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Vinyl chloride biodegradation by methane-oxidizing bacteria and ethene-oxidizing bacteria in the presence of methane and ethene

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VINYL CHLORIDE BIODEGRADATION BY METHANE-OXIDIZING BACTERIA AND ETHENE-OXIDIZING BACTERIA IN THE PRESENCE OF METHANE AND ETHENE

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Vinyl chloride (VC) is a known human carcinogen that is found from contamination in groundwater, and is mostly contributed by the manufacture and use of higher-chlorinated ethenes [tetrachloroethene (PCE), trichloroethene (TCE) and ethanes (DCA, TCA)] as solvents in commercial, industrial, and military operations [1]. Under anaerobic conditions the reductive dechlorination biodegradation process converts PCE to TCE, TCE to *cis*-1,2-DCE, *cis*-1,2-DCE to VC, and VC to ethene. A variety of bacteria can attenuate chlorinated ethenes anaerobically, many organisms were reported to dechlorinate PCE only as far as *cis*-DCE, including *Dehalospirillum multivorans*, and *Geobacter loveleyi* [23, 24]. Only strains of *Dehalococcoides* are known to dechlorinate past cDCE [29] For example, *Dehalococcoides ethenogenes* strain 195 can completely dehalogenate PCE and TCE to ethene with H_2 as an electron donor [25]. Although PCE and TCE dechlorination can occur within most anaerobic environments, DCE and VC dechlorination occur almost only under sulfate-reducing and methanogenic conditions [2].

When these anaerobic processes involving *Dehalococcides* spp. fail to go to completion, the more toxic secondary pollutant VC will accumulate in groundwater zones, where significant amount of methane and ethene may also be generated. In some situations, the groundwater returns to aerobic conditions down gradient of the source with methane, ethene, VC, and possibly cis-DCE still present. Natural attenuation of VC could occur via oxidation by aerobic microorganisms as a VC plume intersects with groundwater containing molecular oxygen [26].

Aerobic oxidation is typically mediated by microorganisms that utilize oxygenase enzymes. The non-specific characteristic of the enzymes allows microorganisms to fortuitously oxidize chlorinated ethenes to CO_2 without yielding energy for microbial growth. Recent studies

have revealed that the initial reaction of ethene-oxidizing bacteria to degrade ethene and VC is catalyzed by a 4-component alkene monooxygenase (AkMO). The first evidence was derived from a VC-assimilating bacterium, *Mycobacterium aurum* L1, reported to convert VC to chlorooxirane. The same degradation pattern was observed from an ethene-grown L1 culture, suggesting that AkMO is responsible for the initial attack on both VC and ethene [31]. Two other VC-assimilating bacteria, *Mycobacterium* strain JS60 and *Nocardioides* sp. strain JS614 were also capable of oxidizing ethene to epoxyethane [3]. Strong evidence provided by these diverse isolates indicates that AkMO was the initial enzyme in the pathway that converts VC and ethene into VC epoxide (chloroxirane) and epoxyethane (ethylene oxide), respectively [3, 4].

The genes involved in ethene and VC oxidation from *Mycobacterium* strain JS60 and *Nocardioides* sp. strain JS614 were identified to be AkMO genes (*etnABCD*), and confirmed to be in a close relationship with propene monooxygenase (*amoABCD*) [5].The EtnABCD enzyme from VC and ethene-assimilating bacteria form a distinct clade within the family known as soluble di-iron monooxygenases (SDIMOs) [6]. Studies to date have shown that all SDIMOs accept at least some alkenes as substrates, regardless of whether the alkene acts as a carbon and energy source of the bacterial host. This characteristic has made these enzymes very attractive for bioremediation of chlorinated ethenes [7].

Alkenes oxidation results in formation of an epoxide, which is stabilized by the addition of a coenzyme M molecule by epoxyalkane: coenzyme M transferase (EaCoMT)[8,9]. Experiments with *Mycobacterium* JS60 showed that CoM is an important cofactor given that CoM-dependent epoxide-degrading activity was present in cell extracts of ethene-and VC-grown cells, and absent from acetate-grown cells. Furthermore, heterologous expression of the JS60 EaCoMT gene (*etnE*) confirmed that 2-hydroxyethyl-CoM was produced from epoxyethane and

CoM in the presence of EaCoMT in cell extracts of the recombinant strain [10]. Numerous studies have demonstrated that VC can be readily degraded under aerobic conditions by many pure and mixed cultures expressing monooxygenase enzymes when grown on a variety of primary substrates, including alkanes, alkenes, and aromatic hydrocarbons [11]. The proposed VC and ethene biodegradation pathways in aerobic assimilators are shown in Figure 1[12]. The oxidation of VC and ethene is initiated by an alkene monooxygenase (EtnABCD), yielding epoxyethane from ethene and chlorooxirane from VC. Then, an epoxyalkane-coenzyme M transferase (EtnE) converts these epoxides to hydroxyalkyl-CoM derivatives. These enzymatic chlorooxirane detoxification systems have given advantage to etheneotrophs because toxic compounds can actually promote cell growth [21, 35]. The following biochemical reactions are proposed by presumed gene function analogous to the propene catabolic genes expressed in *Xanthobacter Py2*: The CoM conjugates can either be converted via a SDR family alcohol dehydrogenase into 2-ketoethyl-CoM, or by the spontaneous release of HCl. The next intermediate could be malonate semialdehyde forming via the conversion catalyzed by a reductase/carboxylase, which is later oxidized to malonate by a bifunctional aldehyde/alcohol dehydrogenase. Finally, acetyl-CoA is generated by a decarboxylase, a CoA synthetase, and a CoA transferase to enter TCA cycle [12].

Methane-oxidizing organisms are known to utilize methane as sole carbon and energy source by methane monooxygenase (MMO) [13, 14]. The initial oxidation of CH_4 to methanol can be carried out by two forms of MMO: a membrane-bound “particulate” MMO (pMMO) or a soluble MMO (sMMO) that resides in the cytoplasm. These enzymes can catalyze the insertion of an oxygen atom into a wide variety of nongrowth compounds, including n-alkanes, n-alkenes, and aliphatic and aromatic substances [32]. But sMMO is much more nonspecific with respect to

potential substrates than pMMO. Research has shown that methanotrophs could oxidize ethene and dechlorinate chloromethanes, therefore, it is reasonable to assume that methanotrophs could also oxidize chlorinated ethenes. Oxidation of VC by MMOs results in the formation of chloroethene epoxide, which has a half-life of about 1 minute in aqueous solutions. This unstable intermediate would rearrange to chloroacetaldehyde, and form glycoaldehyde after hydrolysis, which can be catabolized by other heterotrophic bacteria [14, 15].

For this reason, there is great interest in investigating the roles played by methane-oxidizing bacteria (methanotophs) and ethene-oxidizing bacteria (ethenenotrophs) in oxidative VC degradation in groundwater. Under aerobic conditions, etheneotrophs are able to metabolize epoxyethane and chloroozirane via ethene biodegradation pathways. On the other hand, methanotrophs are able to utilize methane as their sole carbon and energy source through the enzyme methane monooxygenase (MMO), which can also cometabolize VC. However, lacking an energy-producing chlorooxirane detoxification system, methanotrophs are reported to be more susceptible to the potentially toxic effects of chlorooxirane than etheneotrophs. In the absence of methane and ethene, bacteria that use VC as a carbon and energy source (VC-assimilating bacteria) could also participate in VC biodegradation [12]. Furthermore, research has shown that ethene-assimilating bacteria like *Pseudomonas aeruginosa*, *Mycobacterium* strains JS622, J2623, JS624, and JS625 are capable of spontaneously adapting to growth on VC as a carbon source. As the result, we hypothesize that etheneotophs are more advantageous than mathnotophs in terms of aerobic VC bioremediation.

The overall objective of this study was to understand how the interactions of the substrates and the presence of these microbial groups affect VC degradation in a scenario where methane, ethene, VC, and oxygen are all present in a groundwater system. For cometabolism to

occur, enough primary substrates and cofactors must be provided to sustain an active population of microorganisms that possess the cometabolizing enzymes. These variables have added great complexity and cost to cometabolic biotransformations.

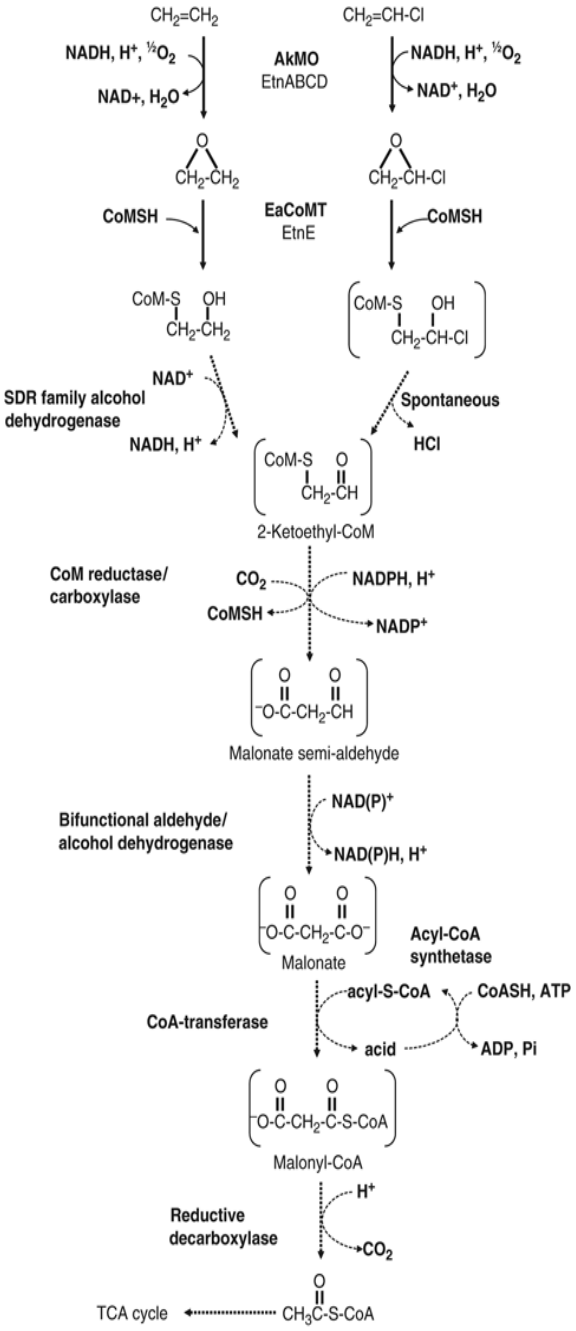

Figure 1 Proposed VC and ethene biodegradation pathway. Adapted from [12].

CHAPTER 2

LITERATURE REVIEW

Cometabolic biodegradation processes have been extensively studied in the past thirty years because of their potential usefulness in biotransforming many of the CAHs found in naturalsystems. However, cometabolic activity is also an unavoidable side reaction that may be harmful to the microorganisms. To understand the factors controlling VC degradation in the presence of both substrates (methane and ethene) and both microbial groups (methanotrophs and etheneotrophs), cometabolic activities of methanotrophs, etheneotrophs, and both microbial groups are discussed respectively. In addition, competitive inhibition between the primary (carbon and energy source) and secondary substrates (pollutants), and the toxic effects from the intermediate compounds on methanotophs and etheneotophs are reviewed in this chapter.

2.1 VC cometabolism by methanotrophs

Several reports showed that chlorinated ethenes are not degraded in the absence of methane in groundwater [16]. In the presence of oxygen and methane, methanotrophs can cometabolize chlorinated aliphatic compounds very rapidly, on the order of days.

In small-inoculum experiments, a pMMO expressing methanotrophic mixed culture was added to the sample bottles, in which 34% of methane and 94 to 99% of the chlorinated aliphatic hydrocarbons (CAHs) (t-DCE, c-DCE, and VC) were in solution. The transformation yield ((T_y), defined as the mass of CAH degraded per unit mass of growth substrate consumed, is useful in characterizing the finite value of CAH cometabolism that occurs in a culture system). The highest observed T_y expressed as moles of CAH per mole of methane for VC was 0.20 mol of VC/ mol of methane when the equilibrium aqueous concentration of VC and methane were 17 µM and 30 µM respectively. However, MMO failed to oxidize both VC and methane when the

VC concentrations were high at 86 µM and 340 µM. This can be due to competitive inhibition, the availability of reducing power and/or the toxic effect of the transformation product [17].

Large-inoculum experiments were done under different growth condition in order to confirm the high yields for VC found in the small-inoculation experiments. The maximum T_y values for VC was 0.25 mol of VC/mol of methane, which is very similar to the result obtained from small-inoculum experiments. However, when comparing the two experiments, maximum T_y value of small-inoculums was found in the bottle containing the lowest methane concentrations (30 µM) and the lowest VC concentration (17 µM), while with the large inoculums, much higher methane concentration (180 µM) together with highest VC concentration (260 µM) gave the maximum transformation yield. The results suggested that factors such as reducing-energy availability, competitive inhibition, and transformation toxicity need to be corrected to reach its balance, so that the maximum values of T_y could be obtained [17].

Fogel et al. utilized a mixed culture containing methane-oxidizing bacteria (CL-M) isolated from lake sediment to demonstrate its ability to degrade TCE and other chlorinated ethenes to CO_2. It is observed that acetylene inhibits 100 ng/ml of TCE degradation completely, suggesting that it is methanotrophs that are responsible for TCE oxidation among the methane-utilizing culture since acetylene is a specific inhibitor of methane monooxygenase. The fact that 12mM methanol can cause 50% inhibition, indicating that methanotrophs are very sensitive to inhibition by methanol. This mixed culture of methanotrophs is observed to degrade VC from 600 ng/ml to less than 0.2 ng/ml in 1 day. Obligate methanotrophs are typically the major component in the mixed culture, although one or more heterotrophic organisms could be present as well. Therefore, methane-oxidizing bacteria are benefited by the presence of these

heterotrophic organisms since they can consume the potential inhibiting organic compounds produced by the methanotrophs [14]. The authors conclude that mixed cultures are more stable than pure cultures in terms of environmental remediation efforts.

