Provectus' Antimethanogenic ERD/ISCR Technologies

Provectus' technologies are unique in their ability **to actively control the production of methane** in a safe, reliable and predictable manner (US Patent Office Scalzi *et al*, 2013, 2014). 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.

Provect-CH4™ is a food-grade, natural source of mixed statins, including Monacolin K (otherwise known as Lovastatin™), that are used to prevent methane (CH_4) production by inhibiting the growth and proliferation of methanogenic Archaea. In environmental remediation applications, it can be used as a supplement to conventional enhanced reductive dehalogenation (ERD) and *in situ* chemical reduction (ISCR) amendments rendering them safer and more effective. These include:

- Oils
- Emulsified Oils
- 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)

Provect-IR™ is a unique mixture of reagents combined into a single product that optimizes the *in situ* reductive dechlorination of chemicals present in soil, sediment, and groundwater. It acts by promoting synergistic interactions between:

- Provect-CH4™ (a proprietary source of Monacolin-K and other natural statins that act as methanogenic inhibitors)
- Multiple hydrophilic, nutrient rich organic carbon sources
- Zero-Valent Iron (ZVI)
- Chemical oxygen scavengers
- Vitamin and mineral sources

This distinctive, patented combination of natural and food-grade chemicals promotes ISCR conditions for fast and effective destruction of targeted constituents of interest (COIs) such as chlorinated solvents, organochlorine pesticides, and other halogenated compounds. Provect-IR is the only ISCR reagent to simultaneously inhibit the production of methane during the requisite carbon fermentation processes. This promotes more efficient use of the hydrogen donor (>30% more efficient which reduces amendment requirements) while avoiding negative issues associated with elevated methane in groundwater, soil gas, and indoor air (Mueller *et al*, 2014).

Provect-IRM™ employs this proven ISCR technology to facilitate established adsorption and precipitation reactions for heavy metal immobilization. Because it inhibits the activity of methanogens during the synergistic ISCR processes, this greatly reduces the biosynthesis of highly toxic, mobile methylated (organo) metals, which is a very negative consequence of conventional ISCR reactions / immobilization practices. As such, all metal species are more quickly sequestered for safe, long-term, stable immobilization of heavy metal contaminants. It is composed of multiple reagents in a single product:

- Provect-CH4™ (a proprietary source of Monacolin-K and other natural statins that act as methanogenic inhibitors)
- Multiple, hydrophilic, nutrient rich organic carbon sources (plant materials, kelp, calcium propionate) → 390 g H /lb product.
- Small (*ca*. 10 to 25 micron) ZVI → 25 square feet reactive ZVI surface area / lb product
- Chemical oxygen scavengers to help maintain reduced conditions during mixing
- Integrated vitamins, minerals mineral sources (yeast extracts) specially selected for the growth and development of anaerobes
- Potassium magnesium sulfate to help promote formation of iron-sulfide minerals, where needed
- Powdered activated carbon (PAC) to help sequester organo-metal complexes

IMPACT OF STATINS ON ARCHAEA NON-METHANOGENIC BACTERIA

Provect-CH4 contains a number of statins including Monacolin K that specifically inhibit methanogenic Archaea because bacterial cell walls are predominantly comprised of murein (peptidoglycan) whereas Archaea cell walls are composed of pseudomurein – a structural analogue of murein. Pseudomurein is biosynthesized via activity similar to that of HMG-CoA reductase which yields cholesterol in humans. In the presence of Monacolin K and other statins, HMG-CoA reductase is inhibited, pseudomurein biosynthesis pathway is interrupted, and methanogens are restricted from growth and proliferation (**Figure 1**). Statins are also known to inhibit the activity of F420 enzyme systems which are also exclusive to Archaea (**Figure 2).**

