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26 July 2013

Alan Seech, Ph.D
Director - Soil & Groundwater Remediation
FMC Environmental Solutions

via email: Alan.Seech@fmc.com

Reference: KB-1® Plus Information for Regulatory Submissions

Dear Dr. Seech:

SiREM is pleased to provide the following information related to product use and safety of SiREM's bioaugmentation culture KB-1® Plus. KB-1® Plus is a natural, non-pathogenic, anaerobic microbial consortium that has been proven to rapidly and completely degrade chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), cis-1,2-dichloroethene (cDCE), 1,1-dichloroethene (1,1-DCE) and vinyl chloride (VC). 1,1,1-trichloroethane (1,1,1-TCA), 1,1-Dichloroethane (1,1-DCA), 1,2-dichloroethane (1,2-DCA), 1,1,2,2-tetrachloroethane (TECA), 1,1,2-trichloroethane (1,1,2-TCA), chloroform (CF), dichloromethane (DCM) and other chlorinated solvents to non-toxic, environmentally acceptable, end products such as ethene and ethane. The KB-1® Plus culture has not been genetically modified in any manner and was derived from naturally occurring bacterial populations found in soil and groundwater impacted by chlorinated solvents. This culture is manufactured and handled in an identical process as the KB-1® culture which has been widely used over the past 12 years.

The organisms detected in KB-1® Plus are predominantly members of the dechlorinating genera *Dehalococcoides, Dehalobacter and Dehalogenimonas*; and also include *Geobacter,* a member of the iron reducing group*, Acetobacterium* and methanogens belonging to the to the genus *Methanomethylovorans, Methanocorpusculum sp.* Together these microbial groups comprise the majority of the microbes found in KB-1® Plus. *Dehalococcoides, Dehalobacter* and *Dehalogenimonas* species have been detected at numerous sites across the United States and Europe, indicating that these bacteria are widespread but not ubiquitous. The organisms detected in KB-1®Plus are not expected to represent human health hazards.

Product -Specific Information

1. Product manufacturer name, address, phone number, and contact person.

SiREM
130 Research Lane, Suite 2
Guelph, Ontario
Canada, N1G 5G3

Sandra Dworatzek
519-515-0839

2. *Identity of specific ingredients (including CAS#) and concentrations of ingredients contained in the product and purpose of each.**

Ingredients for growth media of culture – it is a dilute mineral salts medium.

Chemical Name	Chemical Formula	CAS#	Concentration (grams/liter)	Purpose
Potassium Phosphate Dibasic	KH_2PO_4	7758-11-4	0.27	pH buffer
Potassium Phosphate Monobasic	K_2HPO_4	7778-77-0	0.34	pH buffer
Ammonium Chloride	NH_4Cl	12125-02-9	0.535	Nitrogen source
Calcium Chloride	$CaCl_2$	10035-04-8	0.07	Calcium source
Magnesium Sulfate	$MgSO_4$	10034-99-8	0.125	Magnesium source
Ferrous Chloride	$FeCl_2$	13478	0.02	Iron source
Sodium bicarbonate	$NaHCO_3$	144-55-8	2.0	pH buffer
Ferrous Ammonium Sulfate	$(NH_4)_2Fe(SO_4)_2$	7783-85-9	0.4	Iron, nitrogen and sulfate source
Sodium sulfide	Na_2S	1313-84-4	0.12	Reductant
Sulfuric acid	H_2SO_4	7664-93-9	as required to neutralize to pH 7	pH adjustment during preparation
Resazurin	$C_{12}H_6NNaO_4$	62758-13-8	0.001	Redox indicator
Boric Acid	H_3BO_3	10043-35-3	0.0006	Nutrient source
Zinc Chloride	ZnCl	7646-85-7	0.0002	Nutrient source
Sodium Molybdate	Na_2MoO_4	10102-40-6	0.0002	Nutrient source
Nickel II Chloride	$NiCl_2$	7791-20-0	0.0015	Nutrient source
Manganese Chloride	$MnCl_2$	13446-34-9	0.002	Nutrient source
Copper II Chloride	$CuCl_2$	10125-13-0	0.0002	Nutrient source
Cobaltous Chloride	$CoCl_2$	7791-13-1	0.003	Nutrient source
Disodium Selenite	Na_2SeO_3	10102-18-8	0.00004	Nutrient source
Aluminum Trisulfate	$Al_2(SO_4)_3$	10043-01-3	0.0002	Nutrient source
Vitamins	Various	Various	0.01 maximum	Nutrient source

3. *Microbial Characterization of KB-1 Plus Culture*

Dehalococcoides

The first member of this genus to be described (*i.e., Dehalococcoides ethenogenes* strain 195) was isolated from a bioreactor (35°C) initially seeded with digested sludge from a wastewater treatment plant in Ithaca, New York. (Freedman and Gossett, 1989, Di Stefano et al., 1991, Maymó-Gatell et al, 1997). Bacteria with 16S rDNA sequences affiliated with *Dehalococcoides* were also found in a hot spring in Yellowstone National Park (Hugenholtz et al., 1998). Natural and anthropogenic halogenated compounds serve 3 purposes for anaerobic bacteria: i) carbon or energy source, or both, ii) substrates for co-metabolism, and iii) terminal electron acceptors during anaerobic respiration (Holliger et al., 2003). *Dehalococcoides* strains have different dechlorination activities (He et al., 2003). Strain 195 dechlorinates tetrachloroethene to ethene Maymó-Gatell et al., 1997). Strain CBDB1 is the closest isolated relative in the KB-1® Plus D*ehalococcoides,* at >98% similarity, as determined using the National Center for Biotechnology Information (NCBI) online BLAST tool. Strain CBDB1 does not use any non-chlorinated electron acceptors, uses H2 as an electron donor, and dechlorinates chlorobenzenes, certain chloroethenes, and some polychlorinated dibenzodioxin congeners (Adrian et al., 2000, Bunge et al., 2003).