2.2 Competitive inhibition and product toxicity of CAHs to methanotrophs

Transformation capacity (T_c) is a defined as the amount of cometabolic compound degraded (dS_c,μmol/L) divided by the amount of cells that are inactivated during this process(dx, mg/L). Chang et al. [33] conducted a study in which four methane-oxidizing cultures (a mixed methanotrophs culture grown under both chemostat and batch conditions, a pure culture of CAC1, and a pure culture of *M. trichosporium* OB3B grown under batch conditions) and 18 individual chlorinated and nonchlorinated aliphatics compounds were utilized to test the hypothesis that "the product toxicity of chlorinated aliphatic hydrocarbons to methane-oxidizing cells decreases in proportion to their chlorine content" [33].

In an effort to accurately reflect the product toxicity associated with cometabolic degradation reactions, formate (20 mM) was amended into the cultures to secure the availability of reducing energy without exerting competitive inhibition effects that would occur if methane is added during the reaction. Similar results observed with the four different cultures supported the hypothesis, indicating that within similar carbon structure groups (methane, ethanes, and ethenes), the highest T_c values were found in nonchlorinated compounds. However, 1,1-DCE appeared to have lower T_c values /higher toxicity than TCE, cDCE and tDCE. In addition, the results of degradation kinetics for TCE, cDCE, and VC showed no direct relationship between the degradation rate and chlorine content [33].

2.3 VC cometabolism by etheneotrophs

Numerous organisms are known to grow on ethene according to previous research, including several strains of *Mycobacterium* [19, 20]. To date, *Mycobacterium*, *Nocardioides*, and *Pseudomonas* strains are reported to grow on VC and assimilate ethene with the same enzymes [21]. In one study, an ethene enrichment culture was used to evaluate aerobic biodegradation of VC in the presence of ethene, ethane and VC. VC removal was most extensive in the bottles containing both ethene +VC, followed by ethane+ethene+VC, and only VC. Ethene biodegradation was fastest when it was the sole substrate, and the highest rate of ethane oxidation occurred in bottles that contained both ethene and ethane. The results indicated that ethene is essential for ethene-utilizing organisms to induce the alkene monooxygenase in order to cometabolize VC and catabolize ethane [22]. This work also confirmed that ethane culture was able to degrade both ethene and VC since alkane monooxygenase has a broader substrate range than AkMO. VC biodegradation by the ethene and ethane grown cultures were reported to be roughly equivalent. Interestingly, ethene was still consumed first by ethane-grown culture fed with ethane, ethene, and VC. This result is consistent with the fact that alkane monooxygenases have broader substrate range than AkMO reported in the literature [22].

The effect of varying ratios of VC to ethene on the rate of removal of each compound was investigation as follows: (1) VC only; (2) ethene only; (3) VC: ethene = 1:3; (4) VC: ethene = 1:1; (5) VC : ethene = 3:1. Initially, VC removal rates were uniformly high, and started to diverge as the experiment progressed. By day 64, the bottles that received equimolar amounts of VC and ethene displayed the highest VC removal rate. In contrast,the rate of VC degradation in the bottles that contained unequal amount of VC and ethene are significantly reduced; approximately 90% reduction occurred in the bottle that contained only VC. The results

suggested that at ethene to VC levels greater than a 1:1 molar ratio, VC degradation was competitively inhibited by primary substrate that have higher affinity to AkMO [22].

2.4 VC cometabolism in the presence of both microbial groups

The first microcosm studies comparing methane and ethene as primary substrates for cometabolism of VC by native methanotrophs and etheneotrophs were done by Freedman et al in 2001 [26]. The sediment and groundwater were taken from monitoring well #3037 at a Superfund site in California. The initial concentration of methane, ethene, and VC were equivalent to what was measured in the anaerobic portion of the groundwater that was closest to the interface with the aerobic zone, where ethane and methane were an order of magnitude lower than that of the VC: 200µg/l methane, 112µg/l ethene, and 2600 µg/l VC.

The whole period of microcosm operation lasted 150 days, during the first 76 days of microcosm operation a noticeably slower rate of VC consumption occurred when just CH_4 was present. Beyond day 75, VC biodegradation was fastest with just ethene present in the bottle. The results indicated that ethene may be a more effective primary substrate for alkene monooxygenase(s). The results of cumulative amounts of VC, ethene and methane consumed in the microcosm bottles indicated the presence of methane alone with ethene inhibited ethene biodegradation, thereby inhibiting VC consumption. The fact that VC alone did not inhibit ethene consumption, but did inhibit methane uptake suggests that methanotrophs are more susceptible to the presence of VC than are etheneotrophs. The results from these microcosms confirm that ethene and methane promoted cometabolism of VC even when VC aqueous phase concentration was an order of magnitude higher than that of the ethene and methane [26].

A series of microcosm studies evaluating ethene's role in VC cometabolism were done by Begley in 2011. The purpose of this study was to establish the feasibility of a full-scale

aerobic biostimulation treatment system at a demolition debris landfill in Carver, Massachusetts. By 2002, the site was assessed to have a detached VC plume 3000' long, 40' wide and 30' thick, located 50' below the water table with persistent low levels of VC (2 to 27 µg/L) extending 4600' downgradient from the landfill.

A mixed site groundwater sample was used to generate a series of microcosms to test for aerobic VC degrading activity. The groundwater contained 50 µM of methane and was amended with 1 ml of 100% O_2, MSM, 13µM ethene and 0.5 µM VC. VC degradation was complete in 21 days when groundwater was amended with minerals and 13µM ethene, compared to 9% VC degradation from unamended groundwater, and 25% VC degradation from the groundwater that was amended with mineral nitrogen, phosphorus, and trace elements after 21 days. In this ethene-supplemented microcosm study, VC degradation lagged in the first 12 days, then the concentration of VC started to decrease to nondetectable levels by day 21 after ethene consumption. The result indicated that VC- degrading activity could be simulated by the addition of ethene, and VC is consumed slower than ethene in this groundwater sample. However, rapid degradation of methane was observed in both unamended and amended groundwater samples, but the cometabolism of VC was limited. The results suggested that the addition of oxygen did not stimulate the native methanotrophic bacteria to degrade VC in the site groundwater [27].

Another microcosm experiment was done with the groundwater from well 63-I at the Carver site in MA. The concentrations of methane, ethene and VC contained in the groundwater are 195, 0.35 and <2 µg/L respectively. The relative abundance of etheneotroph and methanotroph were estimated by qPCR analysis of functional genes, showing that methanotophs and etheneotrophs are both present in the samples, and the methanotrophs are more than etheneotrophs by 2-3 orders of magnitude. The screening also showed that methanotrophs,

etheneotrophs and VC-assimilators were all present in the groundwater sample [28]. Relevant observations from this study are described below.

2.4.1 Behavior with VC and methane mixtures

The lag time for VC utilization decreased from 14 days to 10 days, shorter than that of VC as sole substrate (13days). This suggested that cometabolic oxidation of VC (0.47 µM) was carried out by methanotrophs rather than by VC-assimilators in the presence of 1µM methane.[28].

The lag time for initial methane utilization and the time for 50% methane degradation both increased when 0.47µM VC and 0.45µM of methane were in the aqueous phase of the sample, suggesting that methane utilization by methanotrophs was inhibited by similar aqueous concentration of VC. This phenomenon was overcome if increasing amount of methane (0.45 µM, 1.28 µM, 3.6 µM) was fed to the microcosms. However, VC degradation is enhanced in the presence of higher methane concentration, which supports the idea that VC is primarily oxidized cometabolically [28].

2.4.2 Behavior with VC and ethene mixtures

The initiation of VC consumption was about 16 days for microcosm experiments with VC (0.47 µM) alone and with both VC and ethene (3.0 µM). However, etheneotrophs started to oxidize intensively after day 32, resulting in 77% of VC degradation by day 43, which was the time required for VC-assimilator to oxidize just 50% of VC. Increasing concentration of ethene (1.3 µM, 3.8 µM,10.7 µM) did not affect the lag time for VC use significantly, but did increase the time for 50% VC degradation. These results suggest that there is competition between VC and ethene for the alkene monooxygenase binding site. However, the presence of ethene

enhanced VC cometabolic oxidation because in each test VC was degraded faster in the presence of ethene than when alone [28].

2.4.3 Behavior with methane and ethene mixtures without VC

The time required for methanotophs in the groundwater sample to degrade 50% of 1 µM methane was 20 days, after lagging for about 9 days. When 3 µM of ethene was fed into the bottle contained 1 µM of methane, both lagging period and times for 50% degradation increased to 12 days and 26 days respectively. On the contrary, the times for 50% of 3 µM of ethene decreased significantly from 39 days to 20 days when 1 µM of methane was fed into the groundwater samples. The results are expected because methanotrophs can cometabloize ethene using MMO, which impacts its ability to oxidize methane since methane and ethene are competing for the active site. Another reason for faster ethene loss could be due to the inducing of AkMO gene expression by epoxyethane generated by methanotrophs [28].

2.4.4 Behavior with mixtures of methane, ethene, and VC

In 2010 experiments, VC degradation took only 19 days to reach 50% degradation when tested with 1.3 µM methane and 3.8 µM ethene, it is faster than with only 1.3 µM methane (32 days) or with only 3.8 µM ethene (23 days). It took 44 days to obtain 50% VC degradation with VC alone. VC and ethene oxidation are shown to be a synchronized event, whilst great variability were shown in terms of times to reach 50% methane degradation. For example, it took more than 70 days for methnotrophs to oxidize 50% of methane in the microcosm containing only 0.5 µM methane and 3.8 µM ethene, which indicated that low concentration made methantophs susceptible to VC inhibition [28].

Similar time frames for VC degradation were shown in 2011 experiments, where VC degradation took only 20 days to reach 50% degradation when tested with 1 µM methane and 3

μM ethene, it is faster than with only 1.3 μM methane (31 days) or with only 3.8 μM ethene (38 days). It also confirmed that VC degradation did occur simultaneously with ethene use [28].

From these microcosm results, groundwater scenarios were created with relatively low concentrations of methane, ethene and VC. It is shown that methane utilization by methanotrophs was inhibited when similar or low concentrations of VC and methane were contained in the groundwater samples. In addition, the presence of ethene also impacted the methane utilization by MMO. Nevertheless, ethene degradation is enhanced by the presence of methane. VC degradation is observed to be proceeded via cometabolism by methanotrophs and ethenenotrophs, and it has the highest potential to be fully degraded when methane and ethene are both present [28]. This work concluded that VC cometabolic activity in groundwater system is shaped by the relative abundance of native methanotrophs and etheneotrophs as well as the aqueous concentrations of methane, ethene, and VC at the specific site. The purpose of my work was to further investigate how the known ratios of pure methanotrophs and etheneotrophs cultures behave on cometabolize VC using the gas ratio between methane, ethene, and VC observed at the Carver site.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals, media, bacterial strains, and growth conditions

Ethene (99%) and methane (99.995%) were from Airgas, and vinyl chloride (VC)(99.5%) was from Fluka. All other chemicals either were reagent or molecular biology grade. The recipe for preparing one liter of minimal salts medium (MSM) was listed as follows: 0.95 g KH_2PO_4, 2.27 g K2HPO_4, 0.67 g (NH4)SO_4 per liter of deionized water. One liter of MSM was autoclave sterilized at 121 degrees Celsius (°C) for 30 minutes.

One liter of trace metals solution (TMS) was prepared as follows: 60 g $MgSO_4 \cdot 7H_2O$, 6.37 g EDTA ($Na_2(H_2O)_2$),1 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 2.5 g $FeSO_4 \cdot 7H_2O$, 0.1 g $NaMoO_4 \cdot 2H_2O$, 0.1 g $CuSO_4 \cdot 6H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.52 g $MnSO_4 \cdot H_2O$ per liter of deionized water. TMS was filter sterilized by 0.22µm, GP Express membrane from Steritop, but not autoclaved and was stored at 4°C in a foil wrapped container to prevent photodegradation.

The recipe for preparing one liter of $1/10^{th}$ strength trypticase soy agar plus 1% glucose (TSAG) plates was listed as follows: 3g Tryptic Soy Broth (TSB), 15g Bacto agar, 10g glucose(1%) per liter of deionized water. One liter of TSAG broth was autoclave sterilized at 121 degrees Celsius (°C) for 30 minutes, after cooling at room temperature for about 1 hour, the broth was poured in 2 sleeves of petri dish in the laminar flow hood and stored at 4 °C.

In this work, pure culture mixtures of ethene-oxidizing *Mycobacterium* strain JS622[5] and methane-oxidizing Alphaproteobacterium, *Methylocystis sp.* strain Rockwell (ATCC 49242) [30]were used to investigate the degradation activity of ethene, methane, and vinyl chloride mixtures in serum bottles. *Mycobacterium* strain JS622 stock (1ml) was thawed and added to a modified 2-liter Erlenmeyer flasks containing 500 mL of MSM, 1 ml of filter-sterilized trace

metal solution (TMS), and 100 ml of ethene filter-sterilized by 0.22 μm PVDF membrane from MILLEX. *Methylocystis sp.* strain Rockwell (ATCC 49242) stock (1ml) was thawed and added to a modified 2-liter Erlenmeyer flasks containing 500 ml of MSM, 1 ml of filter-sterilized TMS, and 120 ml of filter-sterilized methane. Both cultures were incubated aerobically on a circular shaker at 200 revolutions per minute (rpm) at room temperature (RT, ~22°C).

