Provect-CH4® Essential Oil (EO)
Advanced Methane Inhibitor / ERD and ISCR Supplement

TECHNOLOGY DESCRIPTION

Provect-CH4® EO represents our latest advancements in the science of applied antimethanogenic reagents (AMRs) for controlling excessive methanogenesis during *in situ* remedial action. Provect-CH4® EO is a proprietary mixture of Essential Oils/Saponins (potentially in combination with red yeast rice extract) that prevents methane (CH_4) production by controlling the growth and proliferation of methanogenic Archaea. This advanced AMR formulation offers the benefits of:

- Expanded mode of action = more effective
- Extended release = increased longevity
- Ease of Use = oil-based reagents more compatible with certain ERD/ISCR amendments, such as EVO and EZVI

In environmental remediation applications, AMRs can be used as supplements to conventional enhanced reductive dehalogenation (ERD) and *in situ* chemical reduction (ISCR) amendments rendering them safer and more effective. These include:

- Oils/Lecithin
- Emulsified Oils/Lecithin
- Sugars (lactate, dextrose, glucose)
- Other carbon sources (*e.g.,* molasses, whey)
- Plant based carbon (*e.g.,* cellulose and hemi-cellulose)
- Carbon + ZVI amendments (conventional ISCR reagents)

With widely varying degrees of success, other approaches such as managing pH and using slower-release, cellulose based carbon sources (lignolytic bacteria are not commonly thought to produce methane) have attempted to manage methane production during remedial applications. However, Provect-CH4® and Provect-CH4® EO are the only reagents designed **to actively control the production of methane** in a safe, reliable and predictable manner (Mueller and Booth, 2016; US Patent Office Scalzi *et al*, 2013, 2014; patents pending). In addition to the safety issues, associated with elevated methane in groundwater, soil gas, and indoor air, this effect also promotes **more efficient use** of the hydrogen donor.

WHAT IS THE PROBLEM WITH METHANE?

There are recognized benefits to methanogens and of limited methanogenesis. For example, i) methanogens are known to play important roles in synergistic microbial ecology, ii) their metabolic activity can help maintain anoxic conditions in treatment zones (through seasonal changes), and iii) the activity of methane mono-oxygenases and other enzymes can stimulate co-metabolic activity of TCE/DCE/VC in redox-recovery zones. Hence, limited production of methane is part of a healthy ERD/ISCR application. However, excessive methane production can be dangerous and represents a costly waste of amendment.

Cost and Efficiency Issues: Production of methane is a direct indication that hydrogen generated from the electron donor amendments was used by methanogens instead of the target microbes (*e.g., Dehalococcoides* spp.), substantially reducing application efficiency. **Table 1** (below) presents a site example where hydrogen demand is calculated for a highly aerobic and oxidized source area measuring approximately 1,850 cubic yards. Hydrogen demand for complete dechlorination of all PCE and TCE mass to ethene within this source area example, including both adsorbed and dissolved contaminants, is less than the amendment consumed to generate 20 mg/L of methane. The same is true of reducing all dissolved oxygen, nitrate, sulfate, and bio-available iron and manganese competing electron acceptors within the hypothetical treatment zone. So, even though this example site is highly oxidized with relatively high total concentrations of PCE and TCE, generating just 20 mg/L of methane constitutes greater than 33% of the total amendment consumption based on moles of H2.

Table 1. Hydrogen Demand for Complete Dechlorination of PCE/TCE in Hypothetical Source Area (Courtesy Troy Fowler, Mueller *et al*., 2014)

Constituent	Groundwater Concentration (mg/L)	Molecular Weight (g/mol)	Moles of H_2 to Reduce Mole Analyte	Moles of H_2 Acceptor In Treatment Area
Contaminant Electron Acceptors (To End Product Ethene)				
Tetrachloroethene (PCE)	10.0	165.8	4	1,393
Trichloroethene (TCE)	7.0	131.4	3	364
cis-1,2-Dichloroethene (cDCE)	0.0	96.9	2	0
Vinyl Chloride (VC)	0.0	62.5	1	0
			Complete Dechlorination (Soil+Groundwater) Subtotal	**1,757**
Native Electron Acceptors				
Dissolved Oxygen	9.0	32	2	199
Nitrate (as Nitrogen)	9.0	62	3	682
Sulfate	50.0	96.1	4	736
Fe^{+2} Formation from Fe^{+3}	20.0	55.8	0.5	63
Mn^{+2} Formation from Mn^{+4}	10.0	54.9	1	64
			Baseline Geochemistry Subtotal	**1,745**
Hydrogen Waste for Methane Formation				
Methane Formed	20.0	16	4	**1,769**
			Initial Treatment Area Hydrogen Usage	**5,271**

