Laboratory Assessment of Antimethanogenic Reagents (AMRs): Provect-CH4® with EVO Substrates

Executive Summary

Laboratory microcosms evaluated the ability of various antimethanogenic reagents (AMRs) to control methanogenesis during the biological reductive dehalogenation of trichloroethene (TCE) as induced by commercially available organic hydrogen donors (OHDs). The AMRs included: i) Monacolin K / Lovastatin (clinical grade), ii) 2-bromoethanesulphonate (BES), iii) Provect-CH4® red yeast rice extract (RYR), iv) Provect-CH4® EGO (essential garlic oil; natural) and v) Provect-CH4® SGO (essential garlic oil; synthetic). The OHD fermentable carbon sources / remedial amendments included: i) lactate, ii) emulsified vegetable oil (EVO), iii) EHC®, iv) EHC®-L emulsified lecithin, v) EHC®-Plus that reportedly contains powdered or activated granular activated carbon (PAC or GAC), vi) Provect-IR® and vii) Provectus' emulsified zero-valent iron (EZVI).

After 19 weeks of incubation in the presence of clinical grade Lovastatin (Monacolin K) or RYR methanogenesis was not reduced as expected given the known abilities of these chemicals to specifically control Archaea. These data indicated that the conditions in the microcosms were not optimized for these antimethanogenic processes (*e.g.,* low pH and 8,000 ppm total organic carbon [TOC] likely presented too much fermentable carbon mass for the statins at the concentrations tested to effectively control Archaea). Conversely, the presence of essential plant oil reduced the amount of methane generated by >97% compared to the control microcosms, without having a discernible negative impact on the rate or extent of TCE removal. These data further validated the concept of using AMR technology (Scalzi *et al*., 2013, 2015, 2016) to complement enhanced reductive dechlorination (ERD) and *in situ* chemical reduction (ISCR) remedial processes, and they documented the ability of certain AMR compounds to effectively control methanogenesis even under stringent test conditions that favored methanogenesis (*i.e.,* lower pH, elevated TOC).

Materials & Methods

Aquifer Microcosm Set-Up: Laboratory studies were performed in Dr. Kevin Finneran's laboratory at Clemson University (Clemson South Carolina, USA) to evaluate the ability of six AMRs (**Table 1**) to control methanogenesis from multiple fermentable OHD sources (**Table 2**). Duplicate microcosms of each test condition consisted of 125 ml amber glass bottles fitted with butyl rubber septa. Each bottle was filled with 50 g of aquifer slurry and 30 ml of supplemental groundwater collected from a regional TCE-impacted aquifer (the site is predominately anoxic with no detectable dissolved oxygen and typical ORP values ranging from -100mV to -250mV; the site pH is generally 5.0 to 6.0; *Dehalococcoides* bacteria have been identified at the site using several techniques hence no inoculant was used in these studies). Addition of each electron donor was normalized to 40 mM lactate (*ca*. 8,000 mg/L). Each electron donor was evaluated with and without the addition of each AMR. Lastly, 10 to 20 micromoles of neat TCE (target of *ca*. 1,314 to 2,628 μg/30 ml bottle

or 44 to 88 mg/L) was added to each sealed bottle. Duplicate control microcosms contained TCE without any additives or amendments to account for abiotic losses.

Table 1. AMR Compounds Tested under Batch Incubation Conditions.

Antimethanogenic Reagent	Description	Vendor
BES	2-bromoethanesulphonate	Sigma Aldrich
Lovastatin	Clinical grade Monacolin-K	Merck
Provect-CH4® EGO Garlic Oil – Natural	Essential Plant Oil	Provectus
Provect-CH4® SGO Garlic Oil – Synthetic	Synthetic Plant Oil Diallyl Disulfide / Trisulfide	Provectus
Provect-CH4® Red Yeast Rice Extract #1	Natural statin source *ca*. 0.4% a.i.	Provectus
Provect-CH4® Red Yeast Rice Extract #2	Natural statin source *ca*. 0.4% a.i.	Provectus

Table 2. Organic Hydrogen Donors / Amendments Tested under Batch Incubation Conditions.