It took about 5 days for JS622 to reach mid-exponential phase (OD_{600}= 0.3-0.35), and about 3 days for ATCC49242 [32]. When both cultures both reach their mid-exponential phase at the same time, they were pelleted separately via centrifugation for 5 min at 6438 x*g*. The cultures were resuspended into 10 mL MSM (OD_{600} ~16.5-17.5). These dense cultures were used to prepare mixed liquid cultures at an initial OD600 of 0.1 for experiments, as described in Section 3.2.

Periodically, frozen stocks were made of each bacterial strain for cell line maintenance. After the removal of supernatant, the culture were resuspended in 10 ml of MSM respectively, and distributed per ml in 1.5 ml cryovials and stored at -80°C.

When growing cultures, culture purity was tested by streaking the cultures on agar plates. For etheneotrophs, a streak plate of JS622 onto $1/10^{th}$ strength TSAG plates reveal only 3 mm diameter-white colonies after incubated at 30°C within 2 weeks. For methanotrophs, a streak plate of ATCC49242 onto $1/10^{th}$ strength TSAG plates reveal no colonies after incubated at 30°C within 2 weeks. Methane-assimilating bacteria can not metabolize C_6 compounds like glucose as carbon and energy source, therefore the absence of colonies on TSAG plates indirectly indicates the purity of the culture.

3.2 Experimental design and preparation

In each experiment seven 160-ml serum bottles were prepared, to which 72 ml of liquid culture (initial OD_{600} = 0.1) was added, leaving with 88 ml of headspace. The bottles were then capped with slotted Wheaton gray butyl rubber septa and aluminum crimp caps. Autoclaved controls were also prepared in parallel to test for abiotic losses. For any particular experiment involving seven bottles, a total of 504 ml of culture volume with an initial OD600 of 0.1 was required. To achieve the initial OD600 in any particular bottle required dilution of dense cultures described previously (Table 1). Five different methanotroph and etheneotroph cell concentration ratios were also investigated: (1) 100% JS622; (2)100% ATCC49242;(3) JS622: ATCC 49242=1:1;(4) JS622: ATCC49242=3:1; (5) JS622:ATCC49242=1:3.

Table 1 Dilution approach used to prepare experimental bottles.

The volume constitution for each culture ratio (ml)							
JS622	504	378	252	126	50.4	25.2	0
ATCC49242	0	126	252	378	453.6	478.8	504
Culture ratios	100%	75%	50%	25%	10%	5%	0%

Note: Prior to each experiment 504 ml each of JS622 and ATCC49242 was prepared at initial OD600 = 0.1, then different volumes were mixed as shown below to provide different percentages of each microbial group as desired.

Once capped and crimped, gases were added to the bottles as follows: (1) methane alone; (2) ethene alone; (3) methane+VC; (4) ethene +VC ; (5) VC alone; (6) methane+ethene; (7) methane+ethene+VC; (8) culture medium control containing methane+ethene+VC. The amount of gas added to each bottle for each set of experiments is described in Table 2. The control bottle

that contained 72ml of MSM and methen+ethene+VC was also prepared in each experiment. (Appendix Figure C1)

Table 2. The gases ratios and culture ratios used in each experiment.

	Culture ratios tested (expressed in terms of JS622 percentage)						
MTH:ETH:VC (µmol)	100%JS622	75%JS622	50%JS622	25%JS622	10%JS622	5%JS622	0%JS622
40:40:40	X		X				X
400:400:20	X	X	X	X			X
400:40:20	X	X	X	X	X	X	X

3.3 Analytical methods

To test the optical density of the liquid cultures, the modified 2-liter Erlenmeyer flask containing the growing culture was inverted couple of times, then 1ml of the liquid sample was taken by 1-ml plastic syringe with a needle (22G 1.5), and added into a disposable cuvette. UV absorption spectra of the liquid culture were performed on a Cary 50 Bio UV-Visible spectrophotometer at 600nm.

The bottles were incubated on a circular shaker at 200 revolutions per minute (rpm) at room temperature throughout the observation period. A 0.25-ml Pressure-Lok gas-tight syringe with a side-ported needle was used to take 0.1 ml headspace samples during each measurement. Three measurements were taken in the first day of incubation, during which the gases were consumed rapidly by the bacteria. Two measurements were taken on the next two days, and one measurement was taken each day approximately after day 4. Gases were analyzed on a Hewlett-Packard 5890 series II gas chromatograph using a stainless steel column (8.0 ft x 1/8 in. x

2.1mm) packed with 1% SP-1000 phase on 60/80 Carbopack B.A flame ionization detector was used witha nitrogen carrier gas flow rate of 30 ml/min and an oven temperature of 90 °C. Each compound forms a peak at its specific retention time (minutes): methane at 1.4; ethene at 1.8; VC at 4.4. Peak areas for each compound determined by GC analysis were calibrated to external standards to give the total mass in the bottle. Standard curves for methane and ethene ranged from 0 µmol to 800 µmol, and ranging from 0 µmol to 50 µmol for VC (Appendix Figure A1, Figure A2, Figure A3).

CHAPTER 4

RESULTS AND DISCUSSION

Under aerobic conditions, both AkMO-expressing *Mycobacterium* strain JS622 and pMMO- expressing *Methylocystis sp.* ATCC 49242 are able to cometabolize VC separately. Different concentrations of methane, ethene and VC were provided in order to test how the metabolism and cometabolism of the two microorganisms would be affected. The behaviors of VC degradation affected by different ratios of the two microbial groups were also examined in our work.

4.1 Biodegradation of equal masses of methane, ethene and VC by JS622

In initial experiments we evaluated the cometabolic activity of ethene-oxidizing *Mycobacterium* strain JS622 in the presence of equal masses of methane, ethene and VC (40 µmol each; Table 2). However, due to the diverse values of dimensionless Henry's Law constants: methane (24.4), ethene (7.15) and VC (1.0) [26], the aqueous concentrations of VC were 5-fold higher than ethene and more than 10-fold higher than methane approximately. The theoretical aqueous concentrations of methane, ethene, and VC were: 18µM, 57µM, and 250 µM. However, the actual amount of gases that were added into the bottles were smaller than the theoretical values. This could be due to the residual air left in the pipeline which took away part of the volume that was taken. This technical error was more severe when the taken gas volume was as small as 1 ml. Due to the high Henry's Law constant of VC, the corresponding VC concentrations were about half-fold smaller than the theoretical values. However, the resulting measured aqueous concentrations between each gas are still meaningful in terms of investigating VC cometabolic rate in the presence of different concentrations of the primary substrates.

We did not expect that ethene-grown *Mycobacterium* JS622 would be able to degrade methane as the AkMO is not known to accept C_1 compounds as substrates. As expected, methane was not degraded in the presence of 10 µM methane (Figure 2a). JS622 rapidly oxidized initial 39 µM of ethene at the rate of 31 µmol/day (Fig. 2b), and 6 µM of VC was degraded at the initial rate of 6 µmol/day (Fig. 2c). Studies have shown that AkMO catalyzes the initial steps of the aerobic microbial VC and ethene biodegradation pathways [5, 6, 10]. The similar chemical structure between ethene and VC apparently allows AkMO to catalyze VC oxidation, even though the organisms are not dependent on VC as a carbon and energy source [21]. In the bottle that contained equal masses of methane and VC, 182 µM VC was degraded at the initial rate of 16 µmol/day, which was faster than VC was being degraded alone, while 13 µM of methane remained intact (Fig. 3d). In the bottle that received equal masses of ethene and VC, 217 µM VC was degraded at the initial rate of 16µmol/day, which was faster when being degraded alone. On the other hand, 40 µM of ethene was degraded at the rate of 23 µmol/day. Ethene was degraded faster than VC even when VC concentration was about 5-fold higher, suggesting that the active site of the JS622AkMO preferentially accepts ethene over VC, but the presence of VC did appear to be slightly competed for the active site of AkMO(Figure 3e). When methane and ethene were both present in the bottle containing JS622, 37 µM ethene was degraded at the rate of 30 µmol/day, and methane was not degraded, which validated the fact that AkMO does not bind to methane given that the initial degradation rate of ethene remained the same. (Figure 3f). This is consistent with degradation behavior seen in other bottles. When all three substrates were present, VC and ethene oxidization rate and degradation pattern by JS622 were similar to that in the presence of ethene, 11 µmol/day for VC (176 µM) degradation, and 22µmol/day for ethene(35 µM) degradation (Figure 3g). Overall, this experiment demonstrates that

Mycobacterium strain JS622 was able to cometabolize VC concentrations that were about 5-fold higher than ethene at similar rate of 13 µmol/day when present with ethene. Furthermore, ethene degradation rate only slightly decreased from about 30 to 20 µmol/day in the presence with VC, and the presence of methane does not inhibit ethene degradation (Table B1).

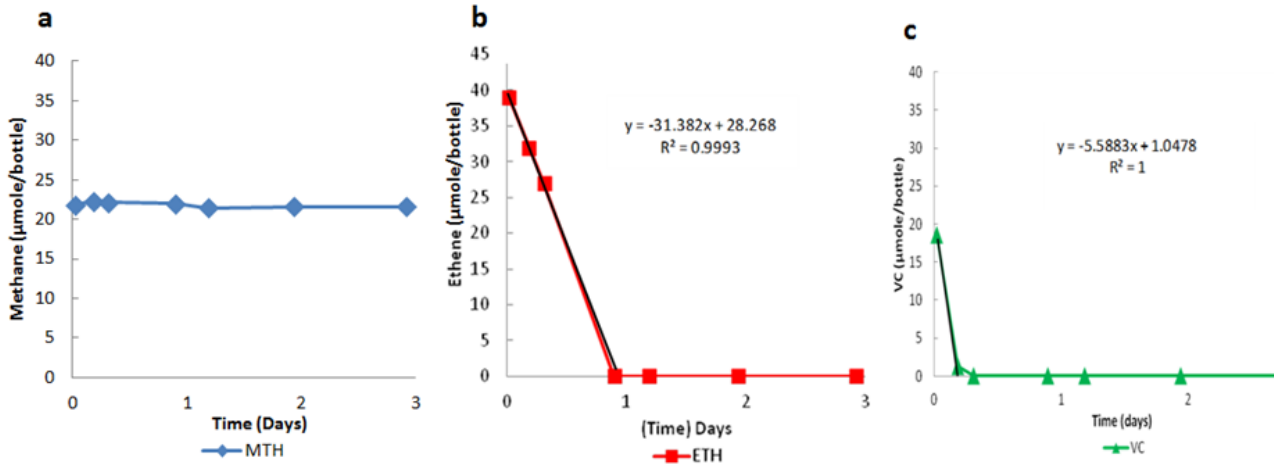

Figure 2 Methane as a single substrate for ethene-grown JS622 cells (a), and degradation of ethene (b)and VC (c)as single substrate by ethene-grown JS622 cells in separate bottles.

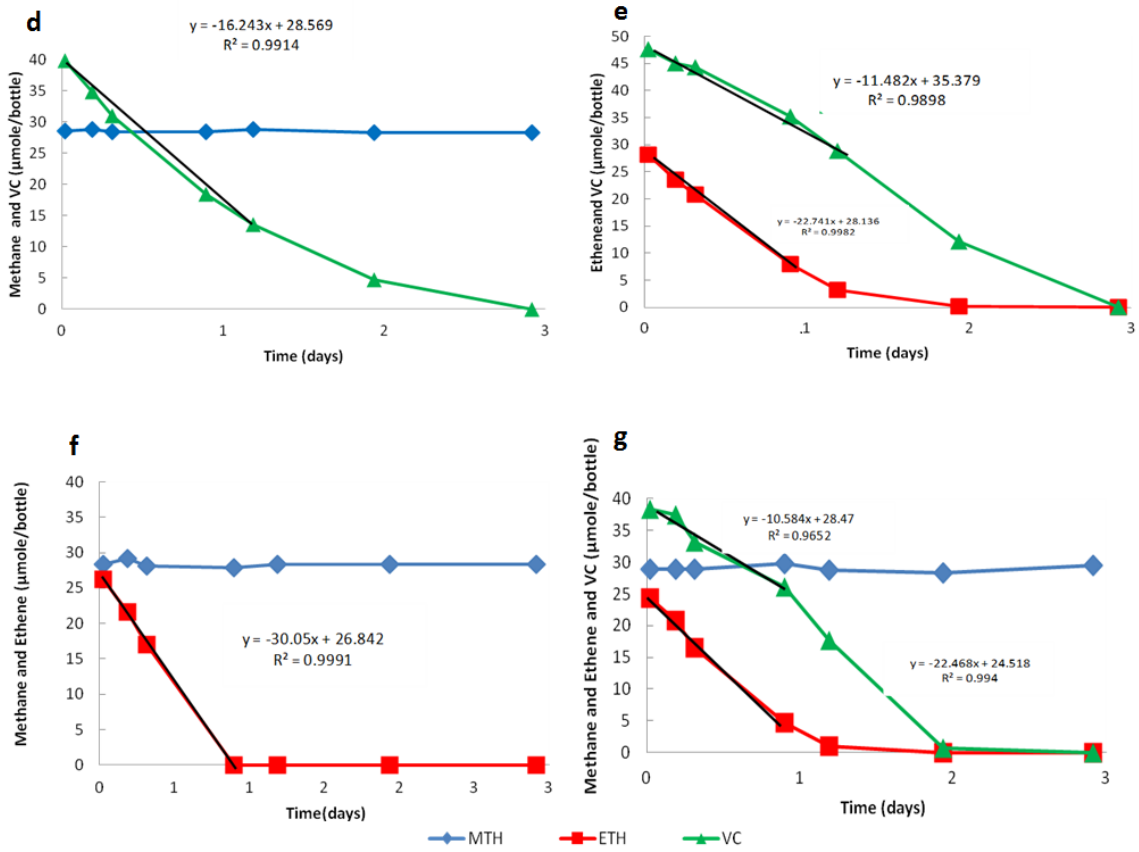

Figure 3 Aerobic biodegradation of methane+VC (d), ethene+VC (e), methane+ethene (f), methane+ethene+VC (g) by ethene-grown JS622.