Figure 1. Schematic of Archaea Cell Walls Containing Pseudomurein

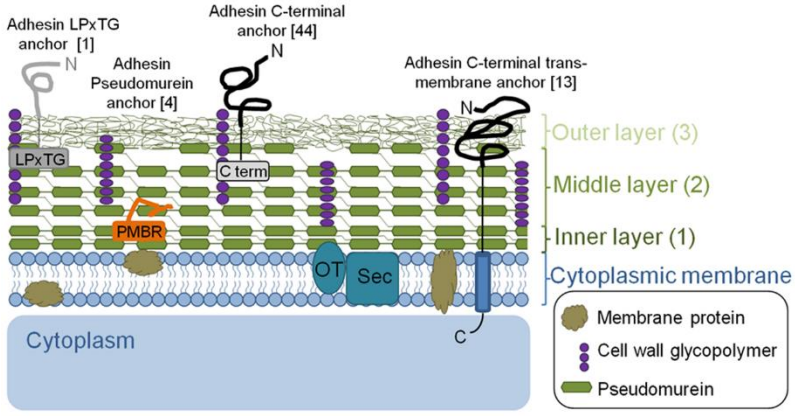
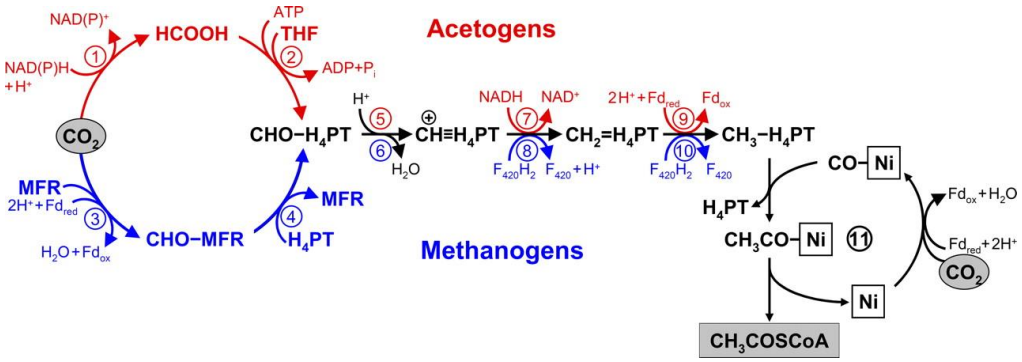


Figure 2. Reductive acetyl-CoA (Wood-Ljungdahl) Pathway



IMPACT OF STATINS ON NON-METHANOGENIC BACTERIA

Since methanogenic Archaea are so uniquely different than other 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 or biodegradation of chlorinated solvents (such as *Dehalococcoides* [DHC] species). A number of independent test methods have been employed to demonstrate that the inhibitory impact of Provect-CH4 statins is selective to methanogens:

- Heterotrophic Bacterial Production using Leucine-^{3}H
- Mineralization of targeted substrates using ^{14}C- radiolabeled compounds (in progress)
- qPCR gene probes for DHC Inoculant SDC-9™ (CB&I – in progress)
- qPCR gene probes for DHC Inoculant KB-1™ (Sirem Labs – in progress)

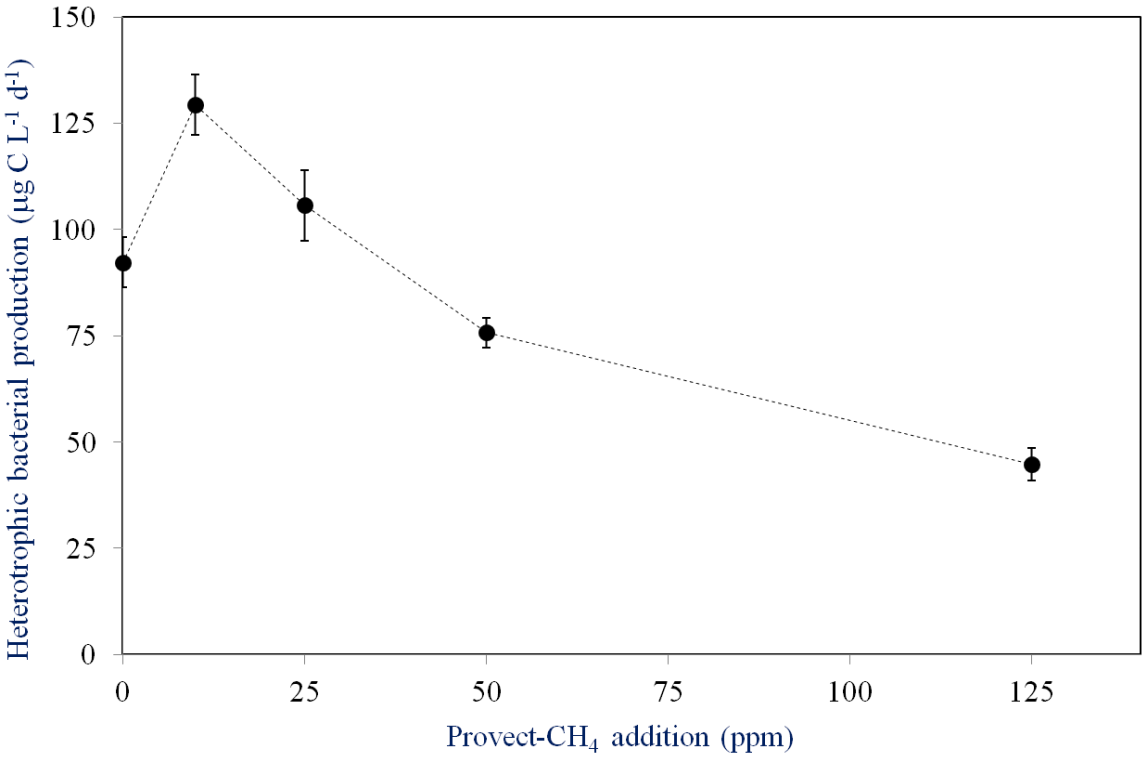
Heterotrophic Bacterial Production using Leucine ^{3}H

