Control[b]	Abdominal aerobic	*Citrobacter freundii*	4 +
		Escherichia coli	4 +
		Pseudomonas sp.	4 +
		Klebsiella oxytoca	4 +
	Abdominal anaerobic	Mixed Anaerobes	3 +
	Fecal Aerobic	*Escherichia coli*	4 +
		Citrobacter sp.	4 +
		Klebsiella sp.	4 +
	Fecal anaerobic	*Clostridium pf*	0
NA	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Escherichia coli*	4 +
		Citrobacter sp.	NQ
	Fecal anaerobic	*Clostridium pf*	0
ST	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Escherichia coli*	4 +
		Klebsiella sp.	4 +
		Citrobacter freundii	4 +
	Fecal anaerobic	*Clostridium pf*	0
AU	Abdominal aerobic	No bacterial growth	0
	Abdominal anaerobic	No bacterial growth	0
	Fecal aerobic	*Citrobacter freundii*	3 +
		Aeromonas sp.	3 +
		Shewanella putrifaciens	3 +
		Pseudomonas sp.	1 +
	Fecal anaerobic	*Clostridium pf*	0

NQ is not quantified.
[a] The relative density of bacterial colonies given are categorized, with 0 being no colonies and 4 being a large number of colonies.
[b] Combined results of two separate tests.

caused by exposure to PCE for green frogs was 7.9 mg/L and the EC_{50} for TCE was 22 mg/L while no increase in the incidence of malformation was seen at the 2.5-mg/L level.

Concentrations of chloroethylenes in the aquifer lanes were well within the range of those measured in the groundwater of contaminated aquifers where concentrations of PCE as high as 75 ppm have been recorded (Environment Canada 1994). Concentrations of PCE and *cis*-DCE in tadpole exposure solutions, in contrast, were in the nd to 100-ppb range, and were reflective of levels in surface waters (Environment Canada 1994). We believe these concentrations accurately reflect a realistic scenario of surface water contamination from groundwater recharge of a wetland. These low chloroethylene levels allowed us to evaluate the effects of the remediation techniques on amphibian health without the confounding influence of chloroethylene toxicity.

Consistent differences were seen among clutches in *Xenopus* in terms of time to metamorphosis and growth. Such interclutch variability is thought to be the result of maternal effects arising from differences in egg size or maternal investment per egg (Parichy and Kaplan 1992). There is evidence to suggest that both maternal and genetic effects can lead to differences in tolerance to suboptimal water chemistry (Pierce and Sikand 1985). This variation in tolerance to water chemistry, growth rates, and time to metamorphosis due to maternal effect has important implications for both field exposure studies and lab toxicity testing. Importantly, it emphasizes the necessity of using multiple clutches in amphibian bioassays.

Chronic exposure to bioremediated effluents had a minimal impact on tadpole survivorship, growth, and development. Levels of chloroethylenes in the effluents were likely too low to have an inhibitory effect on growth and development. In a previous study, chronic exposures of American toad larvae for 30 days at much higher concentrations of PCE (4 ppm) and TCE (4 ppm) did not have an impact on survivorship and rate of development (McDaniel *et al*. 2004). At day 68 in the current study, survivorship was nonsignificantly lower in the aquifer treatment groups compared to controls. By the end of the 100-d exposure, however, these differences had disappeared as most control mortality occurred during metamorphosis; tadpoles exposed to effluents died throughout the entire exposure period. Because of these differing patterns in mortality, control tadpoles developed at higher densities and were, thus, significantly smaller than animals in the AU and NA treatments. This is consistent with tadpole husbandry literature, which suggests growth may be inhibited in crowded conditions (Richards 1958). When density was considered in the statistical model, all differences in body size between treatments disappeared, suggesting size differences were mainly due to differing tank densities. Early mortality in the treatments where tadpoles were exposed to effluents, may have resulted in larger individuals, which were more likely to survive metamorphosis. It is possible that undersized individ-

uals in the control groups may not have had sufficient energy reserves to survive the stress of metamorphosis. Increased body size at metamorphosis has been correlated with increased survivorship of transformed frogs (Alford 1999). In this way, there can be a compensatory effect of density in regard to pesticide-induced mortality and its impact on body size and later survivorship.

