Risk Assessment Summary Conducted Pursuant to the New Substances Notification Regulations (Organisms) [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* NSN-15050: KB-1® Anaerobic Dechlorinating Consortium containing *Dehalococcoides* spp.

This document explains the regulatory decision taken under Part 6 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) and its *New Substances Notification Regulations (Organisms)* [NSNR(O)] regarding the manufacture of KB-1® Anaerobic Dechlorinating Consortium containing *Dehalococcoides* spp. (hereafter referred to as KB-1®) by SiREM, a division of Geosyntec Consultants (Geosyntec), for introduction anywhere in Canada.

KB-1® was notified pursuant to subsection 3(1) of the CEPA 1999 New Substances Notification Regulations (Organisms). Although it contains a mixed population of micro-organisms, KB-1® is a naturally occurring consortium isolated from the environment and, as such, meets the criteria for evaluation as a single substance as defined in Section 3 of CEPA 1999.

Environment Canada and Health Canada have conducted a risk assessment using information submitted by SiREM and other available scientific information in order to determine whether KB-1® meets the criteria as set out in section 64 of CEPA 1999[1].

Regulatory Decision

Based on hazard and exposure considerations, the risk assessment conducted by Health Canada and Environment Canada concluded that the proposed use of KB-1® does not cause harm to the Canadian environment or human health as described in Section 64 of the CEPA 1999. However, a Significant New Activity (SNAc) Notice (SNAc No. 15050) was recommended based on uncertainties regarding possible environmental impacts of the notified organism in activities outside the scope of this assessment. This SNAc Notice outlines information requirements for those activities. The SNAc Notice has been published in the *Canada Gazette* Part I: Vol. 142, No. 28 on July 12, 2008. Any activity not identified in the Notice may proceed after April 18, 2008.

This evaluation did not include an assessment of human health risk in the occupational environment.

[1] In accordance with section 64 of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) a substance is toxic if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

NSNR(O) Schedule: 1 (manufacture of micro-organisms for introduction anywhere in Canada).

Organism Identity: KB-1 Anaerobic Dechlorinating Consortium containing *Dehalococcoides* spp.

Notifier: SiREM (division of Geosyntec Consultants), 130 Research Lane, Suite 2, Guelph, Ontario, N1G 5G3

Date of decision: April 18, 2008

Proposed use: Bioremediation of groundwater contaminated with chlorinated organic solvents.

IDENTITY / STRAIN HISTORY

KB-1® was isolated from soil and water samples taken from a trichloroethene (TCE) contaminated site in Southern Ontario located in the Mixed Wood Plains Terrestrial Ecozone. KB-1® was enriched with progressively increasing TCE concentrations in defined media containing mineral salts, and electron-donor-containing nutrients (mixture of acetate, methanol and lactate) at room temperature under anaerobic conditions. After a year of enrichment, the KB-1® consortium was able to grow on high concentrations of TCE (400 µg/L) (Duhamel *et al*., 2002).

The micro-organisms present in KB-1® are predominately anaerobic, use fermentable electron donors and chlorinated compounds for growth, and tolerate cool to moderate temperatures. To identify these constituents, the notifier used 16S rRNA gene cloning and sequencing to detect operational taxonomic units (OTUs[2]), which were assigned to a specific genus or family using standard bioinformatics. The notifier identified 45 different OTUs in the following genera or families of Bacteria (listed in order of prevalence): *Dehalococcoides*, *Geobacter*, Spirochaetaceae, *Pelobacter, Aminomonas*, Bacteroidetes, *Cryptanaerobacter*, *Acetivibrio, Anaerolinea*, *Chlorobi*, *Desulfovibrio*, *Aminobacterium*, *Syntrophus*, Clostridiales, *Syntrophomonas*, *Dehalobacter*, *Sporomusa*, *Syntrophobacter*, *Acetobacterium*, division OP5, *Bacillus, Cellulomonas*, *Pseudomonas*, *Sulfurospirillium*; and Archaea: *Methanomethylovorans*, Methanomicrobiales, *Methanosarcina*, *Methanosaeta*. Identification of all OTUs identified in the consortium was not possible due to the limited availability of environmental isolates in public databases that would match constituents of the consortium and because constituents present at low concentrations may be not detected, and therefore, remain unidentified. However, the notifier was able to identify the constituents responsible for the dechlorination of TCE, namely, *Dehalococcoides* (50-73%), *Methanomethylovorans* (4-20%), and *Geobacter* (4-12%).