Bacterial production was measured using leucine incorporation as adapted by Montgomery *et al* (2010). Briefly**,** an aliquot of wet sediment (50 µL) or surface water (1 mL) was added to 2 mL microcentrifuge tubes (three live and one killed control) that were pre-charged with tritiated leucine, L-[4,5-^{3}H] (specific activity: 154 mCi mmol^{-1}; final concentration = 20 nM). Samples were incubated (0.5 h for water, 2 h for sediment) at ambient temperature and subsequently processed by the method above.

Results: Studies using marsh creek water (salinity = 25) showed that Provect-CH4 methane inhibitor actually stimulated heterotrophic bacteria production when applied at 10 and 25 ppm (**Figure 3**). The amendment had little discernible impact on heterotrophy when applied at 50 ppm, which is the standard groundwater application rate used to proactively manage Archaea. When applied at 125 ppm in estuarine water (which is the groundwater application rate when used to

damper a dominant and active methanogen population as evidenced by elevated methane concentrations) the amendment slightly reduced (*e.g.,* 40%) heterotrophy.

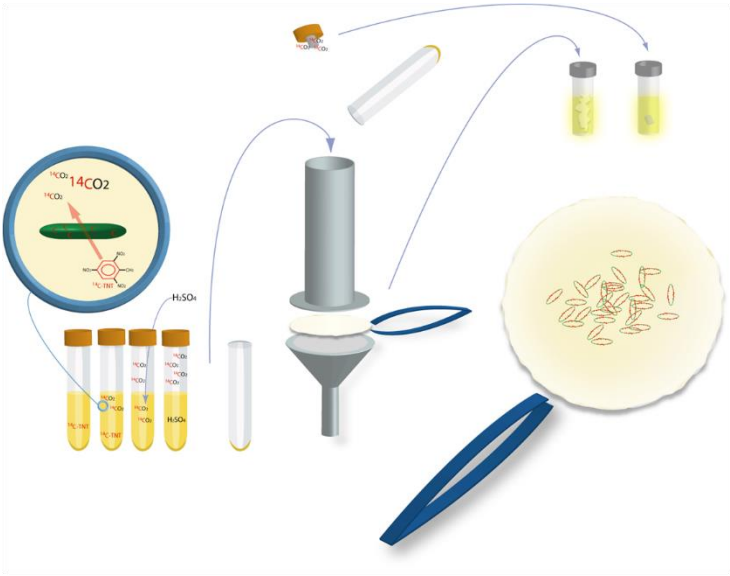
Figure 3. Impact of Provect-CH4 on Bacterial Production (Peale *et al*., 2015).