The survival of many of the bacterial species within the KB-1 culture would be expected to be limited in the aerobic conditions present in the tadpole exposure tanks since most of the dominant bacteria are thought to be obligate anaerobes (*Acetobacterium* sp. and *Dehalococcoides ethenogenes*). There is, however, some evidence to suggest that the effluent did contain live KB-1 bacteria. *Dehaloccocoides* was shown to survive the aerobic conditions present in the aquifer over part of the exposure period, indicating it can survive short periods of aerobic conditions. In addition, DNA from *Dehaloccocoides* was positively identified in the AU effluent (Sandra P. Toquica Diaz, unpublished data). Microfiltration of the effluent indicated numbers of unidentified bacteria in all effluents. However, neither the KB-1 culture nor the aquifer effluents tested positive for any of the pathogenic bacteria species for which they were screened. All the evidence suggests that KB-1 culture itself is not harmful to amphibians. In preliminary trials, American toad embryos, fathead minnows, and *Lemna minor* (duckweed) were exposed to groundwater containing KB-1 culture for between 4 (toads) and 7 (fathead minnow and *Lemna*) days. There were no significant effects on growth or survivorship from exposure to groundwater containing KB-1 culture (unpublished data). Therefore, any effects seen on the tadpoles were more likely a result of reduced water quality associated with either the by-products of bacterial metabolism, bacterial toxins, or with a change in the environmental conditions optimized to promote bacterial growth (Sun *et al*. 2000).

There is no indication that the addition of a microbial consortium initially isolated from a contaminated site (AU treatment) or the enhancement of the indigenous bacterial population (ST treatment) led to the increased risk of infection in *Xenopus*. Abdominal swabs of all *Xenopus* exposed to remediated aquifer effluents were clear of bacterial growth. This is promising in terms of the biosafety of these remediation techniques, and not unexpected. Previous screenings of KB-1 culture tested negative for a series pathogenic to humans, many of which are also pathogenic to amphibians: *Salmonella*, *Listeria monocytogenes*, *Vibrio* sp., *Campylobacter* sp., hemolytic *Clostridia* sp., *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Yersinia* sp., pathogenic yeast and mold, fecal coliforms, and enterococci (Duhamel *et al*. 2002; Peter Dennis, SiREM, Guelph, ON, personal communication), nor did any of our pathogens screens of KB-1 or the effluents reveal any pathogenic bacteria species.

In contrast, abdominal swabs of control frogs tested positive for *Citrobacter freundii*, *Escherichia coli*, *Pseudomonas* sp., and *Klebsiella*, all of which are potential frog pathogens, but are also part of the normal intestinal flora. The presence of high numbers of bacteria in the abdominal cavity of control frogs may indicate a significant bacterial infection. *Citrobacter freundii* is a well-known anuran pathogen and has been found to opportunistically infect open wounds and lesions in farmed frogs, as has *E. coli* (Baldassi *et al*. 1995). While *Citrobacter freundii* was also isolated in the fecal samples of frogs from some of the other treatments, this was to be expected as it is known to be a normal component of the flora of the lower intestinal tract (Hird *et al*. 1983). *Klebsiella* is also both a normal component of the intestinal flora and a potential pathogen that has been related to septicemia (Hird *et al*. 1983). *Pseudomonas* sp. can lead to serious infection (Kaplan 1953). Bacterial infection may help to explain the smaller than average body size of control animals relative to those from other treatments if energy that would otherwise be allocated to growth is used to fight infection. Bacterial infection can lead to anorexia in amphibians (Crawshaw 2000).

It is not clear why bacterial infection may have been present in control individuals and absent in those raised in aquifer effluents. It is possible that populations of pathogenic bacteria were lower in aquifer effluent than they were in the control groundwater. This is supported by the fact that *Pseudomonus aeruginosa* was found in the source groundwater going into the tanks, and not in the aquifer effluents exiting the tanks. In fact, since the source groundwater was used for the control frog exposures, this is likely to have been the source of their *Pseudomonas* infections. All water from the aquifer effluent was passed through a large sand bed that may have filtered a significant proportion of the groundwater indigenous bacteria. Alternatively, the addition of chloroethylenes may have resulted in a changed bacterial community from that originally present in the groundwater. Indeed, it is expected that the addition of PCE at concentrations as high as 30 ppm at 1 m from the injection source had an adverse effect on the groundwater microbial community by reducing its structure and function including potential amphibian pathogens (Fuller *et al*. 1997; Baker *et al*. 2001). While we cannot say whether or not this was due to filtration or to biotic/abiotic conditions in the aquifer, filtration seems less likely as, overall, bacteria were still numerous in the effluent.