Potential Health and Safety Issues: Methane is considered to be a major greenhouse gas. It is explosive, with an LEL of 5% and an UEL of 15%. As a result of the microbial fermentation process, methane will be produced in most situations following the addition of any conventional ERD or ISCR amendment. Excessive and extended production of methane can result in elevated in groundwater concentrations (as high as 1,000 ppm have been reported) which can lead to accumulation in soil gas subsequently impacting indoor air. While this is perhaps more relevant in urban settings where methane can accumulate in basements, under slabs/foundations and/or migrate along utility corridors, excessive methane production has also been observed in more rural settings and other open spaces.

New and Emerging Regulatory Issues: State specific regulations for methane in groundwater have been promulgated, with others pending for soil gas and indoor air. For example, current regulations for methane in groundwater vary from *ca*. 10 to 28 mg CH_4/L (Indiana Department of Environmental Management, 2016). Notably, several ERD projects which intended to use liquid carbon (emulsified oils) sources have failed to receive regulatory approval due to issues associated with excessive production of methane during previous technology applications (Personal Communication - State of California; State of Minnesota). As a result, many remedial practitioners proactively design contingencies for conventional ERD/ISCR implementation in the event that methane exceeds a threshold level ranging from 1 to 10 ppm groundwater. These contingencies often entail expensive and extensive systems for treating methane in soil gas/vapor captured via SVE systems.

MODE OF ACTION – HOW DOES IT WORK?

What is a Methanogen? In the 1970s, Dr. Carl Woese (1928 to 2012) and his colleagues at the University of Illinois-Urbana studied prokaryotic relationships using DNA sequences and they found that microbes that produce methane – or methanogens - are Archaea (Woese and Fox, 1977). The identification of this new Domain of microorganism was very important for many reasons, but from our limited perspective herein this vast difference in genetic composition means that methanogens are significantly different from typical heterotrophic bacteria and eukaryotes. In other words, *Dehalococcoides ethenogenes* are as different from methanogens as you are.

How do Methanogens make methane? Biological methane formation is a microbial process catalyzed by methanogens. As used herein, the term methanogen refers to methane-producing organisms including both methane-producing bacteria and to Archaea (formerly classified as archaebacteria.) The methanogenic pathways of all species of methanogens have in common the conversion of a methyl group to methane. However, the origin of the methyl group varies. Most species are capable of reducing carbon dioxide (CO_2) to a methyl group with either molecular hydrogen (H_2) or formate as the reductant. Methane production pathways in methanogens that utilize CO_2 and H_2, involve specific methanogen enzymes, which catalyze unique reactions using unique coenzymes.