4.2 Biodegradation of equal mass of methane, ethene and VC by ATCC 49242

The same experimental design was used to evaluate the cometabolic activity of pMMO-expressing *Methylocystis sp.* ATCC 49242 in the presence of equal initial masses of methane (40µmol), ethene (40µmol) and VC (40µmol). The calculated theoretical initial aqueous concentrations of methane, ethene, and VC were: 18µM/L, 57µM, and 250 µM.

Methylocystis sp. degraded 11µM of methane at the rate of 75µmol/day (Figure 4a). Methane grown *Methylocystis sp.* produces particulate methane monooxygenase (pMMO) that can bind and fortuitously oxidize ethene and chloroethenes [14, 15]. However, 39µM of ethene was degraded at the rate of 22 µmol/day, suggesting that methane starvation reduced the pool of active enzyme available for the degradation of ethene (Figure 4b). Therefore, when the concentration of ethene was about 3-fold higher than methane, the degradation rate decreased a little more than 3 fold. Gradual degradation of 10 µM of VC (3µmol/day) in the absence of methane shows that *Methylocystis sp.* can also cometabolize VC (Figure 4c). In the bottle that contained 12 µM of methane and 101 µM of VC, 38% of methane was degraded at the rate of 9µmol/day, and 63% of VC was degraded at the rate of 13µmol/day respectively by *Methylocystis sp.*, suggesting that VC inhibits methane degradation greatly. This result is consistent with the microcosm experiments fed with only methane and VC, where methane utilization by methanotrophs was inhibited by aqueous concentration of VC that were more than 10-fold higher than methane (Figure 5d)[17]. In the bottle fed with the 41µM of ethene and 156 µM of VC, *Methylocystis sp.* can only degrade 12% of ethene at the rate of 16 µmol/day and 23% of VC at the rate of 3 µmol/day because no necessary cofactors are generated during the transformation of the cosubstrate (Figure 5e). When 14 µM of methane and 38 µM of ethene

were present in the bottle, ethene was degraded at the rate of 30 µmol/day and methane was 14µmol/day by *Methylocystis sp,* 5-fold slower than methane was being oxidized alone. This result suggests that the presence of ethene not only slowed the rate of methane consumption by its obligate degrader, but also competed for the active site (Figure 5f). In the bottle that contained 12µM of methane, 24 µM of ethene, and 39 µM of VC, 72% of ethene , 41% of VC, and 23% of methane was degraded by *Methylocystis sp.* at the rate of 17 µmol/day, 2 µmol/day and 6 µmol/day respectively (Figure 5g). This result suggested the activity of MMO was strongly inhibited in the presence of concentration of VC that was 10-fold higher, resulting the incomplete degradation of the gases. Interestingly, when the concentrations of ethene were around 24 µM-40 µM, ethene degradation rate were observed to be staying consistent around 20-30 µmol/day.

Overall, these results suggest that the ability of methane-grown *Methylocystis sp.* to cometabolize ethene and VC mixtures is not as efficient in comparison to the ethene-grown *Mycobacterium* JS622 culture. The observation that methane degradation is inhibited by ethene and VC suggests that ethene and VC can compete for the active site of the *Methylocystis sp.* pMMO. Furthermore, it is possible that pMMO may be damaged by the expected generation of epoxyethane and chlorooxirane - epoxide products of pMMO that are known to be toxic to methanotrophs [12] (Table B1).

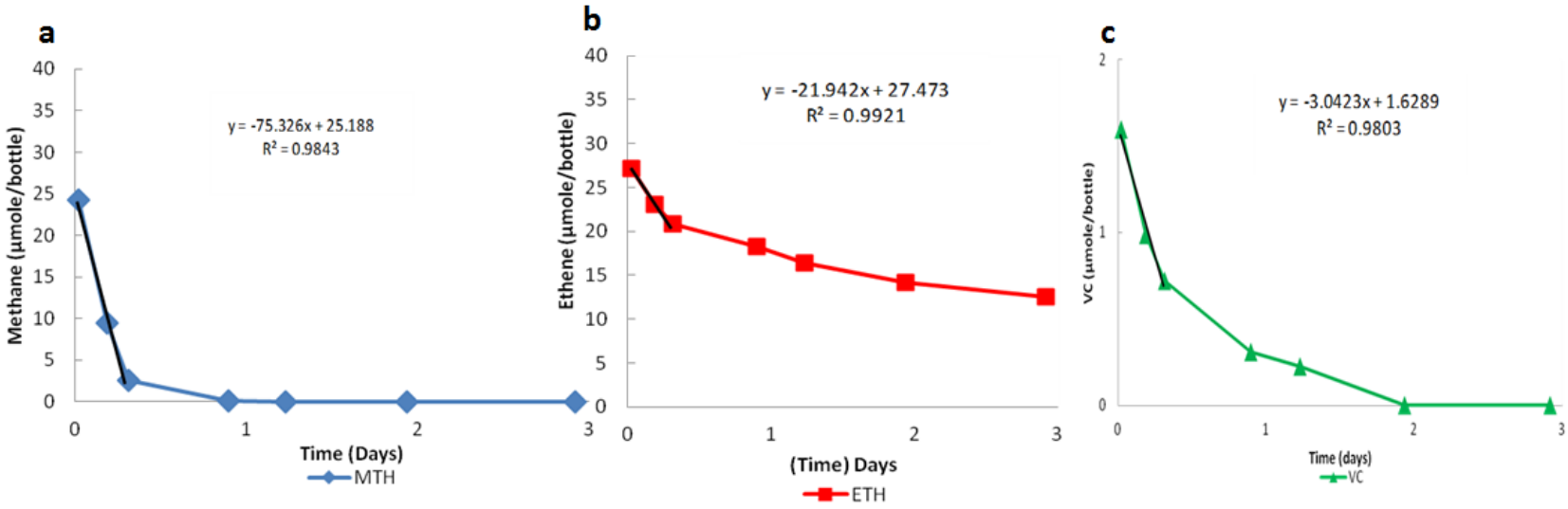

Figure 4 Degradation of methane (a), ethene (b), VC (c)as single substrate for methane-grown *Methylocystis* sp. ATCC49242 in separate bottles.

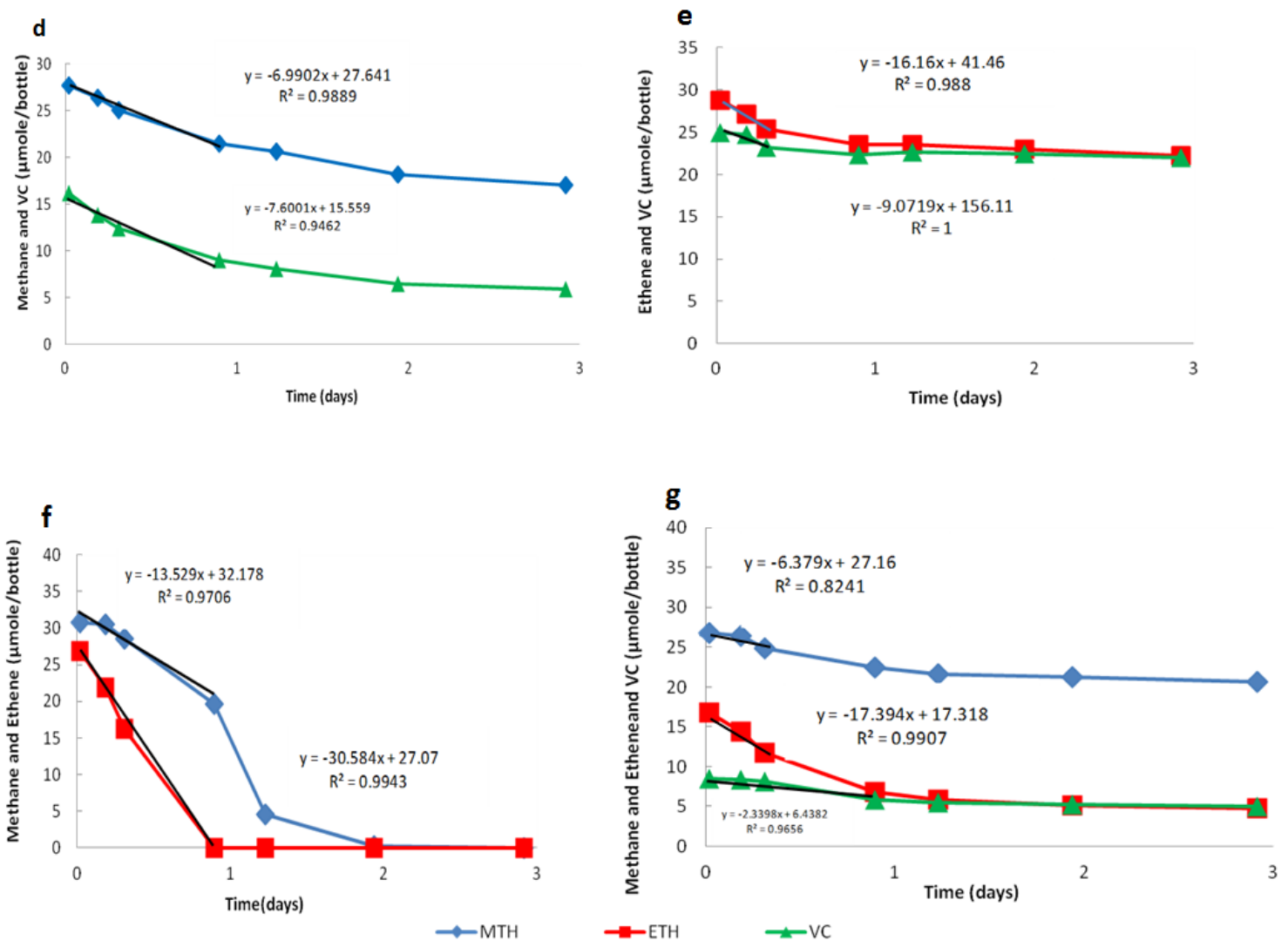

Figure 5Aerobic biodegradation of methane+VC(d), ethene+VC (e), methane+ethene (f), methane+ethene+VC (g) by ATCC49242.

4.3 Biodegradation of equal mass of methane, ethene and VC by mixed cultures of JS622 and ATCC 49242

Having investigated the cometabolic behavior of ethene-oxidizing *Mycobacterium* strain JS622 and methane-oxidizing *Methylocystis sp.* ATCC 49242 in pure cultures, we investigated the effect that the presence of both microbial groups in a single culture will affect VC cometabolism. As an initial basis of comparison, we used equal masses of methane (40 µmol), ethene (40 µmol) and VC (40 µmol). This corresponded to aqueous concentrations of methane (18 µM), ethene (57 µM), and VC (250 µM). The ratio of JS622 to *Methlocystis sp.* was 1:1.

In this mixture, we know that *Methylocystis sp.* will be solely responsible for the degradation of methane as a single substrate . As expected, 12 µM of methane was rapidly oxidized at the rate of 69 µmol/day, which was similar to the rate observed with *Methylocystis sp.* alone (11 µM at 75 µmol/day) (Figure 6a). In a separate bottle, 40 µM of ethene was degraded at the rate of 30 µmol/day because both organisms can degrade ethene (Figure 6b). The rate was the same when 40 µM of ethene was degraded by JS622 alone, suggesting that methanotrophs did not play significantly role in cometabolizing sole ethene. VC (43 µM) degradation occurred at the rate of 6 µmol/day, which was the same rate when degraded by pure JS622 culture, but 3 µmol/day faster than being degraded by pure ATCC49242 culture (Figure 6c). In the bottle that contained 14 µM of methane and 106 µM of VC, VC was completely degraded at the rate of 12 µmol/day and 42% of methane was degraded at the rate of 11 µmol/day. This result indicated that methane degradation was inhibited in the presence of concentration of VC that was approximately 10-fold higher than methane, similar to what was observed when being degraded by pure *Methylocystis sp.* VC cometabolism in the presence of methane was slower when compared to degradation by JS622 alone (182 µM of VC, 12

µmol/day), but improved in the presence of methane by ATCC49242 alone since only 63% of VC was degraded at the rate of 9µmol/day (Figure 7d).

In the bottle that received 40 µM of ethene and 74 µM of VC, ethene was degraded at the rate of 23 µmol/day, and VC was 11µmol/day. With the same initial concentration of ethene (40 µM), ethene was degraded slower (by 2 µmol/day) in the mixed culture compared to consumption by JS622 alone, and 7µmol/day faster when being consumed by ATCC49242 alone at its initial degradation rate (Figure 7e). In comparison to VC cometabolism in the presence of ethene by JS622, 74 µM of VC was degraded slower by 7 µmol/day (VC= 217 µM), only 12% of VC was being degraded in three days by pure culture of ATCC49242.

When 14 µM of methane and 40 µM of ethene were both present, ethene was degraded at the rate of 28 µmol/ day, and methane was degraded at the rate of 13 µmol/day. At the same concentration, the rate of ethene oxidation (30 µmol/day) and methane oxidation (13 µmol/day) weres almost the same comparing to that of being degraded by 100% *Methylocystis sp*, and by 100% JS622 (31 µmol ethene/day).This result suggested that both microbial group metabolize their respective primary substrates without interfering each other (Figure 7f). In the bottle that contained 42 µM of ethene, 147 µM of VC, and 10µM of methane, 38% of ethene, 15% of VC, and 7% of methane was degraded by both bacteria at the initial degradation rate of 15 µmol/day, 2 µmol/day and 3 µmol/day respectively (Figure 7g). The results almost resembled the behavior of pure ATCC49242 culture fed with the three gases, suggesting that ATCC49242 did play a role in ethene and VC cometabolism. Comparing to the three gases being degraded by pure JS622 cultures, the ethene degradation rate was 6µmol/day slower and VC degradation rate was 9 µmol/day slower. These contrasts suggest that the activity of pMMO was inhibited in the

presence of a VC concentration that was 10-fold higher, which also affects its ability to cometabolize ethene and VC (Figure 3g) (Table B1).