OHD / Carbon Amendment	Description	Vendor
None	Control for abiotic losses	N.A.
Bos 100	Powdered Activated Carbon (PAC)	RPI
EHC	Vegetable matter + ZVI	PeroxyChem
EHC-L	Emulsified lecithin	PeroxyChem
EHC Plus	Vegetable matter + ZVI + PAC (assumed)	PeroxyChem
EVO	Emulsified vegetable oil	EOS
EZVI	Emulsified ZVI	Provectus
Lactate	Positive Control	Sigma Aldrich
Provect-IR40	Multiple plant carbon sources + ZVI	Provectus

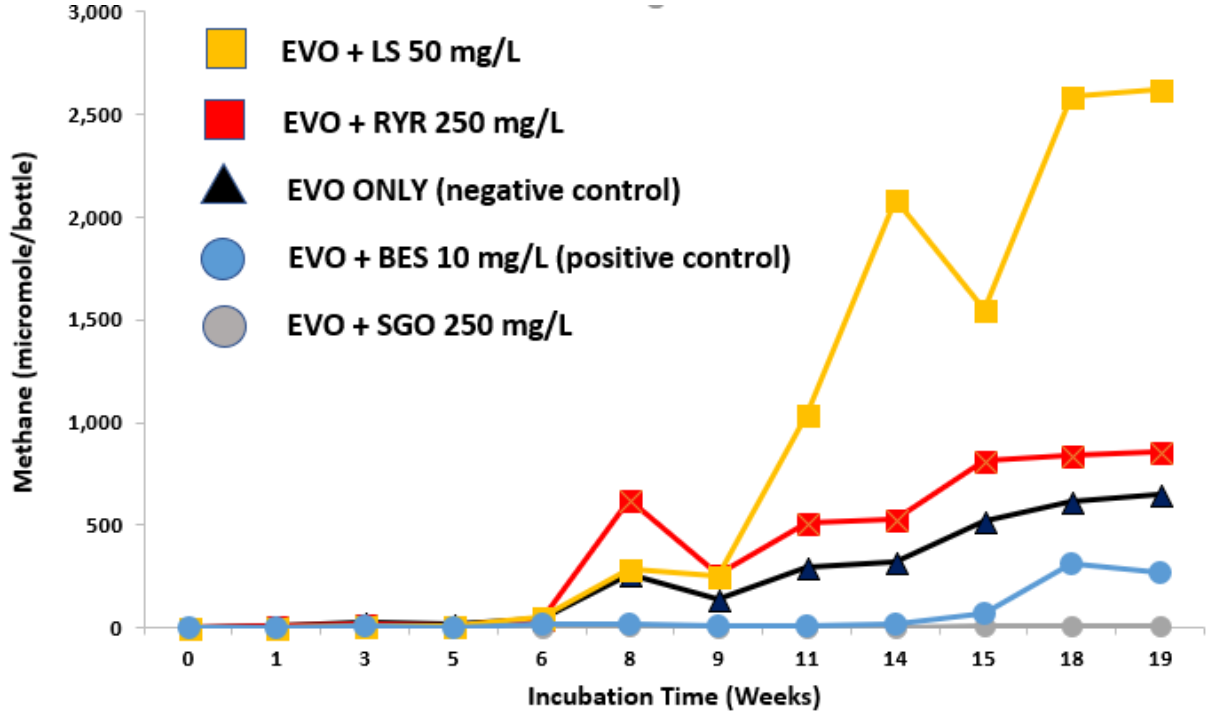
Microcosm Sampling & Analysis: Headspace gasses were analyzed weekly to monitor methanogenesis and biodegradation/removal of TCE following published methods (Amos *et al.,* 2007; Gosset, 1987). In brief, samples (0.2 ml) of headspace were removed from each microcosm with an airtight glass syringe and injected into a Shimadzu 2014 gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 20m GS-Q Plot column. Standard curves were made weekly using known concentrations each of TCE, *cis*-DCE, VC, ethene and methane. Peak area values were plotted against micromole/bottle values and a regression was fitted to the data. All regressions had R^2 values of 0.95 or greater. Using Henry's Law coefficient each peak area was translated to a concentration in micromoles/bottle using the standard curve equation for TCE and each daughter product and subsequently converted to ppmV. Change in concentration over time was tracked and plotted on a graph for each replicated test condition bottle.

Results

Data obtained using EVO as the OHD/fermentable carbon substrate are summarized below as the "average" of the treatment duplicates. Data obtained with other OHDs will be presented in separate Technical Notes. In general, data for AMR responses using other OHDs are either inconclusive or representative of the results reported herein.