As a result of years of research and advanced understanding of *Dehalococcoides* organisms, Loeffler et al (2013) published a formal taxonomic description of the genus *Dehalococcoides.* In honor of the pioneering research by Perry McCarty, *Dehalococcoides ethenogenes*" strain 195 was renamed *Dehalococcoides mccartyi.*

***Dehalobacter* spp.** are a genus of anaerobic bacteria which are Gram negative, non-spore forming, strictly anaerobic, rods that exhibit a single lateral flagellum. The first identified *Dehalobacter* sp. (i.e. *Dehalobacter restrictus*) was isolated from anaerobic sediment in the Rhine River, Germany. *D. restrictus* reductively dechlorinates tetrachloroethene and trichloroethene, and uses only H_2 as the electron donor (Holliger et al., 1998). *D. restrictus* was the first PCE-dehalogenating organism grown in pure culture and was characterized as a small, gram-negative rod with one lateral flagellum, and a low G+C content (Holliger et al., 1998). This organism appears to be an obligate dehaloginator; no other electron acceptors have been found to support growth. *Dehalobacter* have been found in environmental sites and shown to grow by reductive dechlorination of chloro-organic substrates. *Dehalobacter* are also capable of the reductive dechlorination of 1,1,1-trichloroethane (1,1,1-TCA) to 1,1-DCA and chloroethane as well as chloroform biodegradation to dichloromethane (DCM) See Figures 2 and 3 (Grostern et al., 2010). These are the primary biodegradative pathways of interest in the KB-1 Plus culture. In extensive literature searches, *Dehalobacter* species have not been reported as plant, animal or human pathogens. All evidence indicates that this microbial group is an obligate degrader of selected chlorinated compounds.

Dehalogenimonas

The KB-1 Plus culture also contains *Dehalogenimonas* (Dhg) which cooperates with *Dehalococcoides* and *Dehalobacter* in the dechlorination of TeCA to ethene. Dhg is reported to dechlorinate trans-dichloroethene (trans-DCE) to vinyl chloride. The Dhg strain is most closely

related (96% sequence homology [Manchester et al, 2012]) to *D. lykanthroporepellens* which is capable of degrading chlorinated ethanes (Moe et al., 2009).

The genus *Dehalogenimonas* (Dhg) was first described in 2009 (Moe et al., 2009) and belongs to the phylum Chloroflexi. Dhg are closely related, but distinct from, *Dehalococcoides* sharing 90% homology in 16S rRNA gene sequences (Yan et al., 2009). *Dehalogenimonas* share numerous morphological and metabolic traits with Dhc and have been described as strictly anaerobic, mesophilic, non-spore-forming, non-motile and Gram negative cocci with diameters of 0.4-1.1 µm which are resistant to ampicillin and vancomycin (Bowman et al., 2013).

Like Dhc, Dhg uses mainly hydrogen (and in some cases hydrogen sulfide) as an electron donor and halogenated compounds as electron acceptors. Unlike Dhc this group has been observed to primarily use chlorinated ethanes and chlorinated propanes as electron acceptors and have not been reported to exhibit dechlorination against chlorinated ethenes with the exception of trans-DCE (Manness et al., 2012; Manchester et al, 2012). *Dehalogenimonas alkenigignens* dehalogenates a variety of polychlorinated aliphatic alkanes, including 1,2-dichloroethane, 1,2-dichloropropane, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane and 1,2,3-trichloropropane, when provided with hydrogen as the electron donor (Bowman et al., 2013). Dhg typically perform reductive dechlorination by a dihaloelimination reaction in which two chlorines are simultaneously removed instead of the sequential reductive dechlorination more typical of Dhc

Molecular genetic methods to quantify Dhg have been developed potentially allowing in situ tracking of the growth and distribution of Dhg in bioremediation applications using quantitative PCR testing (Yan et al. 2009; Manchester et al., 2012).Given their status as obligate anaerobes with a highly specific metabolism this group of dechlorinating organisms are unlikely to act as pathogens and correspondingly there no reports of pathogenicity associated with Dhg.

Methanomethylovorans species are commonly found in anaerobic biodegradative microbial consortia, for example *Methanomethylovorans* comprise a large proportion of the KB-1® culture (approved for use in NC DWC UIC program, 2005). According to Lomans et al., (1999), *Methanomethylovorans* spp. metabolize methanol, methylated amines, dimethyl sulphide (DMS), methanethiol and in doing so, produce CH_4 and are therefore methanogens. Data concerning *M. hollandica* and *M. thermophila* concurs (Lomans et al., 1999, Jiang et al., 2005). The obligate methylotrophic methanogen, *M. hollandica*, and latter sp. were cultured in media containing dimethyl sulfide and methanol as the sole sources of carbon and energy, respectively (Lomans et al., 1999, Jiang et al., 2005). With exceptions, *Methanomethylovorans* spp. typically inhabit mesophilic conditions (if temperature is greater than 40°C, no growth is observed) and exhibit low salt tolerance (Garcia et al., 2000). There is no evidence to suggest that methanogens such as *Methanomethylovoran*s are pathogenic given their metabolism and their status as strict anaerobes which cannot survive exposure to oxygen.