Mineralization of Reporter Substrates using ^{14}C- Radiolabeled Compounds

Rates of bacterial metabolism of aromatic contaminants were measured by mineralization of ^{14}C-radiolabelled substrates to $^{14}CO_2$. These assays were typically initiated within 2 h of sample collection using a modification of procedures by Montgomery *et al* (2011). As outlined in **Figure 4**, carbon substrates 2,4,6-TNT [ring-^{14}C(U)] (4 mCi mmol^{-1}, American Radiochemical Corporation, 99% purity), 9-^{14}C-phenanthrene (PHE; 55.7 mCi mmol^{-1}), UL-^{14}C-RDX (1.13 mCi mmol^{-1}, Defence R&D Canada), and UL-^{14}C-HMX (1.97 mCi mmol^{-1}), were added in separate incubations to 100 x 16 mm polycarbonate test tubes (*ca*. 0.04 µg mL^{-1}, depending on specific activity). Triplicate live and one killed (2 mL of 2 N H_2SO_4) samples were incubated for 48 hours at room temperature in the dark, and evolved $^{14}CO_2$ was captured on NaOH-soaked filter papers. H_2SO_4 (2 mL, 2 N) was added to end live incubations and to partition any remaining CO_2 into headspace of the tube and to the filter paper trap. Filter paper traps containing metabolized $^{14}CO_2$ were removed, radioassayed and subsequently used to calculate substrate mineralization. Detection limit of the assay was typically 0.01 µg C L^{-1} d^{-1} though average values that were below one standard deviation were considered non-detect (0).

Figure 4. Procedure for Measuring Bacterial Mineralization (Montgomery *et al.*, 2011)



Results: Studies using Delaware bay estuarine water (salinity = 25, Delaware) showed that Provect-CH4 methane inhibitor (at 50 ppm) had little discernible impact on the mineralization of ^{14}C-RDX when incubated under hypoxic (non-aerated; non-anaerobic) laboratory conditions (**Table 1**). When combined with Provect-IR™ the antimethanogenic ISCR reagent increased mineralization of ^{14}C-RDX up to 2-fold. Using estuarine sediment (salinity = 32, North Carolina), 6.25% amendments of Provect-IR™ stimulated RDX mineralization around 5-10 fold. Data from other radiolabeled compounds will be published as they become available.

Table 1. Impact of Provect-CH4 or Provect-IR on Bacterial Mineralization of ^{14}C-labeled RDX (Peale *et al.*, 2015)

Estuarine Media	Sample	Amendment	RDX Mineralization (AVG (SD) µg kg^{-1} d^{-1} or µg L^{-1} d^{-1})
Water	DE-1	None	3.5 (0.36)
		Provect-CH4(50 ppm)	3.1 (0.46)
		Provect-IR (50 ppm)	7.7 (1.8)
Sediment	NC-1	None	1099 (590)
		Provect-IR (6.25%)	4369 (2890)
	NC-2	None	785 (288)
		Provect-IR (6.25%)	4717 (2400)
	NC-3	None	371 (193)
		Provect-IR (6.25%)	5208 (1586)

qPCR Gene Probes for SDC-9 DHC Inoculant

The effect of Provect-CH4 methanogen inhibitor *on Dehalococcoides*-like organisms present in the commercial inoculant SDC-9™ was evaluated by enumerating microbial populations via genetic analysis. In brief, sacrificial anaerobic microcosms were established using clayey soil and groundwater from Monticello, Wisconsin. Each microcosm was inoculated with: a) 1% (w/w) of cattle manure slurry to serve as a source of methanogens, and b) SDC-9 (titre *ca*. 1x10E12 DHC/L) to yield *ca* 1x10E4 DHC/mL. The microcosms were amended with 0.25% (w/w) Provect-IR with or without Provect-CH4 at 50 ppm and incubated for 25 days at room temperature. DHC-like microbes were then quantified by CB&I's Biotechnology Laboratory (Dr. Rob Steffan) using real-time quantitative polymerase chain reaction (qPCR) with a RAPID PCR machine (Idaho Technologies Inc.) and a Lightcycler FastStart DNA Master Hybprobe probe kit (Roche Diagnostics GmbH, Manheim, Germany) and specially developed primers designed to amplify and quantify 16s ribosomal RNA (rRNA) gene DNA (Vainberg *et al*., 2009). *Dehalococcoides* spp. chromosomal DNA was quantified by comparison to a standard curve generated by amplifying serial dilutions of a known concentration of plasmid (pSC-A vector; Stratagene Inc. La Jolla, CA) containing a cloned 16S rRNA gene from the SDC-9 culture.