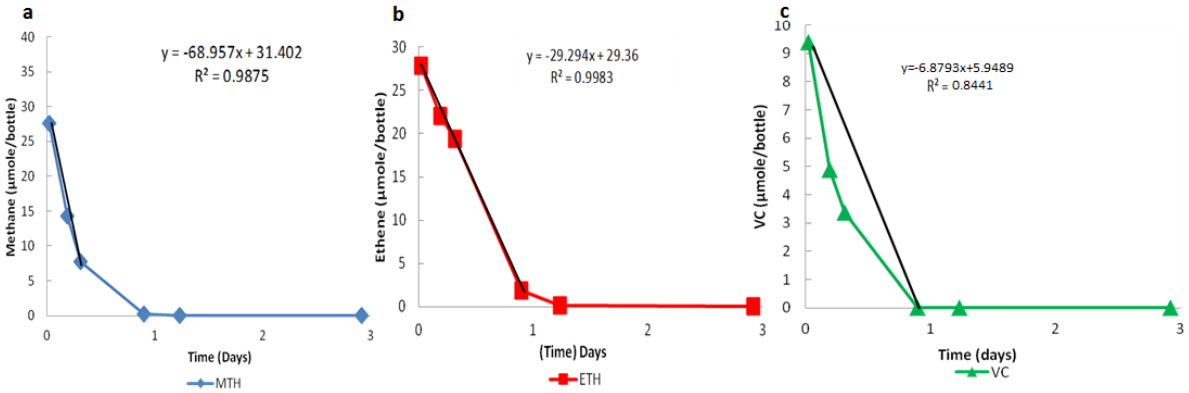

Figure 6 Use of methane (a), ethene (b), VC (c) as a sole substrate for JS622 and ATCC49242 mixture. The slopes represent the degradation rates.

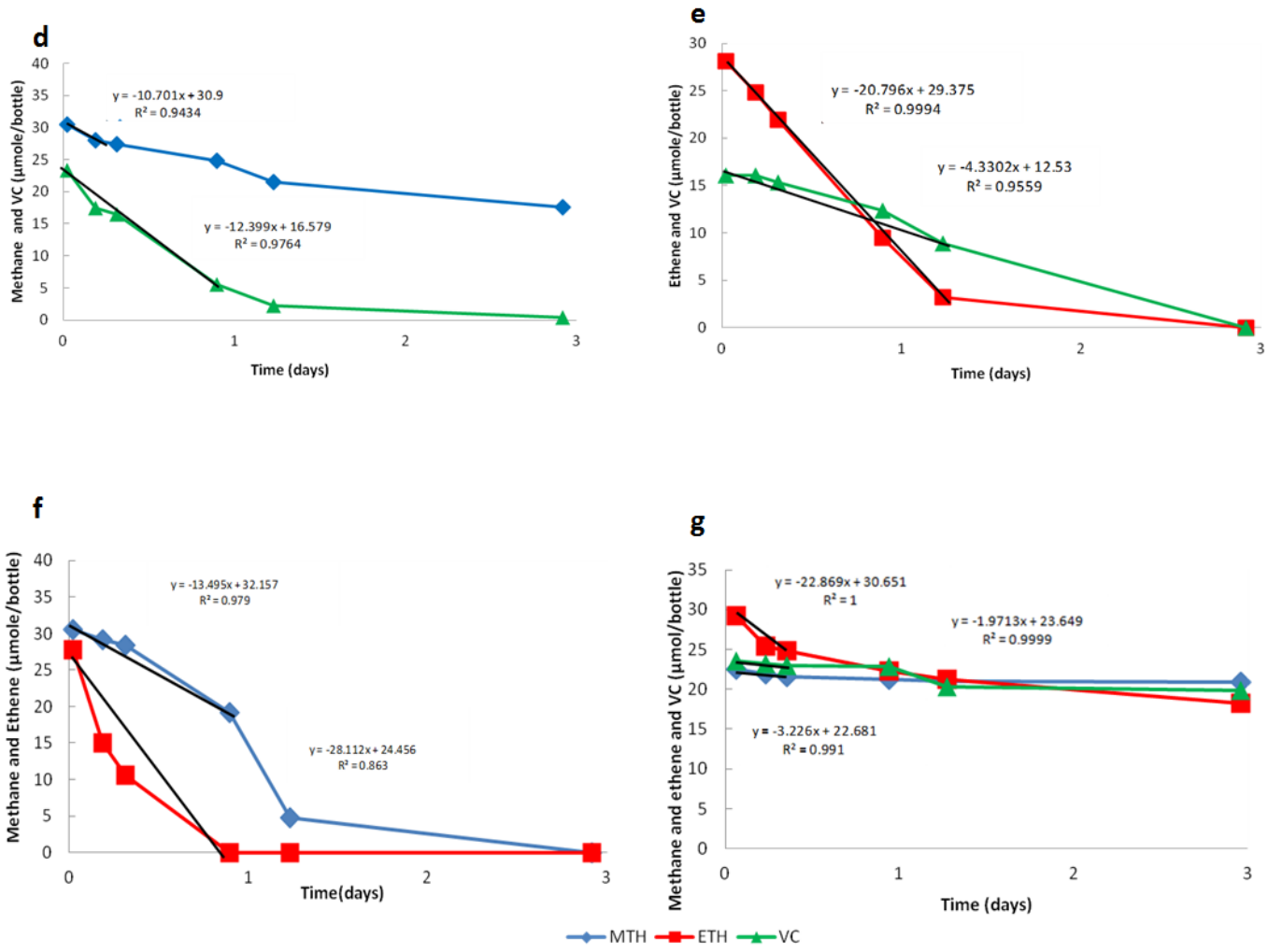

Figure 7 Aerobic biodegradation of methane+VC (d), ethene +VC (e), methane+ethene (f), methane+ethene+VC (g) by JS622 and ATCC49242 mixture.

4.4 Biodegradation of 400 µmol of methane, 400 µmol of ethene and 20 µmol of VC by pure culture of JS622

In March 2010 and March 2011, Bioremediation Consulting, Inc. did microcosmstudies from well 63-I groundwater taken from a VC-contaminated site in Carver, MA to determine how the native mixed population of methanotrophs, etheneotrophs and VC-assimilating bacteria biodegrade VC under varying concentrations of methane alone and ethene alone, and mixtures of these substrates [27]. In 2011 experiments, the microcosms were fed with methane, ethene, and VC in the following concentration: 1.6, 1.6 and 0.08 µmol/bottle, which corresponded to 1µM, 3 µM, and 0.47 µM respectively. The methane and ethene masses tested here were 20 times greater than VC. Therefore, we utilized the same mass ratio of methane, ethene to VC to investigate the behavior of pure culture of methantrophs and etheneotrophs degrading the three gases. In this experiment the theoretical initial aqueous concentrations of methane, ethene, and VC were: 180µM, 570µM, and 125 µM. The reason why we conducted this experiments with the 20-fold higher concentration than Bioremediation Consulting, Inc. was because we were also using much higher cell concentrations in order to obetain the data within reasonable time period.

We did not expect that Ethene-grown *Mycobacterium* JS622 would be able to degrade methane as the AkMO is not known to accept C_1 compounds as substrates. As expected, methane was not degraded in the presence of 170 µM methane (Figure 2a). JS622 rapidly oxidized 476µM ethene at the rate of 197 µmol/day. However, JS622 degraded ethene slowly within the first two days (24 µmol/day) and increased after the second day, resulting a distinct degradation curve compared to that of 40 µmol ethene (Figure 8b). In the bottle that contained 21 µM of VC, it was degraded at the rate of 13 µmol/day because AkMO was already induced (Figure 8c), which was the average degradation rate of VC when pure JS622 culture was fed with 170 µM of

VC (Figure 2c). In the bottle that contained 176 µM of methane and 37 µM of VC, VC was not inhibited by methane and degraded at the rate of 11µmol/day (Figure 9a). In the bottle that received 491 µM of ethene and 49 µM of VC, VC was initially degraded at the rate of 0.5 µmol/day, then increased to the rate of 9 µmol/day, similarly, ethene was initially degraded at the rate of 32 µmol/day at the rate of 204 µmol/day. The highly resembled degradation curves suggested that ethene and VC utilization was a simultaneous event (Figure 9b). When methane and ethene were both present in the bottle containing pure JS622, 507 µM ethene was degraded slow initially at 32 µmol/day, then increased to the rate of 179 µmol/day, The fact that methane was not degraded is consistent with degradation behavior seen in other bottles that ethene metabolism by JS622 was not inhibited by methane(Figure 10a). When all three substrates were present, the VC and ethene oxidation rate and degradation pattern by JS622 were similar to that in the presence of ethene, 54 µM of VC was degraded at the maximum rate of 10µmol/day, and 512 µM of ethene was degraded at the maximum rate of 209 µmol/day (Figure 10b). The fact that ethene and VC degradation rate were very similar in the presence and in the absence of methane, suggesting that methane, no matter at high or low concentrations, did not affect the oxidation of VC and ethene by JS622. Moreover, the fact that the maximum VC cometabolic rate was constantly at the average rate of 10 µmol/day in the presence of ethene or methane no matter VC concentration was 5-fold higher or 10-fold lower, suggested that there is a limit acceptance of VC concentration by JS622 at this cell concentration(Table B1).

4.5 Biodegradation of 400 µmol of methane, 400 µmol of ethene and 20µmol of VC by pure culture of ATCC 49242

In this experiment the same initial aqueous concentrations of methane (180µM), ethene (570µM), and VC (125 µM) was repeated using pure culture of *Methylocystis sp.*.We were interested in investigating the behavior of ATCC49242 under the situation that ethene aqueous concentration was 3-fold higher, but VC aqueous was 3-fold lower.

Methylocystis sp. degraded 151 µM of methane rapidly at the rate of 176 µmol/day (Figure 8m). The cometabolic activity of *Methylocystis sp.* of ethene was not very well; the rate of oxidizing 469 µM of ethene was 28 µmol/day, and only 14% was degraded in 4 days (Figure 8n). This result had shown that pMMO activity was inhibited by the presence of highconcentration of ethene. In the bottle contained 28 µM of VC, VC was cometabolized gradually at the rate of 3 µmol/day because pMMO was induced (Figure 8o). When compared to the VC degradation rate by pure JS622, which was 13 µmol/day for 21µM of VC, the results suggest that AkMO cometabolized VC at a rate greater than that of pMMO. In the bottle that contained 170 µM of methane and 57 µM of VC, methane was metabolized at the rate of 193 µmol/day, and VC was degraded at a rate 6 µmol/day. The fact that the VC cometabolic rate was the almost the same in comparison to its degradation rate alone, and methane metabolic rate was slightly decreased in the presence of VC, suggesting that VC could effectively compete for active binding site of pMMO even when the concentration was about 3-fold lower than methane (Figure 9i). In the bottle contained that 489 µM of ethene and 44µM of VC, at these concentrations both gases are subject to competitive inhibition to pMMO of *Methylocystis sp.*, therefore under starvation and toxic state, it was reasonable that ATCC49242 was only able to cometabolize 14% of ethene at 31 µmol/day and 11% of VC at merely 0.5 µmol/day within 4

days (Figure 9j). In the bottle that contained 167 µM of methane and 494 µM of ethene, it is observed that ethene inhibited the pMMO activity of *Methylocystis sp.* at this relatively high concentration, resulting 26% of ethene degradation and 18% of methane oxidation within 4 days. Interestingly, a slight "hump" was observed on the degradation curve on the second day (Figure 10i). In the bottle that contained all three gases, the initial aqueous concentrations of methane, ethene, and VC were 173 µM, 516 µM, and 66 µM respectively. The result had shown inhibition of the pMMO activity, resulting 20% ethene degradation, 14% methane, and 11% of VC within 4 days (Figure 10j) (Table B1).

4.6 Biodegradation of 400 µmol of methane, 400 µmol of ethene and 20 µmol of VC by different ethenenotroph/methanotroph ratios

In an attempt to evaluate the significance of the two microbial groups on biodegrading methane alone and ethene alone, and mixtures of these substrates at the initial aqueous concentrations of methane (180µM), ethene (570µM), and VC (125 µM), different JS622/ATCC49242 ratios were tested to investigate how the various combined metabolic activities of the two organisms affect the VC biodegradation process.

In the bottles that contained approximately 170 µM of methane, methane degradation rate increased from 98 to 176 µmol/day as the ratios of ATCC49242 increased from 25% to 100% (Figure 8a, 8d, 8g, 8j, 8m). Bottles that received approximately 470 µM of ethene, the average maximum metabolic rate decreased steadily as JS622 ratios deacreased from 100% to 0% as follows: 197 µmol/day , 118 µmol/day, 113 µmol/day , 27 µmol/day. However, the initial ethene degradation rates did not show a direct correlation with the decrease of cell concentrations. Ethene degradation rate was only 27% when being oxidized by pure ATCC49242 culture (0% JS622) due to pMMO inhibition activity (Figure 8b, 8e, 8h, 8k, 8n).