EVO Studies - Methanogenesis: When 40 mMol (*ca*. 8,000 ppm) EVO was supplied as the organic hydrogen donor, methanogenesis was not significant until after approximately 6 weeks incubation time (**Figure 1**). Thereafter, resident Archaea generated around 500 µmol methane (ca. 250 mg/L or 250,000 ppmV) in the absence of any AMR compound (**Figure 1** – black triangle) during the 19-week period. Pure Lovastatin (LS) at 50 mg/L (**Figure 1** – yellow square) seemingly stimulated methane production and had no antimethanogenic response under these test conditions despite its known ability to control Archaea (Liu *et al.,* 2011). Likewise, RYR at 250 mg/L (**Figure 1** – red square) did not reduce Archaea activity (NOTE: the RYR employed was subsequently determined to contain *ca*. 0.1% weight basis statin / monacolin K content. Gordon *et al* (2010) showed that the statin content in RYR extracts can vary significantly in terms of content and activity, with total monacolins ranging from 0.3 to 11.2 mg/unit tested. These data suggested that the high concentration of TOC in the test systems exceeded the ability of statins to influence methanogenesis.

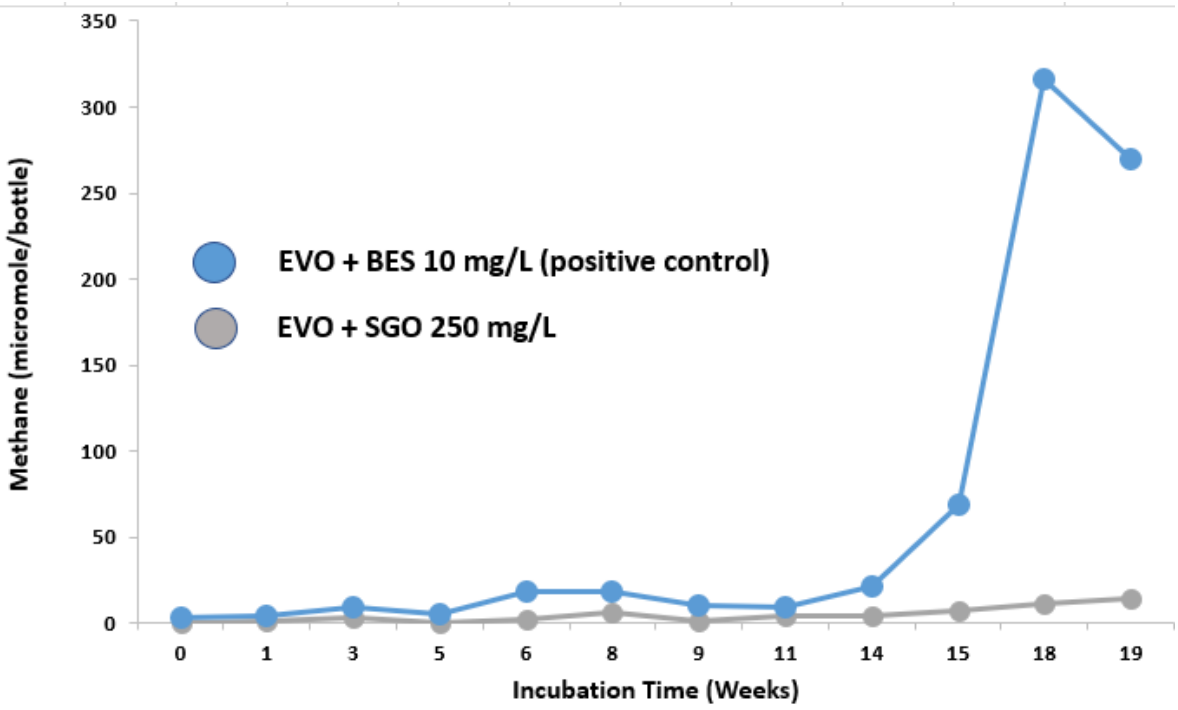
Figure 1. Effect of Select AMRs on Methanogenesis in the Presence of 40 mMol (ca. 8,000 ppm) EVO as the OHD / Fermentable Carbon Substrate (19 weeks incubation time; n=2).



Conversely, both BES at 10 mg/L (**Figure 1** – blue circle) and Provect-CH4® SGO consisting of diallyl disulfide / diallyl trisulfide at 250 mg/L (**Figure 1** – grey circle) effectively reduced methanogenesis without having a negative effect on TCE catabolism (see below). Provect-CH4® EGO (natural garlic oil) exhibited results very similar to those obtained with the synthetic garlic oil, SGO (data not shown).

Zinder *et al* (1984) showed that BES inhibits methanogenesis. A more focused look at these data (**Figure 2**) shows that after 19 weeks of incubation the BES system generated approximately 270 µmol methane (*ca*. 135 mg/L or 137,000 ppmV). This represents a 58% reduction in methanogenesis as compared to the >250,000 ppmV (or >500 µmol or >250 ppm methane in groundwater) generated when EVO was supplied alone, which is a common ERD field application scenario. Essential plant oils – garlic in particular – have been shown to inhibit methanogenesis in ruminants (Pawar *et al*., 2014; Soliva *et al*., 2011). Similar responses were observed with reductive groundwater systems where the Provect-CH4® SGO system generated only 15 µmol methane (*ca*. 8 mg/L or 1,500 ppmV) which represents a 97% reduction in methanogenesis as compared to the EVO control.