Results: The time-zero cell counts in the presence of Provect-IR + 50 ppm Provect-CH4 methane inhibitor was 6-fold greater than the added density of ca. 10,000 DHC/mL, which could be attributed to sample variability. After 25 days of incubation the gene counts of DHC-like microbes was 51,500 DHC/ml, which was greater than the amount added. In the absence of Provect-CH4, there was an apparent doubling of the DHC counts in the anaerobic control (**Table 2**). Additional studies are in progress, but these initial data tend to support the premise that the modes of action of statins are specific to Archaea and that they do not impact all microorganisms uniformly.

Table 2. Impact of Provect-CH4 on qPCR DHC Gene counts (Vainberg *et al.,* 2009)

Treatment	DHC cells / ml Day 0	DHC cells / ml Day 25
Provect-IR with 0 ppm Provect-CH4	9,840	18,400
Provect-IR with 50 ppm Provect-CH4	65,900	51,500

qPCR Gene Probes for KB-1 DHC Inoculant

The effect of Provect-CH4 methanogen inhibitor *on Dehalococcoides*-like organisms present in the commercial inoculant KB-1™ was evaluated by enumerating microbial populations via genetic analysis as conducted by Sirem laboratory (Guelph, Ontario, Canada). Preliminary studies showed that Provect-CH4 at 50 ppm had no effect on the ability of the inoculum to biodegrade TCE under the test conditions evaluated (high inoculum cell density; 2 day incubation time). Future studies will be designed to assess simultaneous impacts on methane production.

SUMMARY

Numerous methods to study the effects of Provect-CH4 on methanogens and non-target organisms were employed to demonstrate that:

- The reagents had a rapid and significant impact on methanogenic activity, reducing the amount of methane in gas samples by ca. 90% within 3 to 5 days treatment time.
- The methanogen inhibitors did not have a detrimental impact on heterotrophy as determined by 3H-Luecine uptake.
- The methanogen inhibitors did not have a negative impact on as determined by the mineralization of ^{14}C-RDX, HMX, TNT or Phenanthrene when incubated under hypoxic (non-aerated; non-anaerobic) conditions;
- The methanogen inhibitors did not have a detrimental impact on heterotrophy as determined by qPCR gene probes.

Overall, these data demonstrate that the inhibitory impact of Provect-CH4 statins is selective to methanogens and that they do not impede the numbers or activities of bacteria, including *Dehalococcoides* spp.

PRIMARY FEATURES

Provect-CH4 is the only ERD/ ISCR supplement 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.

REFERENCES

- Montgomery, M., T., Boyd, T. J., Osburn, C. L. and D. C. Smith. 2010. PAH mineralization and bacterial organotolerance in surface sediments of the Charleston Harbor estuary. Biodegradation 21:257-266. DOI:10.1007/s10532-009-9298-3.
- Montgomery, M. T., Coffin, R. B., Boyd, T. J., Rose, P. S., Smith, J. P., Sachsenmaier, L., Mikan, M., and C. L. Osburn. 2011. 2,4,6-Trinitrotoluene (TNT) and aromatic organic matter metabolism by natural bacterial assemblages at estuarine transition zones (ER-2124). Presentation at the SERDP Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, November 30-December 2.
- Mueller, J., A. Karachalios and T. Fowler. 2014. Controlling Methane Production during ERD and ISCR Applications. Poll. Eng. October Issue. Pages 24-29.
- Peale, J., Scalzi, M., Fowler, T., Montgomery, M. T., Boyd, T. J., and J. G. Mueller. 2015. Antimethanogenic ISCR reagent for safer, more efficient remedial actions. Presentation at the Third International Symposium on Bioremediation and Sustainable Environmental Technologies. Miami, FL, 18-21 May
- Scalzi, M. and McGill, J. 2012. Method for the Treatment of Groundwater and Soils using Mixtures of Seaweed Kelp. US PTO No. 8,147,694 B2 (April 3, 2012).
- Scalzi, M. and A. Karachalios. 2013 and 2014. Inhibition of Methane Production during Anaerobic Reductive Dechlorination. US PTO 13/ 785,840 and CIP 14/268,637
- Vainberg, S., C. Condee and R. Steffan. 2009. Large Scale Production of Bacterial Consortia for Remediation of Chlorinated Solvent-Contaminated Groundwater. J. Ind. Microbiol. Biotechnol. 36:1189-1197.

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 | Email: info@ProvectusEnv.com

Multiple remedial contracting options available via strategic providers

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