The results presented in this paper suggest that bioremediation using the addition of a bacterial consortium known to degrade chlorinated solvents, specifically KB-1™, has little or no impact on amphibian growth, development, or survivorship and was not pathogenic. This work was performed using a large-scale model aquifer to simulate conditions that may exist in field applications of this technique. Although amphibians represent one possible receptor, effects on additional potential receptor species are currently being tested.

Acknowledgments. This project was funded by the Environmental Management of Biotechnology Regulation and Research (EMBRR) program of Environment Canada. The authors thank Sandra Toquica Diaz, Gregg Ferris, Liam Kelly, and Sue Brown for their advice and technical support.

References

Alford R (1999) Ecology: resource use, competition and predation. In: McDiarmid RW, Altig RA (eds) Tadpole: Biology of anuran larvae. University of Chicago Press, Chicago, IL, pp 240–278

Anver MR, Pond CL (1984) Biology and diseases of amphibians. In: Fox JG, Cohen BJ, Loew FM (eds) Laboratory animal medicine. Academic Press, New York, NY, pp 434–445

ASTM (1998) Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) ASTM E1439-98. ASTM International, West Conshohocken, PA

Baker P, Futamata H, Harayama S, Watanabe K (2001) Bacterial populations occurring in a trichloroethylene-contaminated aquifer during methane injection. Environ Microbiol 3:187–193

Baldassi L, Hipolito M, DeSouza FL, DeSouza CWO (1995) Clostridia presence in the bullfrog (*Rana catesbeiana* Shaw, 1802) mouth myiasis lesion. Bol Inst Pesca Sau Paulo 22:95–102

Bantle JA, Dumont JN, Finch RA, Linder G, Fort DJ (1998) Atlas of abnormalities. Oklahoma State University Press, Oklahoma

Bergey DH, Holt JG, Kriet NR, Sneath P (1984) Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, MD

Call DJ, Brooke LT, Ahmad N, Richter JE (1983) Toxicity and metabolism studies with EPA priority pollutants and related compounds in freshwater organisms. U.S. Environmental Protection Agency, Duluth, MN

Crawshaw GJ (2000) Diseases and pathology of amphibians and reptiles. In: Sparling DW, Linder G, Bishop CA (eds) Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola, FL, pp 199–231

Devinny JS, Chang SH (2000) Bioaugmentation for soil bioremediation. In: Wise DL (ed) Bioremediation of contaminated soils. Marcel Dekker, New York, NY, pp 465–488

Duhamel M, Wehr SD, Yu L, Rizvi H, Seepersad D, Dworatzek S, Cox EE, Edwards EA (2002) Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride. Water Res 36:4193–4202

Duhamel M, Mo K, Edwards EA (2004) Characterization of a highly enriched dehalococcoides-containing culture that grows on vinyl chloride and trichloroethene. Appl Environ Microbiol 70:5538–5545

Environment Canada (1994) Tetrachloroethylene. Canadian Environmental Protection Act. Priority substances list supporting document. Environment Canada, Ottawa, ON

Environment Canada (1997) Canadian Environmental Protection Act - New Substances Notification Regulation, Environment Canada, Ottawa, ON

Fort DJ, Stover EL, Rayburn JR, Hull M, Bantle JA (1993) Evaluation of the developmental toxicity of trichloroethylene and detoxification metabolites using *Xenopus*. Teratogen Carcinogen Mutagen 13:35–45

Fuller ME, Scow KM, Lau S, Ferris H (1997) Trichloroethylene (TCE) and toluene effects on the structure and function of the soil community. Soil Biol Biochem 29:75–89

Hird DW, Diesch SL, McKinnell RG, Gorham E, Martin FB, Meadows CA, Gasiorowski M (1983) Enterobacteriaceae and *Aeromonas hydrophila* in Minnesota frogs and tadpoles (*Rana pipiens*). Appl Env Microbiol 46:1423–1425