Figure 2. Effect of BES and Synthetic Garlic Oil on Methanogenesis in the Presence of EVO (19 weeks incubation time; n=2).



EVO Studies – TCE Degradation: The EVO substrate induced complete dechlorination of TCE to ethene over the course of study (**Figure 3**). Noting that the aquifer was initially slightly acidic, that no inoculant was added, and that the AMR dosage was not optimized (see below) there was a slight delay before TCE was also completely dechlorinated to ethene in the presence of 250 mg/L SGO under confined laboratory test conditions (**Figure 4**). Considering the hydrophobic nature of the EVO some partitioning of chlorinated organic carbon compounds likely occurred which could help explain the multiple peaks over the study period.

Figure 3. Effect of EVO (40 mM; 8,000 ppm) on TCE Degradation (19 weeks incubation time; n=2). Note scales and units.

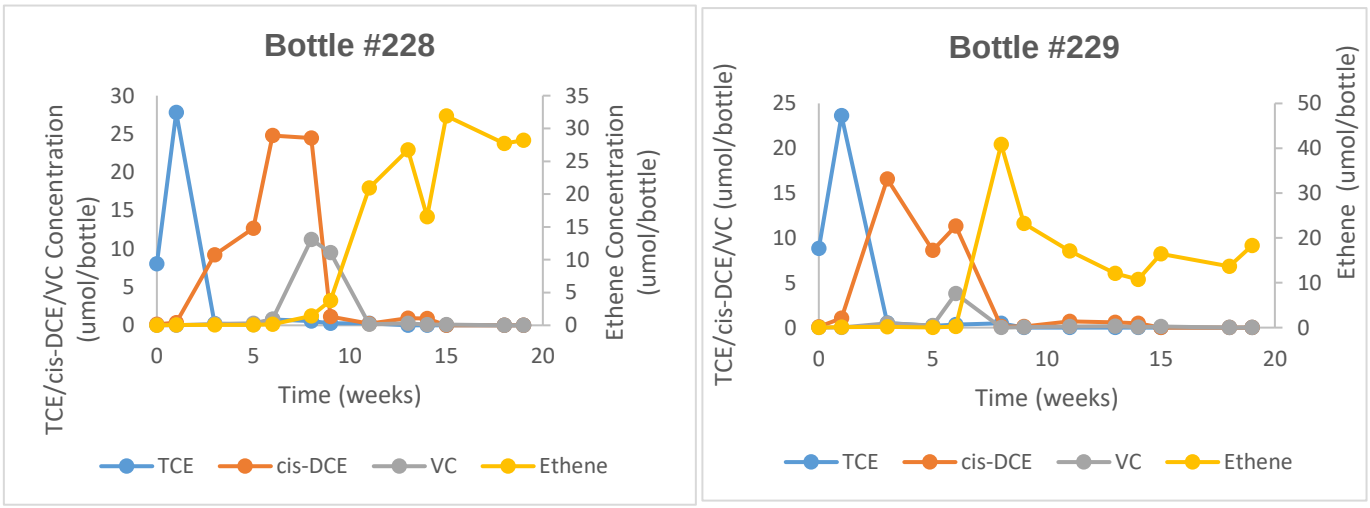
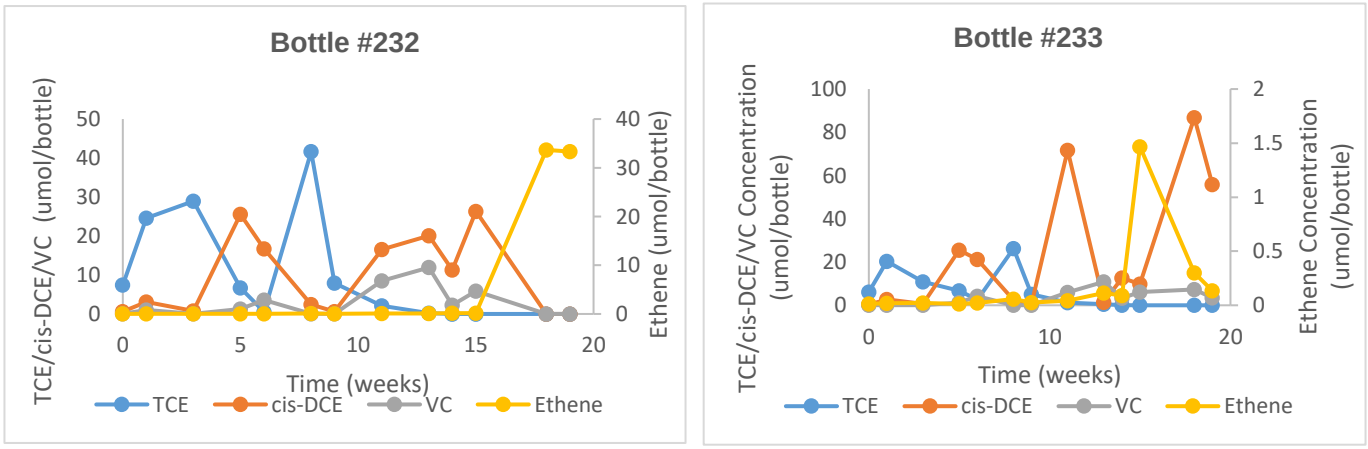