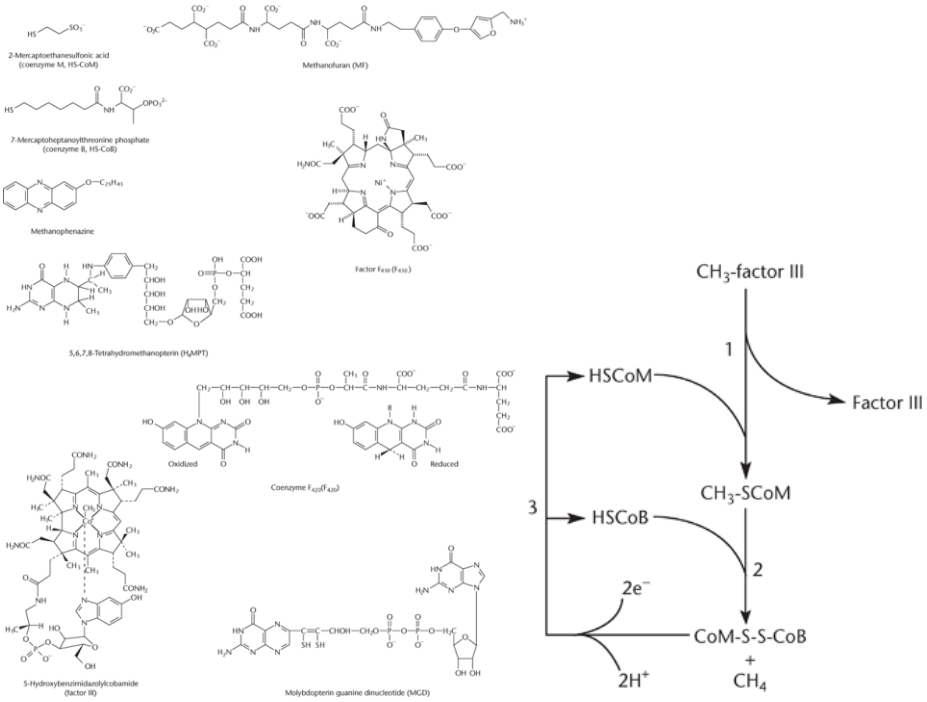


Figure 1. Enzymes and Co-enzymes utilized in methanogenic pathways.

Lessner, Daniel J (Dec 2009) Methanogenesis Biochemistry. In: eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net [doi: 10.1002/9780470015902.a0000573.pub2]

So – How Does Provect-CH4® EO **Inhibit a Methanogen?** Provect-CH4 limits the growth and productivity of Archaea, as well as, acetoclastic methanogenic bacteria, during in situ remediation processes by disrupting enzyme and coenzyme systems unique to methanogens. The various enzyme and co-enzyme systems that are targeted include: i) 4-(β-D-ribofuranosyl)aminobenzene-5´-phosphate (β-RFA-P) synthase, an early step in the biosynthesis of tetrahydromethanopterin (H4MPT), which is a modified folate that is of central importance in growth and energy metabolism of methanogens; ii) Coenzyme F420 (8-hydroxy-5-deazaflavin) NADP oxidoreductase enzyme which plays a vital role in the formation of methane , iii) Coenzyme M (CoM), 2-sulfanylethanesulfonate cofactor the substrate for the methyl reductase which catalyzes the terminal step in all methanogenic pathways; iv) Coenzyme B, 2-[(7-mercapto-

1-oxoheptyl)amino]-3-phosphonooxybutanoic acid, is the second substrate for methyl-coenzyme M reductase, and as a consequence of the reaction; v) Coenzyme A 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is also another enzyme that is very critical in methane production in Methanobrevibactor strains, since Archaea are the only bacteria known to possess biosynthetic HMG-CoA reductase. The saponins/essential oils can be used alone or in conjunction with myriad (organic) hydrogen donors to support desired biological reductive dechlorination reactions while controlling the production of methane during remedial actions, and in other environmental applications. And since methanogens are so uniquely different than bacteria, the inhibitory effect of Provect-CH4 is not observed in microbes that are typically associated with: i) catabolism of organic contaminants (such as pseudomonas species) and/or, ii) halo-respiration/biodegradation of chlorinated solvents (such as *Dehalococcoides* species).

SAFER, MORE EFFICIENT ERD / ISCR TREATMENT

In situ chemical reduction describes the combined effect of stimulated biological oxygen consumption (via fermentation of an organic carbon source), direct chemical reduction with zero-valent iron (ZVI) or other reduced metals. As described by Brown *et al* (2009), the corresponding enhanced thermodynamic decomposition reactions that are realized at the lowered redox (Eh) conditions allow for more effective mineralization of many constituents of interest (COIs). A number of ERD substrates and other accelerated anaerobic bioremediation technologies exist (*e.g.*, emulsified oils, non-emulsified oils, carbon-based hydrogen release compounds, vegetable matter + ZVI amendments) that facilitate biodegradation of related compounds. Provect-IR antimethanogenic ISCR substrate uniquely combines RYR extract with of a variety of specially selected reagents in order to induce genuine ISCR conditions and faciliate the destruction of targeted COIs in a safer, more efficacious manner. Millions of pounds of Provect-IR have been used full–scale, around the world, to ameliorate aquifers impacted by chlorinated solvents, pesticides, heavy metals and other COIs (Provectus Environmental Products, Inc. – http://www.provectusenvironmental.com/)