Geobacter

There a several *Geobacter* species found in KB-1® Plus. One is *Geobacter pickeringii* (94% 16S rRNA homology) which is an iron reducing bacterium isolated from clay in Georgia (Shelobolina et al., 2007). *G. pickeringii* exhibits 1 lateral flagellum and numerous short tubular

projections on the cell surface (Shelobolina et al., 2007). The second was also closely related to *G. pickeringii* (95%) but was even more closely related to Clone EUB74 from granular sludge treating cassava starch wastewater (99%). The third is a 99% match to *G. lovleyi* Strain SZ isolated recently (Sung, 2006) from an anaerobic reductively dechlorinating *Dehalococcoides*-containing mixed culture. *Geobacter* is able to dechlorinate PCE and TCE to cDCE only (Duhamel, 2007) using acetate as electron donor. Therefore this *Geobacter* works with *Dehalococcoides* to effect complete dechlorination to ethene, with *Geobacter* responsible for a significant part of the dechlorination from PCE or TCE to c-DCE, and *Dehalococcoides* responsible for the dechlorination of cDCE to ethene (Duhamel, 2007).

There is no evidence that *Geobacter* sp. are pathogenic or toxic. The first *Geobacter* sp. descibed (i.e., *G. metallireducens*) was obtained from surface bottom sediments of the Potomac River in Maryland, USA (Lovley et al., 1993). *G. chapellei* was isolated from deep subsurface sediments of the Atlantic Coastal plain (Coates et al., 2001). *G. argillaceus* and *G. pickeringii* were discovered in sedimentary kaolin strata in Georgia, USA (Shelobolina et al., 2007). Kaolin is a rock largely consisting of the clay mineral, kaolinite, a material commonly used for porcelain and ceramics production. *Geobacter* spp. were also isolated from a uranium-contaminated aquifer in Rifle, Colorado, USA (Anderson et al., 2003). This particular site was situated on alluvial deposit in the Colorado River floodplains and was adjacent to a former uranium ore processing facility (Anderson et al., 2003). Geobacteraceae microorganisms were also involved in the anaerobic degradation of aromatic hydrocarbons in petroleum-contaminated aquifers (Snoeyenbos-West et al., 2000).

Geobacter spp. oxidize organic compounds to CO_2 using metal ion-mediated electron transport to generate adenosine triphosphate (ATP) or to harvest energy (Bond et al., 2002); therefore, they contribute to the global recycling of carbon (Methe et al., 2003). In particular, *G. metallireducens* reduces amorphic ferric oxide to extracellular magnetite, using ferric iron as the terminal electron acceptor for organic matter oxidation (Lovley et al., 1987). As reviewed by (Lovley et al., 1996), *G. metallireducens* may use humic substances, which are found ubiquitously in terrestrial and aquatic environments, as electron acceptors for the anaerobic oxidation of organic compounds and hydrogen. *G. sulfurreducens* oxidizes acetate completely to CO_2 and water, using metal ions, elemental sulphur, and fumarate as electron acceptors (Methe et al., 2003). *G. metallireducens* and *G. sulfurreducens* encode a form of citrate synthase reported in Eukaryotes (Schnarrenberger and Martin, 2002). The latter sp. encodes genes involved in mediating glycolysis and the tricarboxylic acid (TCA) cycle (Methe et al., 2003).

Geobacter spp. are rod-shaped, non-motile, non-spore forming, strictly anaerobic, and chemoorganotrophic (Lovley et al., 1993). *G. pickeringii* exhibits 1 lateral flagellum and numerous short tubular projections on the cell surface (Shelobolina et al., 2007). *G. argillaceus* has large quantities of elongated tubular projections or blebs, which is an unusual phenotype for *Geobacter* (Shelobolina et al., 2007).

Acetobacterium

The *Acetobacterium* found in KB-1® Plus are related to an uncultured bacterium clone PL-18B2, which was detected in a biodegraded oil reservoir (Grabowski et al., 2005) as well as they are closely related to *Acetobacterium carbinolicum* (99%). There is no evidence that *Acetobacterium* spp. are pathogenic or toxic.

Acetobacterium spp. are obligate anaerobes that ferment a variety of sugars and yield acetate as their primary product. Via fermentation, *A. malicum* degrades the organic solvents, 2-methoxyethanol and 2-ethoxyethanol, to acetate and alcohol (Tanaka and Pfennig, 1988). This sp., in addition to *A.wieringae* and *woodii*, may also metabolise H_2/CO_2 (Tanaka and Pfennig, 1988, Braun and Gottschlk, 1982, Balch et al., 1977). *A. carbinolicum* metabolises primary aliphatic alcohols with 1-5 carbons and similar to *A. malicum*, does not reduce sulphate, thiosulphate, S_0, and nitrate (Eichler and Schink, 1984). *A. tundrae* cannot use 2-methoxyethanol and malate, whereas *A. fimetarium, A. paludosum*, and *A*. *bakii* can (Simankova et al., 2000, Kotsyurbenko et al., 1995). In contrast with *A. fimetarium* and *A*. *bakii*, *A. paludosum* cannot utilise maltose, glucose, xylose, and methanol (Kotsyurbenko et al., 1995). Aside from being unable to hydrolyse gelatine and esculin, *A. wieringae* grows rapidly only on D-fructose and D, L-lactate, and cannot ferment many sugars including glucose, maltose, and sucrose (Braun and Gottschalk, 1982).

Acetobacterium spp. are Gram positive, actively motile (1 to 2 subterminal flagella), nonendospore forming, and oval to rod-shaped (Balch et al., 1977). The main lipid complex of *A. bakii* cells is a saturated aldehyde, while that for psychotrophic *A. fimetarium* are unsaturated fatty acids (Kotsyurbenko et al., 1995).