The average initial aqueous concentration of VC was about 30 µM, but the cometabolic rates of VC did not show an obvious trend as the ratio of JS622 decreased. Pure JS622 degraded VC the fastest at 13 µmol/day, followed by 75% JS622 and 75%ATCC49242 degraded VC at about 11 µmol/day, and 50% JS622 and 0%JS622 could only degraded VC at 3 µmol/day (Figure 8c, 8f, 8i, 8l, 8o). In the bottles that contained about 170 µM of methane and 45 µM of VC, the rate of methane degradation increased from 86 µmol/day to about 200 µmol/day as the ratio of ATCC49242 culture increased to 75%. Pure ATCC49242 degraded methane in the presence of VC a bit slower at 193 µmol/day, which could be due the inhibitory effect from more accumulation of VC since no JS622 were present in the bottle to help degradation. On the other hand, VC degradation rates showed no obvious decreasing trend, but forming the same rank of degradation rates between each cell ratio, where the high average degradation rate (about 11.5 µmol/day) was by pureJS622, 75%JS622 and 75%ATCC49242, finally the slowest VC cometabolic rate was reached by 50%JS622 (6.8 µmol/day) and 0%JS622 (6.4 µmol/day) (Figure 9a, 9c, 9e, 9g, 9i). The results observed from bottles that contained VC alone and methane+VC suggested when the cell concentrations were equal, AkMO and p MMO activities were reduced instead of adding up.

In separate bottles that contained about 500 µM of ethene and approximately 40 µM of VC, ethene degradation deacreased steadly from 205 µmol/day to 31 µmol/day as JS622 raio decreased from 100% JS622 to 0% JS622. However, the highest VC degradation was from pure JS622 (9 µmol/day), the rest of the culture ratios degraded VC at average of 3 µmol/day (Figure 9b, 9d, 9f, 9h, 9i). The fact that VC degradation rates were slower at the presence of high concentration of ethene compared to VC being degraded without primary substrates suggested high concentration of ethene would compete for the binding site of AkMO. Futhermore, VC

degradation rates were smaller when degraded in the presence of ethene than of methane, proved that p MMO did participating in ethene and VC cometabolism, but was inhibited by ethene.

When ethene concentrations was about 500µM and methane was about 170 µM, pMMO was highly inhibited, resulting that methane degradation rate slightly increased from 23 µmol/day to 35 µmol/day as ATCC49242 ratio increased from 25% to 75% . On the other hand, the rate of ethene degradation decreased from 179 µmol/day to50 µmol/day as the ratio of JS622 culture decreased to 0% (Figure. 10a, 10c, 10e, 10g, 10i). At aqueous concentrations of methane, ethene, and VC were about 170µM, 500 µM, and 50µM respectively. As expected, ethene degradation rate decreased from 209 µmol/day to 52 µmol/day, and methane still showed no obvious trend of degradation as ATCC49242 ratio increased (degraded at the rate~18 µmol/day). However, the special rank of VC degradation rates observed from the previous bottles did not repeat in the bottles that contained all the gases, yet the VC cometabolic rate resumed to be led by the decrease of JS622 ratios in the bottles, 100% JS622 had the highest VC degradation rate (10 µmol/day), followed by 9 µmol/day by 75%JS622, then 4 µmol/day by 50% JS622, and only 2 µmol/day by 25% JS622 and 0% JS622 (Figure 10b, 10d, 10f, 10h, 10i).

In summary, no major sign of competitive inhibition to methane degradation by VC at concentration that was about 3-fold lower than methane. The results essentially resembled the combined behaviors of methane and VC being degraded alone. Evidence of strong inhibition to methanotrophs by high concentration of ethene was once again proved when VC cometabolic rates were rather smaller when being degraded together with ethene than with methane. From the steadily decresed ethene degradation rate and ethneotrophs ratios, it is safe to say that ethene was mainly metabolized by JS622. However, the special rank of VC cometabolic rates observed in the bottles contained VC, methane+VC suggested that when JS622 and ATCC49242 were at the

same ratio, ethenenotrophs and methanotrophs were not able to cometabolize as efficient as one of the organism was greater in concentration than the other (Table B1).

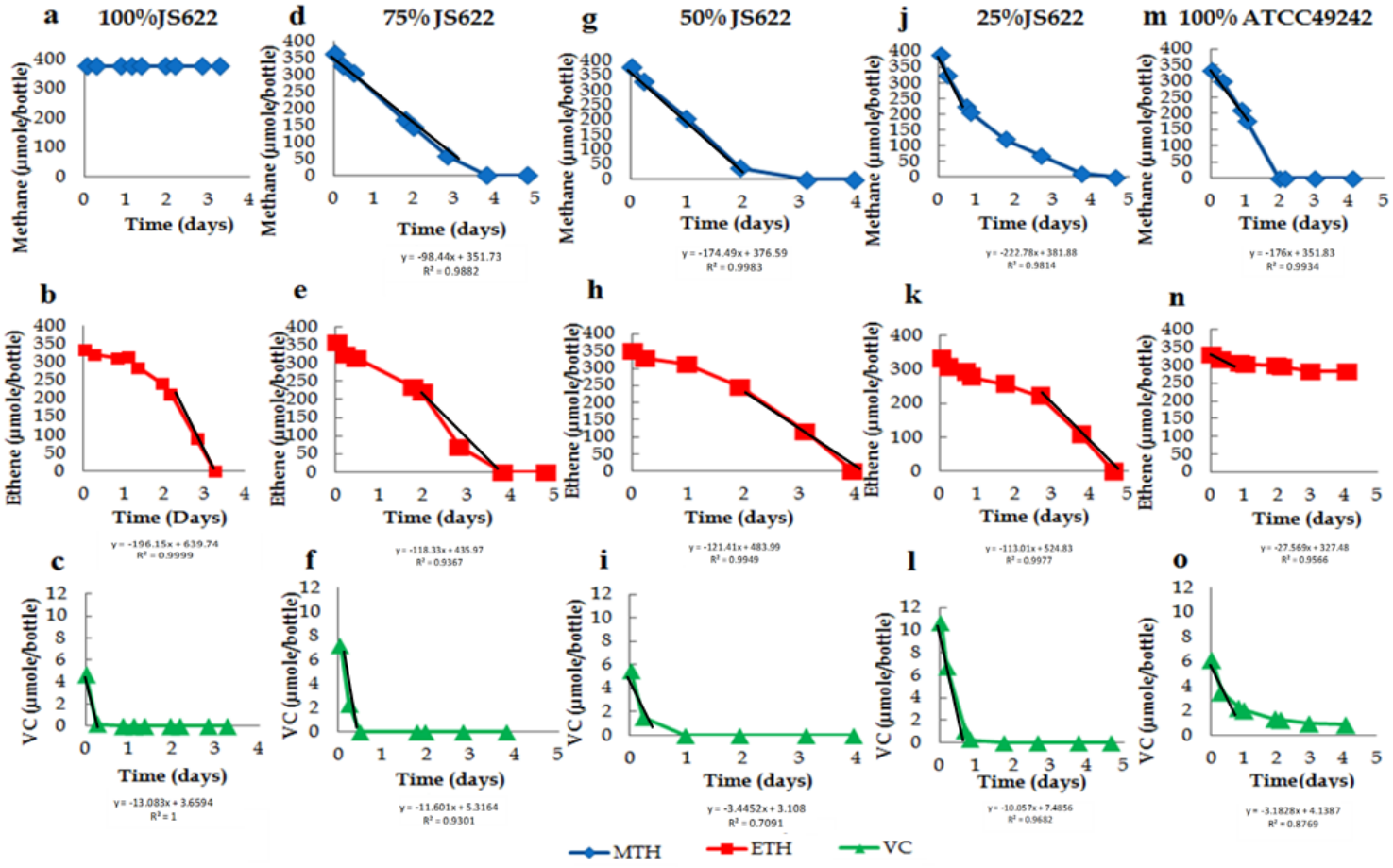

Figure 8 The degradation patterns of methane, ethene, VC as single substrate by different JS622/ATCC49242 ratios.

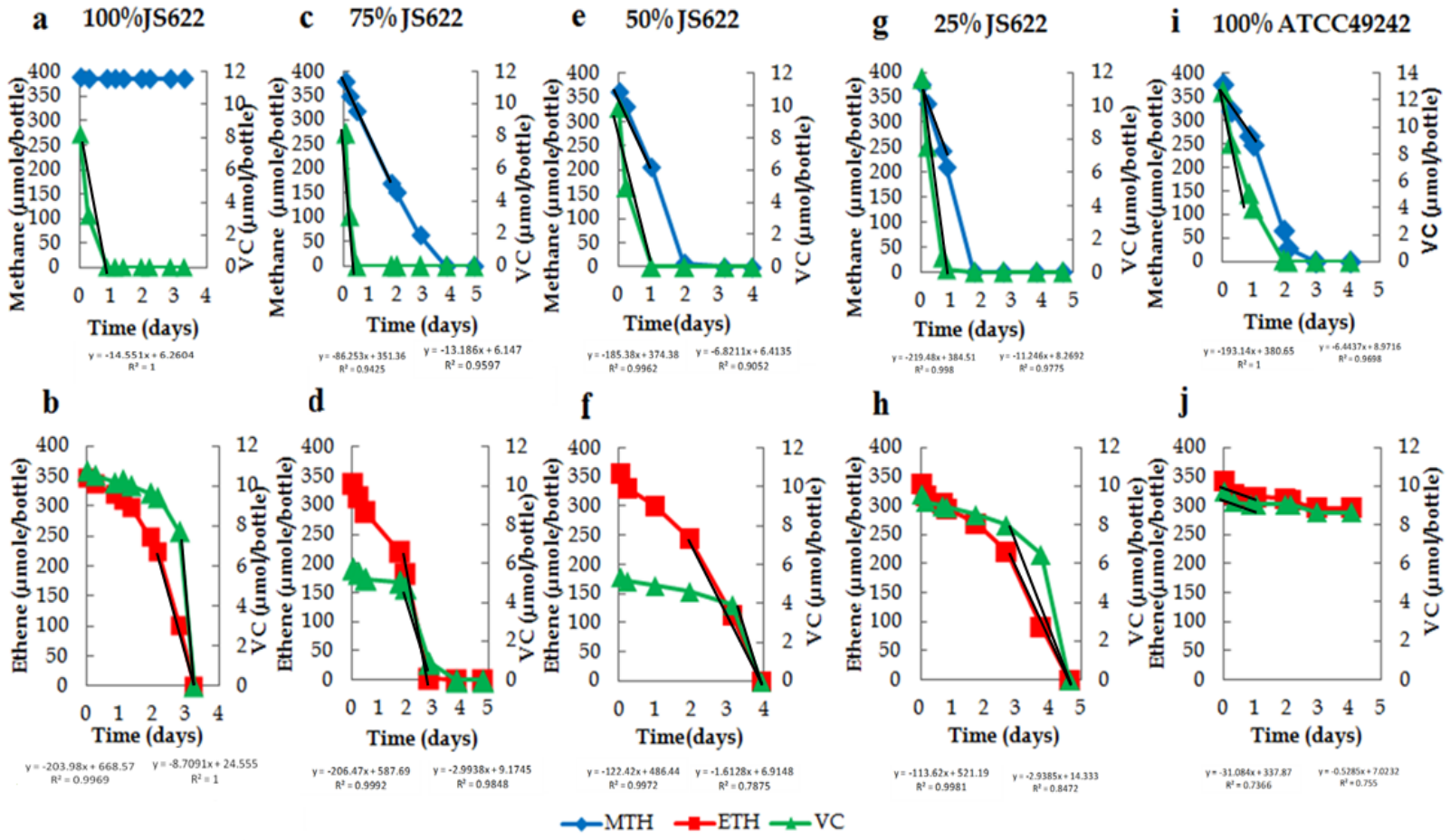

Figure 9 The degradation patterns of methane+VC and ethene+VC by different JS622/ATCC49242 ratios.

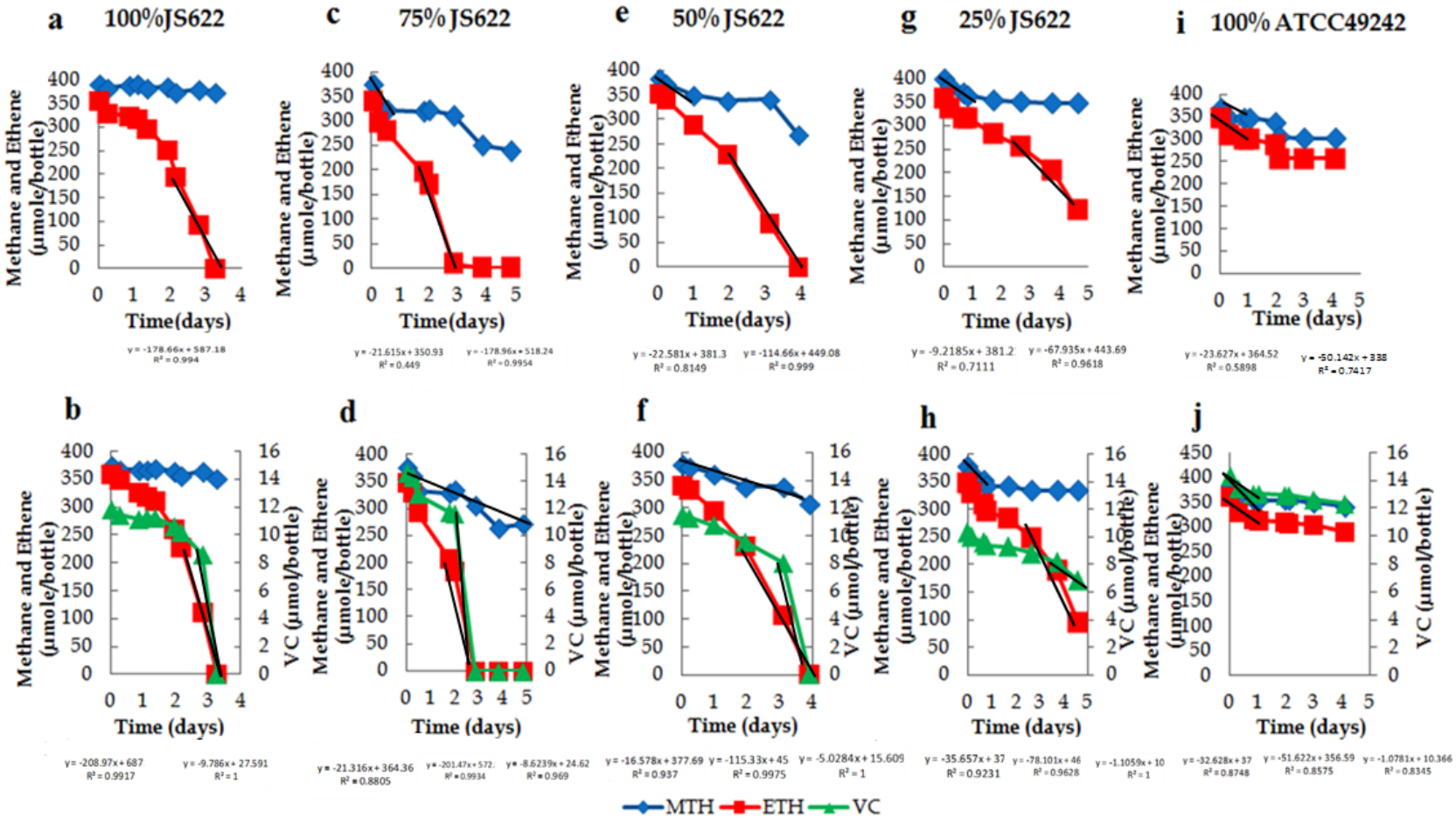

Figure 10 The degradation patterns of methane+ ethene, and methane+ ethene+ VC by different JS622/ATCC49242 ratios.