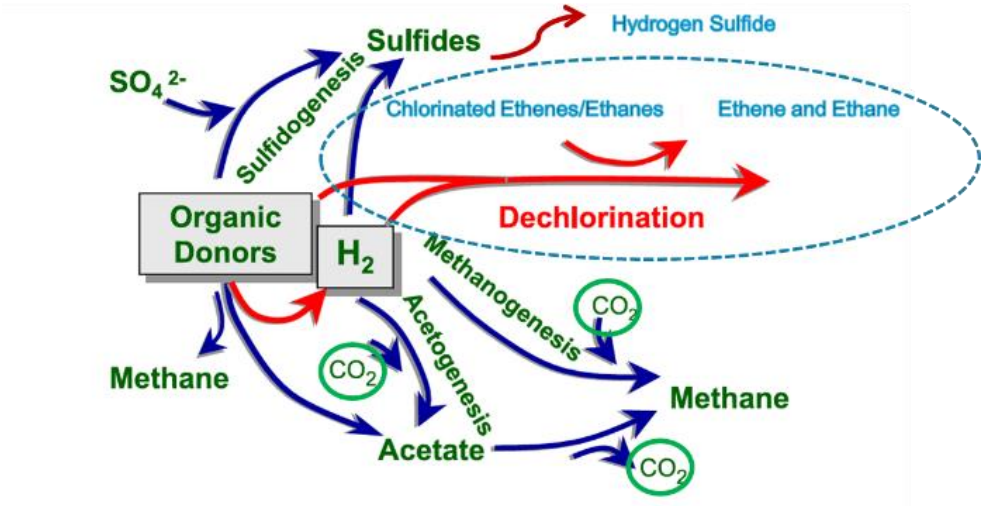


Figure 2. What happens to H2 when methanogens are inhibited?

Methanogens are often the dominant hydrogenotrophs (*i.e.*, consumers of hydrogen) in many environments because they have a lower threshold for H_2 than do acetogens, and because the energy yield from the conversion of CO_2 and H_2 to methane is greater than that for conversion to acetate. However, when methanogens are inhibited, acetogens such as *Clostridium* and many other microbes with a broad range of catabolic abilities will thrive and produce acetyl-

CoAQ / acetate and other VFAs from H_2 and CO_2 via the Wood-Ljungdahl pathway (See **Figure 2**). By inhibiting the growth and proliferation of methane producing Archaea, chlororespiring bacteria can become the more dominant bacterial populations. In an anaerobic environmental remediation setting, halorespiring and other bacteria such as Desulfobacter spp. and Desulfuromonas spp. will more effectively utilize the available hydrogen for dechlorination of targeted COIs, and the VFAs will be fermented to ultimately yield CO_2 (Schauder *et al.,* 1986).

PROOF OF CONCEPT

Independent third-party laboratory studies comparatively evaluated the antimethanogenic potential of multiple essential oils (*e.g.,* Garlic Oil [GO], Cinnamon Bark Oil [CO], Cinnamon Bark Powder [CBP] and lemongrass Oil [LO] as examples of essential oils that can potentially manage Archaea during remedial action. Manure and groundwater samples were collected from a site. The collected samples were added to 125 mL amber glass bottles equipped with PTFE-lined open septum caps (VOA vials). The testing program included 40 vials each filled with 20 g manure slurry and 20 g groundwater. A total of 40 vials were prepared to facilitate replicate analyses 3 time intervals: i) Day 3, ii) Day 7 and iii) Day 12. Five of the vials were used to indicate the onset of anaerobic conditions by measuring pH, ORP and methane. Gas samples from the vial headspace were analyzed for methane in the gas phase using a gas chromatograph (GC) with a flame ionization detector (FID). After these analyses were completed, pH and ORP were also measured. All samples were sacrificial and disposed after completion of the analyses.

Figure 2. Amber glass vials with PTFE lined open septum caps were used sacrificially for the laboratory tests.