Clostridium Species

A small proportion (<3%) of the KB-1 Plus culture is comprised of *Clostridium* species. *Clostridium are* Gram positive, rod-shaped obligate anaerobes (Plorde, 1994). *Clostridium are* commonly detected in environmental samples and in the gut of animals.

While most members of the genus clostridium are non-pathogenic, some specific *Clostridium* species such as *C. perfringens*, *C. difficile*, *C. tetani*, and *C. botulinum* are responsible for gangrene and food poisoning, enterocolitis, tetanus, and botulism, respectively. *C. tetani* and *C. botulinum* make toxins that target nerve endings and cell membrane ionophores, respectively (Coggin Jr., 2006, USDHHS, 2007). The *Clostridium* species detected in KB-1 Plus is almost certainly not a pathogen as routine pathogen testing of KB-1 Plus indicates no *Clostridium perfringens* were detected on blood agar plates, which are used to detect pathogenic members of this genus (see below). Furthermore, the *Clostridium* identified in KB-1 Plus was most closely related in the GenBank database to *Clostridium lactifermentans* which was isolated from the caeca of a chicken and may play a role in the lactate fermentation in chickens, this *Clostridium* may play a similar role fermenting lactate in the KB-1 Plus culture and is not suspected to be pathogenic.

Other microorganisms identified in KB-1 Plus include *Acidaminococaceae* , *Thermanaerovibrio* sp. *Synergistes* sp. *Desulfovibrio* sp. *Anaerovorax* sp. *Spirochaeta* sp. *Methanolinea* sp. *Anaeroarcus* sp., *Desulfobulbos*, *Rhizobium, Sulfurospirillum,* Synergistestales. All are anaerobic environmental microbes that include, nitrogen fixers, sulfate reducers, methanogens and fermenters of variety of compounds. None of these microbial groups is present in high concentrations or is suspected to be pathogenic.

Pathogen Testing Results

The KB-1 Plus culture is screened annually by an external laboratory (GAP EnviroMicrobial Services Inc., London, ON) for a suite of common human pathogens. A copy of recent pathogen test and methods results (March, 2013) are included in Attachment A. The test

results indicate the absence for all tested pathogens. Since starting production of KB-1 Plus (in 2007), pathogen test results have consistently been negative for *Salmonella* sp., *Listeria monocytogenes*, *Vibrio* sp., *Campylobacter* sp., *Clostridium perfringins*, *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Yersinia* sp., Pathogenic Yeast and Mold (includes *Candida albicans*, *Aspergillus fumigatus*), fecal *Streptococci,* fecal coliforms and Enterococci. The absence of fecal related organisms (fecal coliforms, fecal Streptococci, Enterococci) makes it highly unlikely that other fecal related diseases (viruses, etc.) are present in the culture.

4. *Documentation from authoritative technical references that the microorganism(s) are naturally-occurring in the immediate or similar environment.*

The KB-1 Plus culture was derived from soil cores and groundwater derived from several chemically contaminated sites located in North America. The culture is injected into anaerobic groundwater at sites with similar characteristics and microbial communities with the possible exception they lack key dechlorinating microorganisms in particular *Dehalococcoidies*, *Dehalobacter and Dehalogenimonas*.

Dehalococcoidies, *Dehalobacter, Dehalogenimonas*, sulfate reducers (*Desulfovibrio*), methanogens (e.g., *Methanomethylovorans),* and fermenters such as *Clostridium* are microorganisms commonly found in sites under going anaerobic bioremediation. For example, the microbial composition of a site undergoing remediation at Wurtsworth Airforce Base in Michigan (Dojka et al., 1998) consisted of many of these microorganisms as did a trichlorobenzene degrading community enriched from the Saale River Sediment (Germany) sediment (von Wintzingerode et. al., 1999). In general the microorganisms present in KB-1 Plus are widespread in the subsurface environment but are not ubiquitous or in very low concentrations, therefore, some sites require bioaugmentation to improve remediation performance.

5. *Documentation from authoritative technical references of specific degradation products expected.*

Degradation pathways are attached for degradation of chlorinated ethenes, ethanes and methanes (Figures 1, 2 and 3). KB-1 Plus can also promote the degradation of chloroform to dichloromethane (Grostern et al., 2010) and DCM to acetate or formate (Justica-Leon et al., 2012).

7. *Documentation from authoritative technical references of expected migratory potential of microorganisms and degradation products in soil and groundwater.*

Transport of introduced bioaugmentation cultures can be assessed in situ using molecular genetic tests (e.g., Gene-Trac®) on subsurface soil or groundwater samples. By testing monitoring well locations successively further from the point of introduction, information on site-specific microbial transport rates can be obtained. Evidence of microorganism growth and transport occurs when populations are detected (where they previously were not present) at increasing distances from the injection locations accompanied by increasing or stable concentrations of organisms at the injection locations, demonstrating that the injected microorganisms were not merely transferred downgradient.

Using this approach, the typical rate of transport of *Dehalococcoides* bacteria in the bioaugmentation culture KB-1® (a bioaugmentation culture produced by SiREM that is very similar in many respects to KB-1 Plus was estimated to be about 0.2 feet per day from an injection location. Figure 4 shows results from a site in California where 40 L of KB-1® was amended groundwater containing CVOCs. Specifically, KB-1® was added to wells MW-40-22 and MW-40-25 in April 2003. Wells located up to 10 meters down gradient from the point where KB-1® was added (i.e., MW-40-23, MW-40-29, MW-40-24, and MW-40-28) increased in *Dehalococcoides* concentrations over a period of 7 months, indicating growth and transport of these microorganisms at a rate of approximately 2.4 inches/day (~0.2 feet/day) for the first 3 months post-bioaugmentation.