4.7 Biodegradation of 400 µmol of methane, 40 µmol of ethene, and 20 µmol of VC by different ethenenotroph/methanotroph ratios

The last set of experiments showed that high concentration of ethene inhibits MMO activity. Thus, in an attempt to learn the role of ethene played in VC cometabolism, same experiments were repeated using a methane: ethene: VC = 10:1:0.5 gas volume ratio, which corresponds to aqueous concentrations of methane (180 µM), ethene (57µM), and VC (125 µM) respectively. Furthermore, last results have shown that JS622 played a significant role in VC cometabolism. Therefore, two additional smaller ratios of JS622 were tested, giving the seven different concentration ratios of methanotophs and etheneotrophs were prepared as followed: (1) 100% JS622 (2) 75% JS622 (3) 50% JS622 (4) 25% JS622 (5) 10% JS622 (6) 5%JS622 (7)100% ATCC49242.

In the bottles that contained approximately 170 µM of methane, it was expected ATCC49242 was responsible for metabolizing methane, the initial rate of methane degradation increased gradually as follows: 34, 80, 128, 200, 274 µmol/day as the ratio of ATCC49242 culture increased from 25% to 95%. However, it was observed that 100% ATCC49242 degraded methane at a slower rate, 141 µmol/day. An exception to this was observed in experiments with 50% and 25% methanotroph ratios, where ATCC49242 seemed to rapidly oxidize methane around day 2 at the average rate of 500 µmol/day (Fig. 11a,11d, 11g, 11j, 11m), forming similar curves like ethene degradation curves from the last experiments (Fig 8b), while other bottles generated smooth degradation curves. In the bottles that received approximately 30 µM of ethene, small decreases of the ethene degradation rate ranging from 24 µmol/day to 14µmol/day occurred as the ratio of JS622 decreased to 5%. The fact that only 61% of ethene was cometabolized at the rate of 9 µmol/day suggested that pMMO was either failed to work due to

lack of energy source, or the accumulative epoxyethane inhibited pMMO activity (Fig. 11b, 11e, 11h, 11k, 11n, 11q, 11t). Cultures that had been fed with approximately 45μM of VC showed a similar phenomenon observed in the experiment discussed in the last section, which the lowest VC degradation rate was from the bottles that contained 50%JS622/50%ATCC49242, and 100%ATCC49242. The rest of the culture ratios had their VC utilizing rate about 1-4 μmol/day higher (Fig. 11c, 11f, 11i, 11l, 11o, 11r, 11u).

In the bottles that contained about 170 μM of methane and about 50 μM of VC, the rate of methane degradation did not steadily increase as the ratio of ATCC49242 culture increased from 25% to 95%. Like in the bottle contained just methane, 100% ATCC49242 was observed to be degrading methane at a smaller rate, 117 μmol/day. The resulted suggested when VC concentration were about 3-fold lower than methane, small competitive inhibition by VC were observed (Figure 12a-12g). On the other hand, the same lowest VC degradation rate (8 μmol/day) were observed from 50%JS622/50%ATCC49242, and 25% JS622. Higher VC cometabolic rates(2-6 μmol/day higher) were observed in the bottles that contained 75%JS622, 90% and 95% ATCC49242.

In the bottles that contained approximately 35 μM of ethene and 60 μM of VC, VC concentrations were about 2-fold higher than ethene. The rate of ethene degradation decreased from 18 μmol/day to 8 μmol/day as the ratio of JS622 decreased from 75% to 0%. The reason why 100%JS622 degraded at a slower rate (12 μmol/day) was due to the technical error that caused the loss of the expected volume while adding ethene to that bottle. On the other hand, VC degradation rates decreased slightly from 4 μmol/day to 2 μmol/day as the ratio of JS622 decreased from 75% to 0% (Figure 13a-13g).

In comparison to the experiments where the aqueous actual concentrations of ethene and VC were approximately 500 µM and 50µM, we noticed that the average VC degradation rate for about 50µM of VC was 3µmol/day in the presence of ethene (both 500 µM and 30 µM) for the culture ratios of 75%, 50%, and 25% of JS622 (Figure 9d, 9f, 9h). The results suggested that higher concentration of ethene does not necessarily enhance VC cometabolism. Therefore, it was observed that VC cometabolism was more efficient in the presence of ethene concentrations that were 2-fold lower than VC.

In the bottles contained approximately 170 µM of methane and 30 µMof ethene, methane degradation rates increased from 30, 52, 58, 128 to 195 µmol/day as the ATCC49242 ratio increased from 25% to 95%. However, the fact that all the values were about half-fold smaller in comparison to methane being degraded alone, and that only 22% of methane and 70% of ethene were degraded within a week by 100% ATCCC49242, suggesting ethene concentrations that are 3-fold smaller than methane still was even more inhibitory to p MMO activity than VC. By comparing the degradation patterns of methane and ethene in the culture ratios of 50%, 25% and 5% JS622, it was noticed that methane started to decrease rapidly after ethene was completely utilized. The results were the visualization of ethene inhibition to p MMO (Figure 14a-14g).

Similarly, the bottles received about 170 µM of methane, 30 µM of ethene, and 60 µM of VC, the degradation rates and patterns were the combined results of methane alone and of ethene+VC were fed in the bottles. The fact that in a week only 20% of methane, 70% of ethene, and 40% of VC were degraded by pure ATCC49242, suggesting that pMMO was strongly inhibited by the combined toxic intermediates of epoxyethane and chloroxirane. In addition, the rate of ethene degradation did not decrease as the ratio of JS622 decreased, 40 µM of ethene was degraded at the average rate of 16 µmol/day,VC degradation rates appeared to very low at the

average of 5 µmol/day throughout all the culture ratio bottles. No obvious trend and rank were observed from the bottles that contained low concentrations of ethene and VC (Figure 15a-15g) (Table B1).

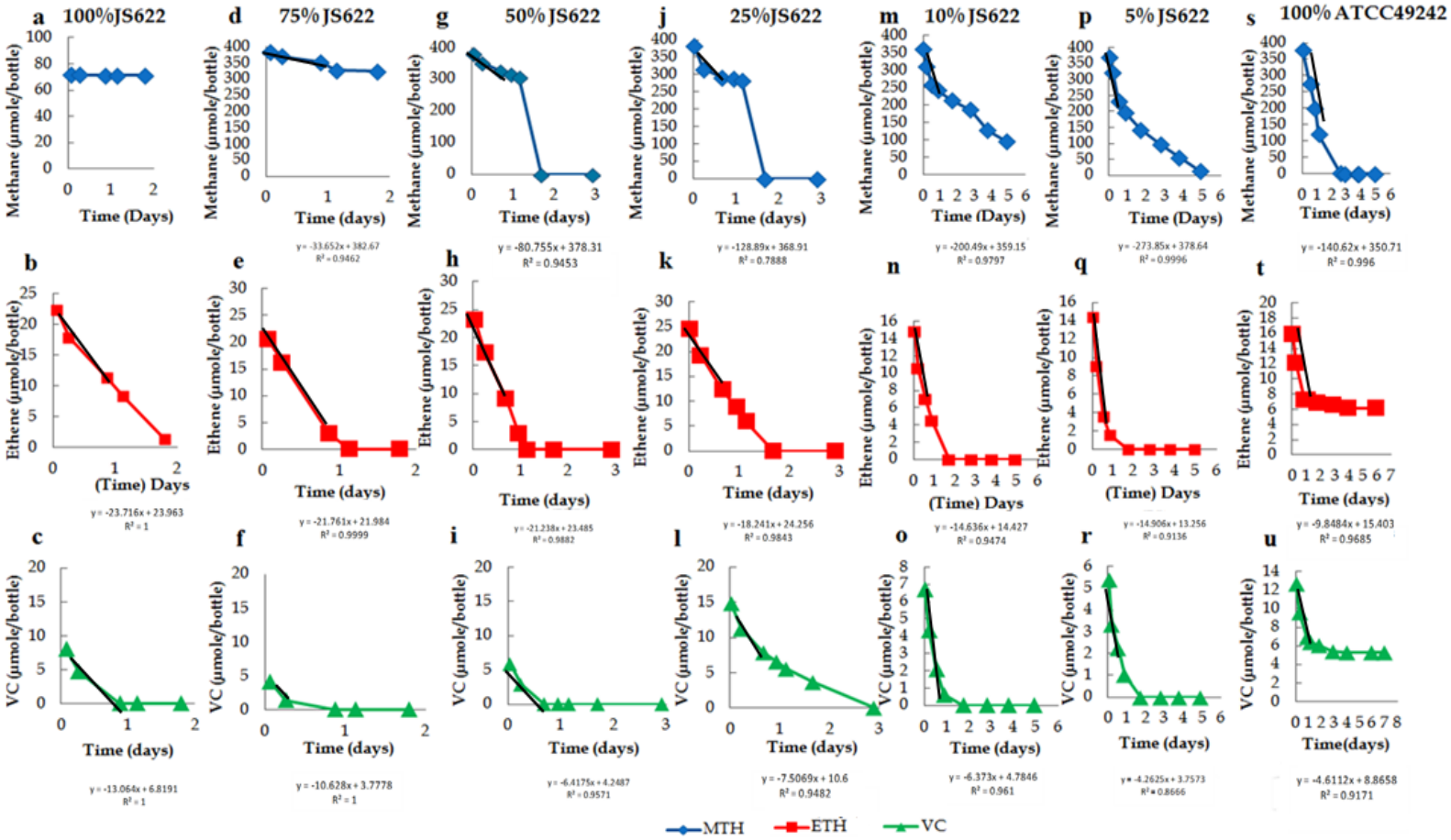

Figure 11 The degradation patterns of methane, ethene, VC as single substrate by different JS622/ATCC49242 ratios.

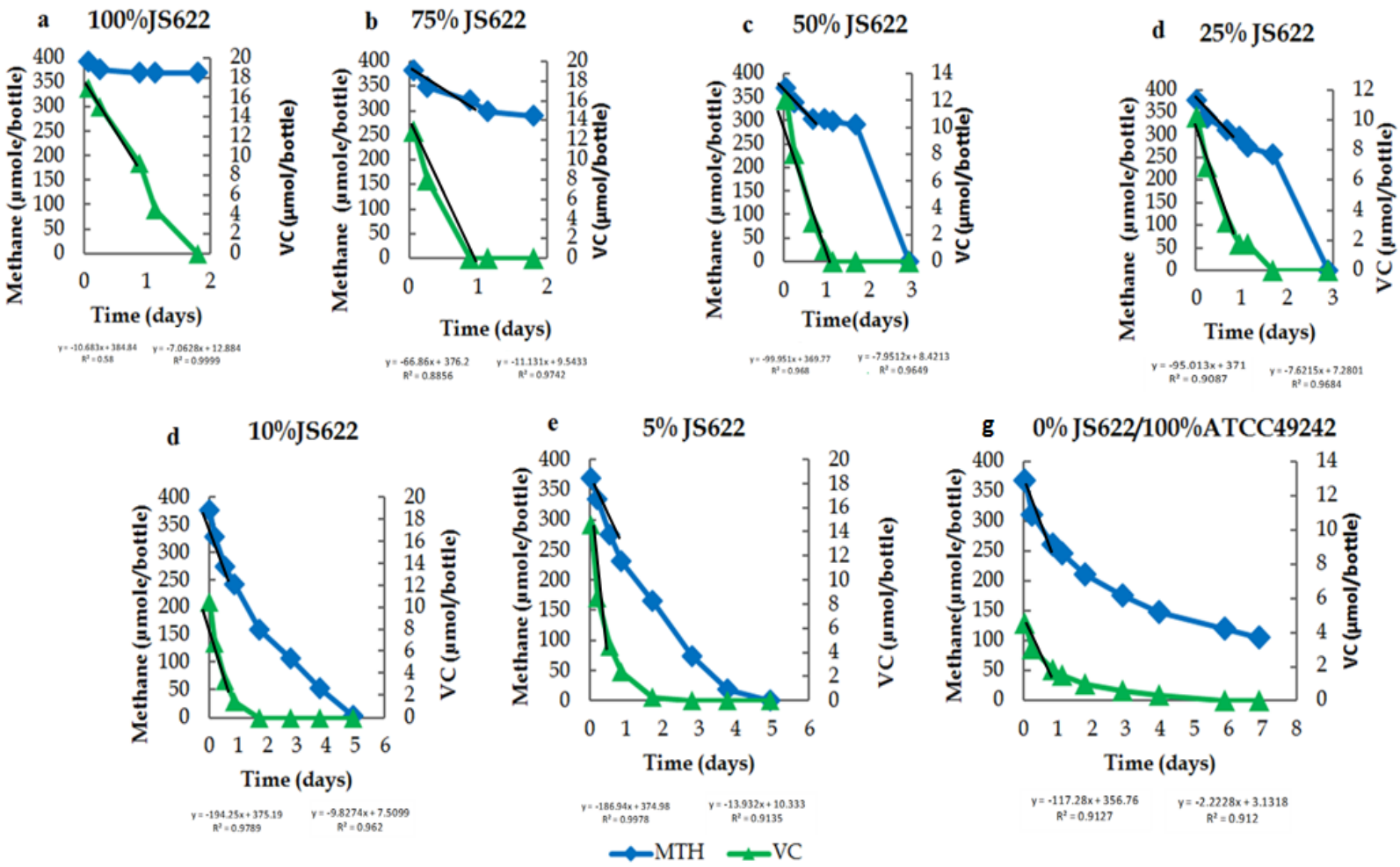

Figure 12 The degradation patterns of methane+VC by different JS622/ATCC49242 ratios in the gas ratio of 20:1.