RESULTS

All essential oils tested were successful in inhibiting methane production: garlic oil (GO) was the most effective as determined by reducing ORP, maintaining pH, and controlling methane production after 3, 7 and 12 days (**Tables 2, 3 and 4**, respectively). After 12 days incubation in the presence of active methanogens under ideal growth conditions, the amount of methane produced in the presence of GO was reduced by 74% as compared to the active control (from 7,920 µg CH_4/L water to 2,030 µg CH_4/L). In fact, the amount of methane generated in the GO microcosms was less than the sterile, killed-cell control system (**Figure 2**).

Additional data from laboratory and field studies evaluated longer reaction times and have shown efficacy up to 60 and 90 days under various aquifer and test conditions (data not shown). These essential oil-based AMR are currently the subject of additional independent studies evaluating performance, efficacy, longevity and microbiology.

Upon approval from various clients to release data, our intent is to make all information freely available.

Table 2. Essential Oil Methane Generation Test Results – 3 Day Reaction Time

Vial #		Reagent Added		Date Analyzed	Time Following Set-up (days)	Time Following Dosing (days)	Methane (ug/L)	pH	ORP (mv)
		Date	Amount (g)						
Manure Slurry Added to Vials: 10/19/2015									
Control #1		10/23/15	--	10/26/2015	7	3	3,675	5.31	-54
GO 4% #1		10/23/15	0.776	10/26/2015	7	3	3,180	5.58	-159
CO 4% #1	dup	10/23/15	0.799	10/26/2015	7	3	3,095	5.61	-100
LO 4% #1		10/23/15	0.799	10/26/2015	7	3	2,910	5.50	-61
CB 4% #1		10/23/15	0.798	10/26/2015	7	3	2,820	5.16	-35
GO 10% #1		10/23/15	1.951	10/26/2015	7	3	2,610	5.27	-119
CO 10% #1		10/23/15	2.001	10/26/2015	7	3	2,710	5.39	-71
LO 10% #1	dup	10/23/15	2.013	10/26/2015	7	3	3,675	5.87	-74
CB 10% #1		10/23/15	2.001	10/26/2015	7	3	2,100	5.17	-26

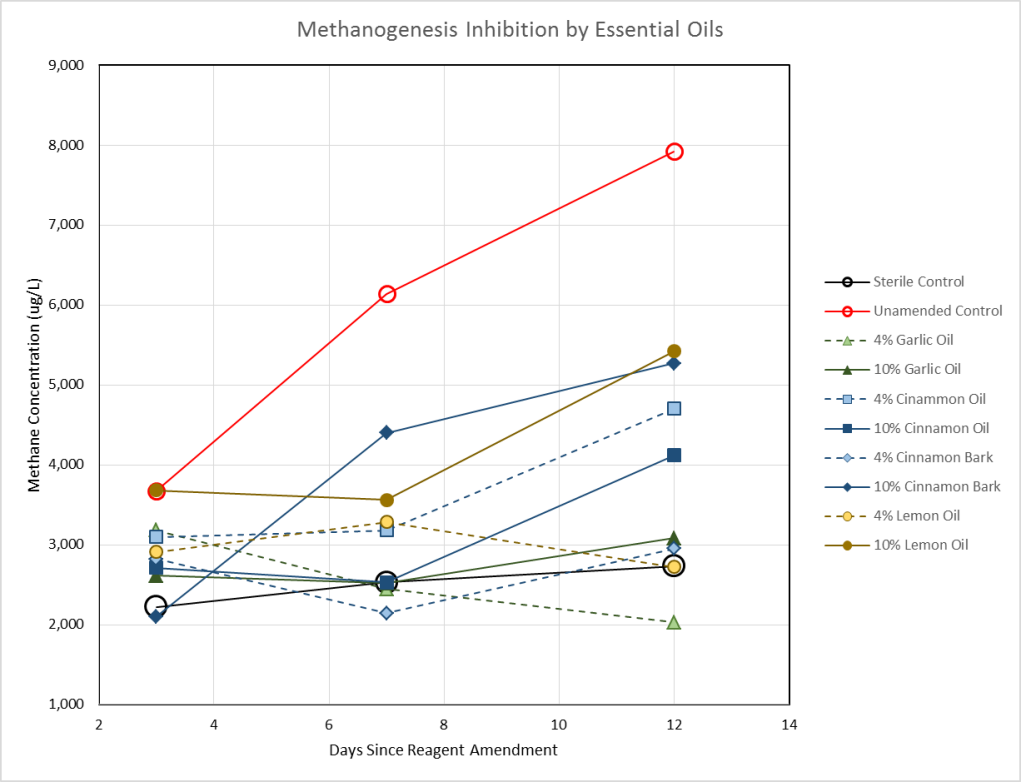
Table 3. Essential Oil Methane Generation Test Results – 7 Day Reaction Time