Laboratory characterization of the relative rates of transport of other predominant microorganisms in the bioaugmentation culture KB-1® has also been performed to better understand the relative movement of these organisms in natural environments. The retention of *Methanomethylovorans*, *Geobacter* and *Dehalococcoides* were measured in soil column studies using qPCR to determine their relative concentration of the microorganisms at the influent and at the effluent and obtain breakthrough curve. Results are summarized in Figure 5. In this study *Geobacter* moved through the matrix most quickly, *Methanomethylovorans* movement was slowest, and *Dehalococcoides* migrated at a rate between these two microorganisms. Although the different microorganisms demonstrated variable migration rates it does suggest a (~3 fold) range. It would be a reasonable assumption that *Dehalobacter* and *Dehalogenimonas* (the other key dechlorinators in KB-1 Plus) may also move at rates in situ approximating this range and may move somewhat faster or slower than 0.2 feet per day often observed for *Dehalococcoides*. If required, site specific migration rates for *Dehalobacter* and *Dehalogenimonas* could be determined using targeted genetic tests (i.e., Gene-Trac® Dhb/Dhg).

The migration of biodegradation products is related to the groundwater flow velocity and the characteristics of the aquifer including the fraction organic carbon. It is, therefore difficult to generalize the rate of migration of the breakdown products as migration is highly dependent on site specific factors.

8. *Complete description of the use of the product at the site (e.g., application of the product to soil and/or groundwater, aeration of soil. Procedures needed to maintain growth and chemical degradation).*

The KB-1 Plus culture is typically injected into anaerobic groundwater. KB-1 Plus is commercially produced by SIREM and delivered to the site in specially designed shipping containers. SiREM requires that specific geochemical conditions (e.g., neutral pH, the presence of fermentable electron donor and reducing redox conditions) be established to promote growth of the culture and degradation of the target contaminants.

SiREM ships all required equipment and materials to the site (with the exception of a gas cylinder which is sourced locally) prior to the scheduled injection date. The bioaugmentation technician inspects the vessel integrity upon arrival at the field site and confirms that all valves are closed and all connections are secure. The pressure in the vessel and vessel weight are checked to ensure that they have not changed since leaving the laboratory. The vessel is shipped under slight positive pressure (1 pound per square inch [psi]).

The injection technician proceeds by placing the injection tubing in the well/drive point to the desired injection depth and purges the well/drive point with argon or nitrogen gas to displace oxygen from the well column and maintain an inert gas blanket in the well above the water table. A five minute purge is recommended.

The culture is then injected into well/drive point using the compressed gas. There are four stainless steel stems on SiREM vessels: the pressure relief and pressure gauge port (to monitor pressure and pressure relief for safety); the purge port (for purging or venting the vessel); the inoculation port (culture line into subsurface); and the pressurization port (connected to compressed gas to pressurize the head space of the vessel and push culture into the subsurface) shown in Figure 6. The bioaugmentation technician connects the tubing so that the compressed gas is used to pressurize the vessel (typical maximum pressure required is 30 psi for injection depths up to 30 feet below ground surface) and push the culture into the injection tubing and into the well/drive point at the desired depth interval. The required volume is metered into the ground and the injection equipment moved to the next injection as required until all locations are completed. The bioaugmentation technician facilitates the return of all materials and equipment to SiREM following completion of the bioaugmentation.

9. Approximate concentration of each ingredient following release into soil or groundwater.

The typical application of culture results in a 1 in 35,000 dilution in groundwater (1 liter of culture per 35,000 Liters of groundwater) so that the chemical ingredients in the media are highly diluted and the target *Dehalococcoides*, *Dehalobacter and Dehalogenimonas* concentrations are approximately 10^5 to 10^6 gene copies per liter range.

KB-1 Plus has a proven record of success for enhancing reductive dechlorination at contaminated sites and has been applied at multiple sites and states. To date there have been no reports of adverse impacts related to human/animal/plant health at any of these locations. Ongoing quality control measures preclude the introduction of contaminating organisms in future production batches, ensuring that the ongoing safety of future KB-1 Plus bioaugmentations.

Copies of all the cited references can be provided upon request. We hope you find the information in this letter useful and if you require additional details please contact me at 519-515-0839) or email at sdworatzek@siremlab.com.

Sincerely,

Sandra Dworatzek

Sandra Dworatzek, M.Sc.
Senior Manager, SiREM

/with attachments

References

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Figures:

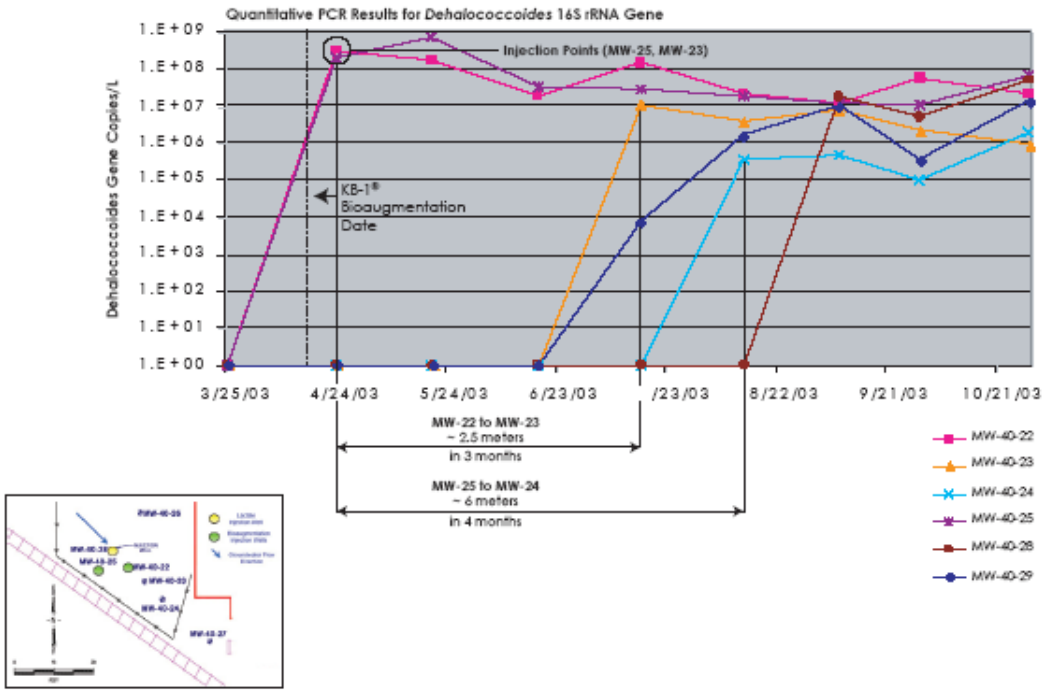

Figure 4: Spread of *Dehalococcoides* after bioaugmentation at a Site in California indicating average spread of ~0.2 feet per day

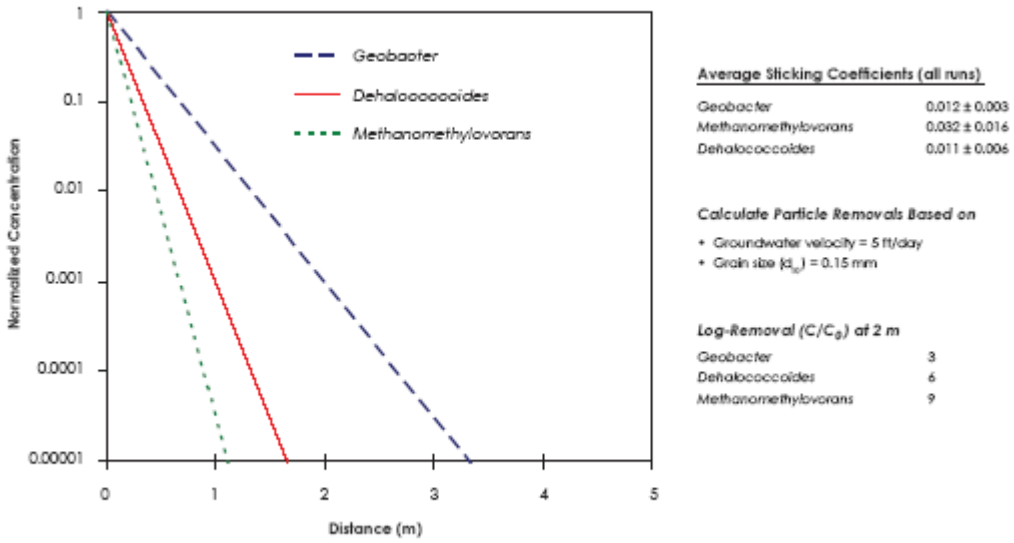

Figure 5: Relative migration (removal) of introduced microorganisms in groundwater columns

Attachment A: Pathogen Testing Results and Methods

CALA FINAL RESULTS FORM

GAP EnviroMicrobial Services Ltd.
1020 Hargrieve Road. Unit 14
London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 1 of 6	**ANALYSIS FINISH DATE:** 07-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	MF Results (CFU per 1 mL)							Benchsheet Page Reference	Quality Control Comments
		Total Coliforms (TCMF-0001)	Background (TCMF-0001)	Detection Limit	Fecal Coliforms (FCMF-0001)	Detection Limit	*E. coli* (ECMF-0001)	Detection Limit		
939		<1	<1	1	<1	1	<1	1	Page 63	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager **MANAGER APPROVAL:** S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

CALA Testing Accreditation No. A 2914

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Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 2 of 6	**ANALYSIS FINISH DATE:** 08-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	Heterotrophic Plate Count		Fecal Streptococci		Benchsheet Page Reference	Quality Control Comments
		CFU per mL HPCMF-0001	Detection Limit CFU per mL	CFU per mL MF, mENTER, 35°C, 48h	Detection Limit CFU per mL		
939		3	1	<1	1	Page 63	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

SIGNATURE: J Patterson

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

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GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 3 of 6	**ANALYSIS FINISH DATE:** 08-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Clostridium perfringens*		*Pseudomonas aeruginosa*		Benchsheet Page Reference	Quality Control Comments
		CFU per mL CLOSP-0001	Detection Limit CFU per mL	CFU per mL SOP #46	Detection Limit CFU per mL		
939		<1	1	<1	1	Page 63 & Reverse	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

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GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab	
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb	
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2	
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2	
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151
PAGE: 4 of 6	**ANALYSIS FINISH DATE:** 11-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	Yeast		Mold		Benchsheet Page Reference	Quality Control Comments
		CFU per mL FUNGIID-0001	Detection Limit CFU per mL	CFU per mL FUNGIID-0001	Detection Limit CFU per mL		
939		<1	1	<1	1	Page 63 Reverse	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager	**MANAGER APPROVAL:** S. Verhoeven (Technical Manager)
SIGNATURE: J Patterson	**SIGNATURE:** S Verh **DATE:** 13-Mar-13

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CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