The stability of the consortium was assessed using Denaturing Gradient Gel Electrophoresis (DGGE) and quantitative Polymerase Chain Reaction (qPCR) to identify significant shifts that could affect the safety or performance of the consortium. DGGE was used to evaluate the fundamental stability of the consortium and indicated that the composition of the consortium remained stable over time, throughout multiple transfers,

[2] An OTU is broadly defined as a dataset that is assumed to represent a valid taxon for the purposes of phylogenetic analysis.

between batches, and among samples. These data were complemented by targeted qPCR experiments that sought to quantify the populations of micro-organisms responsible for the functionality of the consortium and compare their relative percentage in different samples. The experiments indicated that although some variability was observed between samples, the predominant OTUs remained relatively constant. Minor shifts are to be expected due to the complex nature of a consortium growing in a batch system. Stringent production methods and quality assurance and quality control (QA/QC) procedures are in place to prevent the introduction or loss of micro-organisms that could be detrimental to the performance and safety of KB-1®.

History of Use

For more than a decade KB-1® has been handled in both academic and industrial research institutions in Canada and abroad without incident. A safe history of field use of KB-1® has been established in the United States and Europe (United Kingdom, Denmark and Sweden). The notifier cited several projects to further demonstrate the history of safe use of KB-1®. These projects included the use of KB-1® in PCE and TCE contaminated municipal water supply wells in Abilene, Kansas; Kelly Air Force Base in Texas (Major *et al*., 2002); Launch Complex 34 in Florida (Battelle, 2004); and a former aerospace facility in California (Barros *et al.*, 2007).

HAZARD CONSIDERATIONS

Environmental Hazard Characterization

Analysis of data derived from molecular methods used for identification (i.e., 16S rRNA sequencing and cloning) of constituents of KB-1® indicated that none of the recovered OTUs in KB-1® are closely related to known environmental pathogens. Furthermore, pathogen-screening trials conducted on influent groundwater and KB-1®-treated effluents were negative, suggesting that the introduction of KB-1® does not result in a proliferation of known pathogens (Lesage *et al*., 2006). DGGE analyses were used to evaluate the effect of KB-1® on indigenous microflora. These studies indicated that the introduction of KB-1® into a contaminated site does not adversely affect the native microbial communities and that any perturbation to the native microbial community was restricted to the area of application only (Toquai-Diaz, 2006), which is likely to be already significantly affected by the chlorinated pollutants present. Overall, the benefits of quickly restoring the groundwater quality through KB-1® addition outweigh the minimal impacts on the indigenous microbial community structure.

Ecotoxicological testing conducted with KB-1®-treated effluents indicated an absence of ecological effects from the KB-1® consortium. Data from tests conducted on aquatic and terrestrial species provide no evidence of any adverse effects resulting from the introduction of KB-1® into a TCE-contaminated aquifer. (Lesage *et al*., 2006; McDaniel *et al*., 2007). However, the notifier did not provide data on aquatic invertebrates or terrestrial vertebrates.

Although 16S rRNA sequence data indicates that the consortium potentially contains metal accumulators and pesticide-degrading micro-organisms (OTUs identified as *Bacillus* and the *Pseudomonas* were closely related to metal accumulating species), these

micro-organisms are naturally occurring in the Canadian environment and are not expected to pose additional hazards.

Human Health Hazard Characterization

The major constituents of KB-1® (*Delhalococcoides*, *Methanomethylovorans*, and *Geobacter*) are of low potential virulence, are not closely related to known human pathogens, and there is no evidence to date, that implicates them in cases of human infection.

Some of the minor constituents of the consortium, including Spirochaetes, Bacteriodetes, Clostridiales, *Pseudomonas*, *Bacillus*, and *Cellulomonas* were identified to be in the same genus or family as known human pathogens. Phylogenetic analyses of these OTUs suggest that these isolates are distinct from their pathogenic relatives and are more closely related to environmental, non-pathogenic isolates. Moreover, routine pathogen screening including: total coliforms, *Campylobacter* spp., *Clostridium perfringens*, *Pseudomonas aeruginosa*, fecal streptococci, *Salmonella* spp., *Listeria monocytogenes*, *Cryptosporidium parvum, Shigella sp*., *Staphylococcus aureus, Vibrio* spp., *Yersinia enterocolitica*, *Escherichia coli*, and *Bacillus anthracis* as well as yeasts and moulds such as *Candida albicans* and *Aspergillus fumigatus* are performed at 6-month intervals on KB-1® to ensure the absence of specified pathogens in the consortium.

Antibiotic susceptibility testing revealed that KB-1® is susceptible to a number of antibiotics including erythromycin, cefoperazone, tetracycline and ofloxacin, despite the major constituent (*Delhalococcoides)* being resistant to ampicillin (Ritalahti and Löffler, 2004). In the unlikely event that KB-1® leads to infection in humans, treatments are available.