Vial #		Reagent Added		Date Analyzed	Time Following Set-up (days)	Time Following Dosing (days)	Methane (ug/L)	pH	ORP (mv)
		Date	Amount (g)						
Manure Slurry Added to Vials: 10/19/2015									
Control #2		10/23/15	--	10/30/2015	11	7	6,140	5.18	-62
GO 4% #2		10/23/15	0.774	10/30/2015	11	7	2,445	5.27	-169
CO 4% #2		10/23/15	0.798	10/30/2015	11	7	3,180	5.57	-122
LO 4% #2	dup	10/23/15	0.801	10/30/2015	11	7	3,285	5.19	-73
CB 4% #2	dup	10/23/15	0.804	10/30/2015	11	7	2,145	4.96	-45
GO 10% #2		10/23/15	1.948	10/30/2015	11	7	2,520	5.08	-163
CO 10% #2		10/23/15	2.019	10/30/2015	11	7	2,530	5.43	-113
LO 10% #2		10/23/15	2.008	10/30/2015	11	7	3,560	5.54	-96
CB 10% #2		10/23/15	1.999	10/30/2015	11	7	4,400	5.54	-43

Table 4. Essential Oil Methane Generation Test Results – 12 Day Reaction Time

Vial #		Reagent Added		Date Analyzed	Time Following Set-up (days)	Time Following Dosing (days)	Methane (ug/L)	pH	ORP (mv)
		Date	Amount (g)						
Manure Slurry Added to Vials: 10/19/2015									
Control #3		10/23/15	--	11/4/2015	16	12	7,920	5.23	-74
GO 4% #3		10/23/15	0.778	11/4/2015	16	12	2,030	5.12	-173
CO 4% #3		10/23/15	0.812	11/4/2015	16	12	4,700	5.77	-116
LO 4% #3		10/23/15	0.808	11/4/2015	16	12	2,720	5.08	-61
CB 4% #3		10/23/15	0.802	11/4/2015	16	12	2,950	4.96	-46
GO 10% #3	dup	10/23/15	1.946	11/4/2015	16	12	1,635	5.23	-198
CO 10% #3		10/23/15	2.017	11/4/2015	16	12	4,120	5.46	-131
LO 10% #3		10/23/15	2.004	11/4/2015	16	12	5,420	5.10	-43
CB 10% #3	dup	10/23/15	1.999	11/4/2015	16	12	5,270	5.03	-22

Figure 3. Methanogenesis Inhibition by Essential Oils



PRIMARY FEATURES AND POTENTIAL BENEFITS:

Provect-CH4® and Provect-CH4® EO patented AMRs are the only ERD/ISCR supplements that will rapidly improve remedial performance while simultaneously preventing or significantly minimizing the production of methane. The benefits are notable:

- More Efficient = More Cost Effective: Production of methane is a direct indication that the hydrogen generated from the organic carbon amendments was used by methanogens and the amendment has been wasted because it was not utilized by acetogens or dehalorespiration. By inhibiting the growth and proliferation of methane producing Archaea, chlororespiring bacteria can become the more dominant bacterial populations.

- Safer: Methane is explosive with an LEL of 5% and an UEL of 15%. Production of methane will result from the addition of any conventional ERD or ISCR amendment: excessive and extended production of methane can result in elevated in groundwater concentrations (as high as 1,000 ppm have been reported) which can lead to accumulation in soil gas subsequently impacting indoor air. State specific regulations for methane in groundwater have been promulgated, with others pending for soil gas and indoor air.