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GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab			
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb			
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2			
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2			
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151		
PAGE: 5 of 6	**ANALYSIS FINISH DATE:** 11-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**		

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Salmonella* sp. P/A per 10 mL	*Listeria monocytogenes* P/A per 10 mL	*Vibrio* sp. P/A per 10 mL	*Yersinia* sp. P/A per 10 mL	Benchsheet Page Reference	Quality Control Comments
939		Absent	Absent	Absent	Absent	Benchsheets	QC Passed

CALCULATED BY: J. Patterson	**POSITION:** Lab Manager	**MANAGER APPROVAL:** S. Verhoeven (Technical Manager)	
SIGNATURE: JPatterson		**SIGNATURE:** SL Verh	**DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

FINAL RESULTS FORM

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London, ON N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150

GAP JOB #: A10284	**DATE SAMPLED:** 05-Mar-13	**REPORT TO:** Geosyntec/Sirem Lab			
CLIENT: Geosyntec/Sirem Lab	**COLLECTED BY:** Jen Webb	**ATTENTION:** Jen Webb			
	DATE RECEIVED: 06-Mar-13	**ADDRESS:** 130 Research Lane, Suite #2			
PROJECT: P.O. TL500534	**RECEIVED BY:** J. Patterson	Guelph, ON N1G 5G2			
	ANALYSIS START DATE: 06-Mar-13	**TEL:** 519-822-2230 x307	**FAX:** 519-822-3151		
PAGE: 6 of 6	**ANALYSIS FINISH DATE:** 12-Mar-13	**EMAIL:** jwebb@siremlab.com	**EMAIL:**		

LAB #	SENDERS #	MATRIX	SAMPLE DESCRIPTION	COMMENTS
939		Aqueous	KB-1/WBC-2/ACT-3	

TEST RESULTS *These test results relate only to the samples submitted and the analysis requested*

LAB #	SENDERS #	*Campylobacter* sp. P/A per 10 mL	*Bacillus anthracis* P/A per 10 mL			Benchsheet Page Reference	Quality Control Comments
939		Absent	Absent			Benchsheets	QC Passed

CALCULATED BY: J. Patterson **POSITION:** Lab Manager

MANAGER APPROVAL: S. Verhoeven (Technical Manager)

SIGNATURE: J Patterson **SIGNATURE:** S Verh **DATE:** 13-Mar-13

< = Less Than; > = Greater Than; ~ = Approximate; TNTC = Too Numerous To Count; OBSC = Obscured; NR = No Result; LA = Laboratory Accident
CFU = Colony Forming Unit; PFU = Plaque Forming Unit; MF = Membrane Filtration; MPN = Most Probable Number; SP = Spread Plate
Accredited Method Codes: TCMF-0001, ECMF-0001, ECMF-0002, HPCMF-0001

5 Mar 2013

GAP EnviroMicrobial Services Ltd.

GAP EnviroMicrobial Services Ltd.
1020 Hargrieve Road, Unit 14; London, Ontario; Canada N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150 www.gapenviromic.com

CHAIN OF CUSTODY

Client: Geosyntec / SIREM Lab	ANALYSES REQUESTED
Client Contact: Jennifer Webb	GAP Project # A 10284
Address: 130 Research Lane, Suite #2, Guelph, Ontario, N1G 5G2	Invoice to:
Tel: 519-822-2230 x307 Fax: 519-822-3151	
Email: jwebb@siremlab.com	
Client Project # P.O. # TL500534	
GAP Contact: Water Works #	

Fecal streptococci; Pseudomonas aeruginosa; bacillus anthrasis; fecal coliforms

SAMPLE IDENTIFICATION	# of containers	Matrix	Please check analyses requested	Date/Time Collected	Volume Sampled	Lab Sample No.
KB-1 / WBC-2 / ACT-3	1	aqueous	✓ ✓ ✓ ✓	5 mar 13		939

Turnaround Time:

☒ Normal (5 to 10 business days)

☐ *Rush: Date _____

* Double Rates Apply for Rush Analysis

Report Options:

☐ Fax

☒ Email

☐ Mail

Special Instructions / Comments:

Sampler (Print & Sign Name)

Collected by: Jon Webb

Signature: J-Webb

Laboratory Information

Rental Equipment (Completed by Sampler)

Equipment Standard Operating Procedure Read and Understood Initial ☐

Laboratory Use Only

Sample Information

Sample Temperature 8.1 °C Temp

Condition Acceptable Upon Receipt (Y/N) _____

Received at Lab by: JPatt

Date: 6 mar 13 Time: 10:30 am

Please read the reverse side of this form for sampling and shipping instructions.

5 Mar 2013

GAP EnviroMicrobial Services Ltd.

1020 Hargrieve Road, Unit 14; London, Ontario; Canada N6E 1P5
Tel: (519) 681-0571 Fax: (519) 681-7150 www.gapenviromic.com

CHAIN OF CUSTODY

Client: Geosyntec / SIREM Lab
Client Contact: Jennifer Webb
Address: 130 Research Lane, Suite #2, Guelph, Ontario, N1G 5G2.
Tel: 519-822-2230 x307 | Fax: 519-822-3151
Email: jwebb@siremlab.com
Client Project # | P.O. #: TL500534
GAP Contact: | Water Works #

ANALYSES REQUESTED

GAP Project # A 10284

Invoice to:

SAMPLE IDENTIFICATION	# of containers	Matrix	Total Coliforms	Escherichia coli	heterotrophic plate count	Yeast & Mold	Salmonella sp.	Listeria monocytogenes	Vibrio spp.	Yersinia spp.	Campylobacter spp.	Clostridium perfringens	Date/Time Collected	Volume Sampled	Lab Sample No.
			Please check analyses requested												
KB-1 / WBC-2 / AO-3	1	aqueous	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	5 Mar 13		939