No cases of allergic or adverse immunological reactions resulting from exposure to KB-1® or its principle constituents have been reported.

EXPOSURE CONSIDERATIONS

Environmental and Human Exposure Characterization

KB-1® will be manufactured by SiREM in Guelph, Ontario. An estimated 5,000 L (approximately 7 x 10^{15} micro-organisms) will be produced during the first year of production, 10,000 L the second year and 20,000 L the third year. The notifier provided a description of proper storage, handling, manufacturing and disposal procedures limiting human and environmental exposure during the manufacture and application of the consortium and described methods for monitoring the consortium in the environment, if required.

KB-1® is formulated to be applied directly to depths between 1.5 and 60 m below the surface to bioremediate soil, aquifers and groundwater contaminated with chlorinated ethenes in habitats that would be very similar to the original habitat of KB-1®. Many KB-1® constituents have a natural, broad geographic distribution in Canada. Thus, it is reasonable to assume that the receptive environment will allow survival of KB-1®.

KB-1® consortium persists once injected into an anaerobic aquifer, as indicated by resumption of reductive dechlorination following periods of starvation ranging from several months to a year (Toquia-Diaz, 2006). This indicates that KB-1® constituents may subsist at low densities once bioremediation is complete; however, active growth is limited by the availability of key metabolites (e.g., chlorinated compounds and the addition of appropriate electron donors) (Lesage *et al*., 2006), which is consistent with previous work done on *Dehalococcoides* (Cupples *et al*., 2004). Some constituents, specifically members of the genera *Bacillus* and *Clostridium*, can form environmentally resistant spores. The formation of spores by some consortium constituents might occur in sub-optimal conditions such as extreme temperature, changes in pH or lack of nutrients. Spores are resistant and do not require nutrients to persist for extended periods.

The dechlorinating strains are capable of colonizing areas beyond the initial injection site, but there is little evidence of independent motility; KB-1® constituents appear to disperse by groundwater advection and colonization. Molecular analyses of samples from monitoring wells indicate the typical transport rate for KB-1® is approximately 6 cm per day (Major *et al*., 2002). Anaerobic saturated groundwater exists only below 1.5 m below the surface, and transport into drinking water supplies, soils or surface water near the application site is possible, but potential for exposure to the consortium in these compartments is limited by the anaerobic nature and nutritional requirements of the consortium constituents.

The organisms expected to be exposed to KB-1® consortium are indigenous micro-organisms inhabiting contaminated anaerobic aquifers (Bacteria, Archaea, and Fungi). Due to the nature of application, the anaerobic nature of the principal constituent organisms, and their specific nutritional requirements, the potential for exposure of humans is relatively low. It is unlikely that the general population will be exposed to KB-1®. There is potential for the Canadian fauna and flora to be exposed to KB-1 should the treated groundwater enter soils or surface water near the application site, but as mentioned, the survival of the consortium in these compartments is severely restricted by its growth requirements.

Based on the available information, exposure of humans and environmental species to KB-1® is limited due to the restrictive nutritional and growth parameter needs of the main constituents. It is unlikely that surface waters of lakes, streams, rivers, irrigation waters or drinking water, which are predominately aerobic, would contain sufficient quantities of the required nutrients and chlorinated solvents necessary to sustain or increase the numbers of the major constituents of KB-1®.

RISK CHARACTERIZATION

Based on the hazard and exposure considerations, the risk assessment conducted by Environment Canada and Health Canada concluded that KB-1® does not cause harm to the Canadian environment or human health as described in section 64 of CEPA 1999.

The organisms most likely to be exposed to KB-1® are indigenous micro-organisms inhabiting contaminated anaerobic aquifers (Bacteria, Archaea, and Fungi). The habitats at proposed sites of injection are very similar to the habitat from which the consortium was first isolated, so it is reasonable to assume that many KB-1® constituents or close relatives would already be indigenous at the application site, and that perturbation of the receptive environment would consequently be minimal. The predominant constituents in the KB-1® consortium are not known to be pathogenic, and are not related to known human or environmental pathogens. In addition, tests conducted on aquatic and terrestrial species provide no evidence of any adverse effects related to the KB-1® consortium. Furthermore, the predominant constituents in the KB-1® are expected to be limited to anaerobic environments where chlorinated compounds and specific electron donors are present. These characteristics limit the risk of KB-1® to humans, and Canadian flora and fauna.

However, because it is probable that not all constituents of KB-1® have been identified, and because ecotoxicology studies were not provided for aquatic invertebrates and terrestrial vertebrates, some uncertainties remain related to the hazard to environmental species associated with KB-1®. A Significant New Activity (SNAc) Notice was therefore proposed to require notification prior to any use of KB-1 other than its injection into subsurface contaminated groundwater.

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