Figure 4. Effect of EVO (40 mM; 8,000 ppm) + SGO (250 mg/L) on TCE Degradation (19 weeks incubation time; n=2). Note scales and units.



Discussion

The amount of methane produced (*e.g*., >28 mg/L and >250,000 ppmV) when Archaea are uncontrolled can lead to issues associated with: i) induced vapor migration, ii) reduced remedial efficacy, iii) failure to meet regulatory compliance, and iv) increased liability / potential health and safety issues. Accordingly, remedial practitioners (*e.g.,* Eklund *et al*., 2014; Mueller *et al*., 2014; Mueller and Booth, 2016) and various regulatory agencies (*e.g*., IDEM, 2015; MI DEQ, 2016; NJ DEP, 2017; US EPA, 2015) have noted the importance of considering the potential consequences of excessive methanogenesis associated with a chosen remedial design and its field implementation.

Anaerobic microbiomes can be complex, and biodiversity increases tolerance to prophylaxis. For example, Zhou *et al.* 2011 showed that BES exhibited greater control of methane production with isolated, single cultures as compared to that which can occur in natural aquifer systems where some individual Archaea strains can sustain very high rates of methanogenesis. These studies were not designed to optimize AMR efficacy (*e.g*., dose/response) and the statin compounds at the concentrations tested did not elicit the characteristic response of controlled methanogenesis. As noted above, this could be due to the high amount of TOC exceeding the ability of statins to influence Archaea. For example, typical ERD field applications target <500 TOC mg/L as higher concentrations favor methanogens (US AFCEE, 2004). Likewise, lower pH favors methanogens over *Dehalococcoides* and related microbes (Yang *et al*., 2017).

Conclusions

Laboratory studies assessed the dynamics of complete dechlorination versus methanogenesis as induced by multiple organic hydrogen donors at high concentration in the presence and absence of various AMRs. Changes in the amount of monitored constituents in headspace gasses collected from duplicate microcosms over a 19-week incubation period - under the given test conditions - validated the concept of using AMR technology to complement ERD and ISCR remedial processes. Specifically, under test conditions that favor methanogens (*i.e*., 8,000 ppm and lower pH);

1. Essential plant oil (*i.e.,* garlic oil) and synthetic garlic oil at 250 mg/L both reduced the amount of methane generated by >97%, without having an overall negative effect on the rate or extent of TCE removal.
2. BES at 10 mg/L served as an effective “positive control” which reduced methanogenesis >58%.
3. Microcosm test systems were not optimized for the AMR processes (for example, at *ca*. 8,000 ppm TOC the microcosms likely contained too much fermentable carbon for RYR at 250 mg/L to effectively control Archaea). Future R&D will focus on AMR dosage optimization and efficacy.
4. The current regime for AMR application employs both RYR and GO/SGO targeting 100 to 250 mg/L of each in the groundwater. This strategy allows for ample RYR/statin dosing, multiple AMR modes of action, and extended longevity (>19 weeks) for controlled methanogenesis.

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Acknowledgements and Disclaimers

These data are part of an ongoing research project at Clemson University that has proceeded with support from multiple parties including Provectus, PeroxyChem, and others. Data were shared with permission of the Finneran Research Group. ***However, technical review and approval of this document do not indicate an endorsement for or against any of the materials, products, or methods reported by either the Clemson University Finneran Research Group, or Finneran Environmental, LLC.*** Please contact Dr. Finneran via email (ktf@clemson.edu) with any questions or comments regarding the information presented in this Summary Report.

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