Turnaround Time:
☒ Normal (5 to 10 business days)
☐ *Rush: Date _____
* Double Rates Apply for Rush Analysis

Report Options:
☐ Fax
☒ Email
☐ Mail

Special Instructions / Comments:

Sampler (Print & Sign Name)
Collected by: Jen Webb
Signature: J. Webb

Laboratory Information
Rental Equipment (Completed by Sampler)
Equipment Standard Operating Procedure Read and Understood Initial ☐

Laboratory Use Only
Sample Information
Sample Temperature 8.1 C Temp
Condition Acceptable Upon Receipt (Y/N) _____
Received at Lab by: JPatt
Date: Mar 6/13 Time: 1030 am

Please read the reverse side of this form for sampling and shipping instructions.

GAP EnviroMicrobial Services
a division of Conestoga-Rovers & Associates Ltd.

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METHOD FOR DETECTION OF MICROBIAL SPECIES.

Our laboratory is accredited by the Canadian Association for Environmental Analytical Laboratories (CAEAL), and all of the proper quality control procedures were conducted and documented when performing these methods.

***Vibrio* sp.**
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9260H and Health Canada method MFLP-72.
Sample was enriched in 90 mL of alkaline peptone water (APW), and incubated stationary at 35°C. At 6-8 h and 24 h, 0.1 mL of APW enrichment broth was added to Thiosulfate Citrate Bile Sucrose (TBCS) agar, and incubated at 35°C for 18 to 24 h. Confirmation using TSI slant, catalase, oxidase, Gram-strain, API 20E.

***Campylobacter* sp.**
Based on Madden *et al.* (2000) J. Microbiol. Methods 42:115-119.
Sample was added to 90 mL of modified Cefoperazone Charcoal Desoxycholate broth (mCCD) broth, and enriched under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 24 and 48 h. A 0.1 mL sample of mCCD broth was then added to modified Cefoperazone Charcoal Desoxycholate agar (m CCDA), and incubated under microaerophilic conditions at 42°C for 48 h. Confirmation using microscopic examination, Gram-stain, blood agar, catalase test, oxidase test, API Campy strip.

Pseudomonas aeruginosa
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9213 E.
Sample was processed by membrane filtration, and the filter was transferred onto mPAE agar. mPAE agar is prepared as described for mPA agar, with the following additions (g/L): $MgSO_4 \cdot 7H_2O$, 5.0; sodium deoxycholate, 0.1. Plates were incubated at 41.5 °C for 46 to 50 h. Confirmation using Gram-stain, oxidase test, Milk agar and Acetamide agar (Atlas, Handbook of Microbiological Media, 1993).

***Salmonella* sp.**
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9260B.
Sample was added to 90 mL of Tetrathionate broth, and incubated at 35°C. After 24 and 48 h, a 0.1 mL sample of enrichment broth was added to Brilliant Green agar, and incubated for 24 hours at 35°C. Suspect colonies were inoculated into a Lysine Iron agar slant, and incubated at 35°C for 24 h. Further confirmation by Gram-stain, oxidase test, catalase test and API 20E.

GAP EnviroMicrobial Services
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Listeria monocytogenes
Sample was added to 90mL of UVM Listeria Enrichment broth (Difco), and incubated 30°C for 48 h. A 0.1 mL sample of enrichment broth was streaked onto Listeria Selective agar (Difco), and incubated at 30°C for 24 h. Confirmation using blood agar, Gram-stain, catalase test, oxidase test, TSI agar and API Listeria.

Yersinia enterocolitica
Method based on that of Bhaduri *et al.* (1997) Appl. Environ. Microbiol. 63:1657-1660.
Sample was processed by membrane filtration onto Yersinia selective (CIN) agar (Oxoid), and incubated at 28°C for 24 h. Confirmation using Gram-stain, oxidase test and API 20E.

***Clostridium* sp.**
Sample was added to 90 mL of Thioglycollate medium (BBL), and incubated anaerobically at 35°C for 24 h. A 0.1 mL sample of enrichment broth was added to Clostrisel agar (BBL), and incubated anaerobically at 35°C for 48 hours. Confirmation by Gram-stain, blood agar, skim mild broth and species identification (16S rDNA analysis).

Total yeast and mold
Based on Standard Methods for the Examination of Water and Wastewater (20th Ed.) method 9610 D.
Sample was processed by membrane filtration, and the filter was transferred onto Rose Bengal agar (Difco). Plates were incubated at 26°C for 7 days.

Fecal Streptococci (Enterococci)
Sample was processed by membrane filtration, and the filter was transferred onto mEnterococcus agar (BBL). Plates were incubated at 35°C for 48 h. Confirmation using Ethyl Violet Azide (EVA) broth and incubation at 35°C for 24 h, and Gram-stain.

Fecal coliforms
Sample was processed by membrane filtration, and the filter was transferred onto mFC agar (BBL). Plates were incubated at 44.5°C for 20 h.

Aerobic Plate Count
Sample dilutions were prepared in Phosphate Buffered Water, and spread plated onto R2A agar (Difco). Plates were incubated at 28°C for 4 days.

Anaerobic Plate Count
Sample dilutions were prepared in Phosphate Buffered Water, and spread plated onto R2A agar (Difco). Plates were incubated in anaerobic jars, using the BBL GasPak system (H_2 + CO_2 generator envelops) at 28°C for 4 days. Anaerobic conditions were monitored using a BBL GasPak disposable anaerobic indicator strip.