- Ease of Use: Green and sustainable. All components integrated in a single package. Logistics with no surprises.

- Patented Technologies: Technology end users and their clients are fully protected from all Patent and other legal issues.

EXAMPLE APPLICATION GUIDELINES (50 ppm AMR in groundwater):

In an aquifer setting, a targeted dose of 50 - 150 ppm is generally recommended: site specific treatability studies can be conducted to help validate potential effectiveness and optimize the design specifications

Treatment Zone Dimensions

Width of targeted zone (perpendicular to gw flow)		150	ft
Length of targeted zone (parallel to gw flow)		125	ft
Depth to top of treatment zone		10	ft
Depth to bottom of treatment zone		20	ft
Treatment zone thickness		10	ft
Calculated Volume		187500	ft3

Methane Inhibitor Calculations

Estimated Porosity		35	%
Calculated impacted liquid		65625	ft3
Methane Inhibitor for Project	ROUNDED TO NEAREST 25 lb	200	lbs

* PLEASE CONTACT US (info@provectusenv.com) FOR PRICING INFORMATION AND DELIVERY QUOTES.

LITERATURE CITED:

Alberts, A., J. Chen, G. Kuron, V. Hunt, J. Huff, C. Hoffman, J. Rothrock, M. Lopez, H. Joshua, an E. Harris; 1980. Mevinolin: a Highly Potent Competitive Inhibitor of Hydroxymethylglutaryl-coenzyme A Reductase and a Cholesterol-Lowering Agent. Proceedings of the National Acadaemy of Sciences of the United States of America 77:3957-3961.

Brown, R., J. Mueller, A. Seech, J. Henderson and J. Wilson. 2009. Interactions between Biological and Abiotic Pathways in the Reduction of Chlorinated Solvents. Remediation Journal Winter 2009, pages 9-20.

Henderson, G., G.E. Naylor, S.C. Leahy and P.H. Janssen. 2010. Presence of novel, potentially homoacetogenic bacteria in the rumen as determined by the analysis of formyltetrahydrofolate synthetase sequences from ruminants. Appl. Environ. Microbiol. 76:2058-2066.

Miller, T.L. and M.J. Wolin. 2001. Inhibition of growth of Methane-Producing Bacteria of the Rumen Forestomach by HydroxymethylglutarylSCoA Reductase Inhibitors. J. Dairy Sci. 84:1445-1448.

Mueller, J.G. and J.G. Booth. 2016. Managing Excessive Methanogenesis during ERD/ISCR Remedial Action. Remediation Journal-Summer Issue; Pages 53-71.

Mueller, J.G., A. Karachalios and T. Fowler. 2014. Controlling Methane at ERD and ISCR Applications. Pollution Eng. October 2014, Pages 24 – 29.
http://www.provectusenvironmental.com/marketing/articles/Controlling_Methane_at_ERD_and_ISCR_Applications_PE-OCT2014.pdf

Scalzi, M. and A. Karachalios. 2013; 2014; 2016. Inhibition of Methane Production during Anaerobic Reductive Dechlorination. US PTO 9,221,699 and CIP 14/268,637.

Schauder, R., B. Eikmanns, R.K. Thauer, F. Widdel and G. Fuchs. 1986. Acetate Oxidation to CO2 in Anaerobic Bacteria via a Novel Pathway not Involving Reactions of the Citric Acid Cycle. Arch. Microbiol. 145:162-172.

Woese, C.R. and G.E. Fox. 1977. Phylogenetic Structure of the Prokaryotic Domain: the Primary Kingdoms. Proceedings of the National Academy of Sciences of the United States of America **74** (11): 5088–5090.

CONTACT US FOR A COMPLIMENTARY SITE EVALUATION

PROVECTUS ENVIRONMENTAL PRODUCTS, INC.

2871 West Forest Road, Suite 2 | Freeport, IL 61032

Tel: (815) 650-2230 | Fax: (815) 650-2232 | Info@Provectusenv.com

Multiple remedial contracting options available via strategic providers

Turn-Key, Risk-Reward, Pay-for Performance, Remedial Guarantees/Warranties