Iwamoto T, Tani K, Nakamura K, Suzuki Y, Kitagawa M, Eguchi M, Nasu M (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. FEMS. Microbiol Ecol 32:129–141

Kaplan HM (1953) The care and diseases of the frog. Proc Anim Care Panel 4:74–92

Lendvay JM, Loeffler FE, Dollhopf M, Aiello MR, Daniels G, Fathepure BZ, Gebhard M, Heine R, Helton R, Shi J, Krajmalnik-Brown R, Major CL Jr, Barcelona MJ, Adriaens P (2003) Bioreactive barriers: a comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. Environ Sci Technol 37:1422–1431

Lorah MM, Olsen LD (1999) Natural attenuation of chlorinated volatile organic compounds in a freshwater tidal wetland: Field evidence of anaerobic biodegradation. Water Resources Res 35:3811–3827

Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW (2002) Field demonstration of successful bioaugmentation to acheive dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36:5106–5116

McDaniel T, Martin P, Ross N, Brown S, Lesage S, Pauli B (2004) Effects of chlorinated solvents on four species of North American amphibians. Arch Environ Contam Toxicol 47:101–107

Meteyer CU, Loeffler IK, Fallon JF, Converse KA, Green E, Helgen JC, Kersten S, Levey R, Eaton-Poole L, Burkhart JG (2000) Hind limb malformations in free-living northern leopard frogs (*Rana pipiens*) from Maine, Minnesota and Vermont suggest multiple etiologies. Teratology 62:151–171

Négrel P, Petelet-Giraud E, Barbier J, Gautier E (2003) Surface water-groundwater interactions in an alluvial plain: Chemical and isotopic systematics. J Hydrol 277:248–267

Nieuwkoop, Faber (1994) Normal table of *Xenopus laevis* (Daudin). Garland Publishing Inc, New York

Ouellet M (2000) Amphibian deformities: Current state of knowledge. In: Sparling DW, Linder G, Bishop CA (eds) Ecotoxicology of amphibians and reptiles. SETAC Press, Pensacola FL, pp 617–661

Parichy DM, Kaplan RH (1992) Maternal effects on offspring growth and development depend on environmental quality in the frog *Bombina orientalis*. Oecologia 91:579–586

Pierce BA, Sikand N (1985) Variation in acid tolerance in wood frogs: Genetic and maternal effects. Can J Zool 63:1647–1651

Richards CM (1958) The inhibition of growth in crowded *Rana pipiens* tadpoles. Physiol Zool 31:138–151

Ross N, Deschenes L, Bureau J, Clément B, Comeau Y, Samson R (1998) Ecotoxicological assessment and effects of physicochemical factors on biofilm development in groundwater conditions. Environ Sci Technol 32:1105–1111

Ross N, Millar K, Brown S, Steer H, Robinson C, Grande P, McDaniel T, Martin P, Pauli B, Lesage S (2002) Assessing the biosafety of three bioremediation approaches in a tetrachloroethylene-contaminated environment, Paper 1C-04. In: Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, May 20–23, 2002

Sokal RR, Rohlf FL (1999) Biometry, 2nd ed. WH Freeman and Co, New York, NY

StatSoft Inc. (2003) STATISTICA (data analysis software system). Tulsa, OK

Sun Y, Bartlett C, Johnson CD, Klecka GM, Clement TP (2000) Natural attenuation of chlorinated ethene compounds: Model development and field-scale application at the Dover site. J Contam Hydrol 42:113–140

Tobias CR, Harvey JW, Anderson IC (2001) Quantifying groundwater discharge through fringing wetland to estuaries. Seasonal variability, methods comparison, and implications for wetland-estuary exchange. Limnol Oceanogr 46:604–615

Todd DK (1980) Groundwater hydrology. John Wiley & Sons, New York, NY

US EPA (1996) U.S. EPA Test Method 82 60B: Volatile organic compounds (VOCs)-GC/MS. EPA report # SW-846 ch 4.3.2. United States Environmental Protection Agency, Washington, DC

US EPA (1997) Point to consider in the preparation of TSCA biotechnology submissions for microorganisms. Office of Pollution Prevention and Toxics, Washington, DC

Westrick JJ, Mello JW, Thomas RF (1984) The groundwater supply survey. J Am Water Works Assoc 76:52–59

Attachment G:

Photographs of KB-1® Vessels and Shipping Overpacks