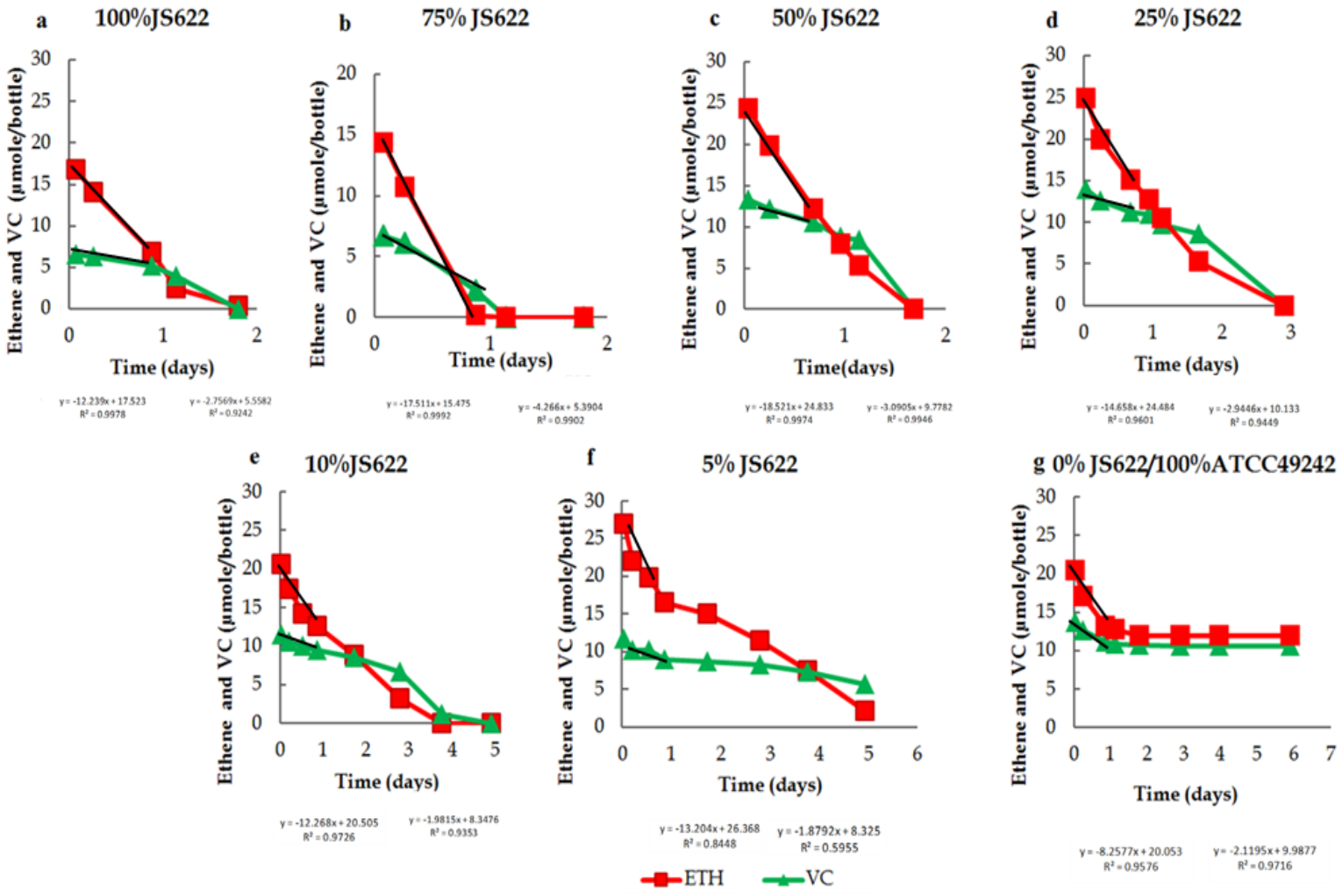

Figure 13 The degradation patterns of ethene+VC by different JS622/ATCC49242 ratios in the gas ratio of 2:1.

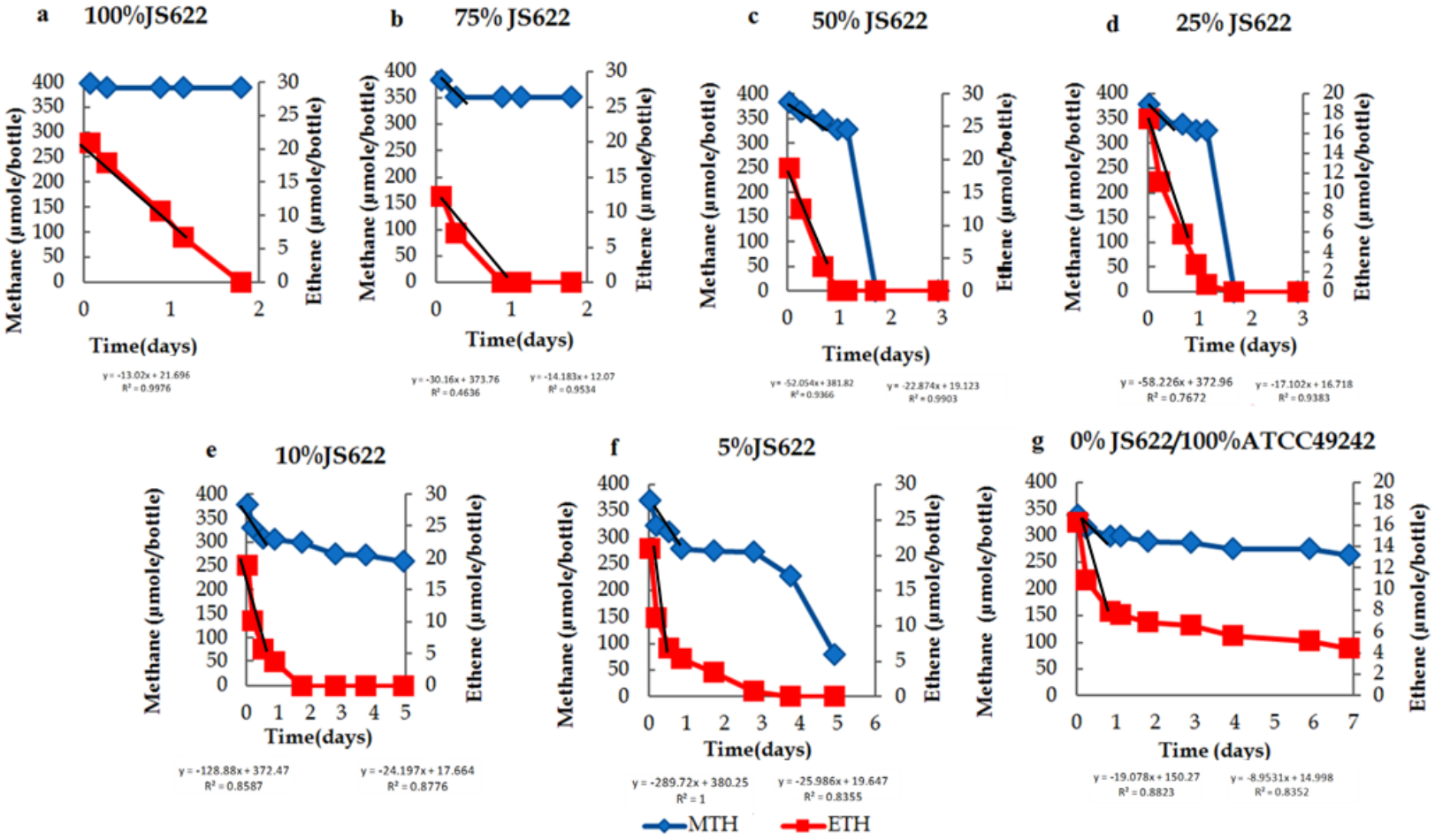

Figure 14 The degradation patterns of methane+ethene by different JS622/ATCC49242 ratios in the gas ratio of 10:1.

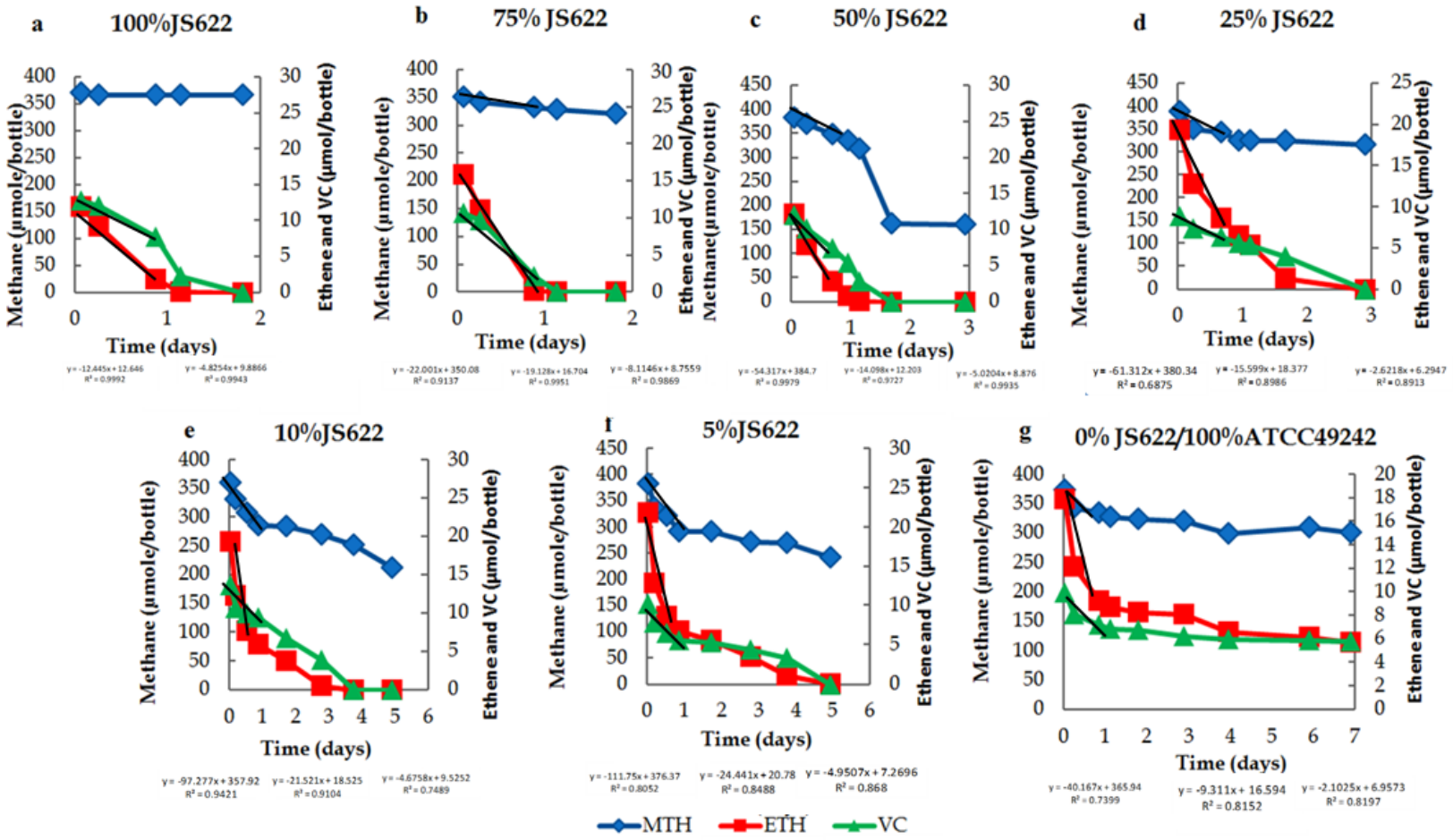

Figure 15 The degradation patterns of methane+VC and ethene+VC by different JS622/ATCC49242 ratios in the gas ratio of 20:2:1.

CHAPTER 5

SUMMARY AND CONCLUSIONS

From this work it was learned that the relative aqueous concentration of between each gas is the essential element that determines the behavior of microorganisms. These experiments validated the fact that methane degradation by *Methylocystis sp.* was readily inhibited in the present of higher(~170μM) and lower aqueous concentrations of VC and ethene (~50 μM). But, the presence of methane would not affect AkMO activity. As the result etheneotrophs do have higher advantage over methanotrophs for aerobic VC bioremediation since these organisms can tolerate the toxic effects generated via biodegradation pathway. In addition, VC degradation did occur simultaneously with ethene uptake.

In the two set of experiments with high concentration of methane (170 μM), it was observed that VC cometabolic activity was greatly reduced (as great as 10 μmol/day difference) when the ratio of JS622 to ATCC49242 was 1:1 specifically in the bottles that contained VC alone and methane+VC. The result suggested that if there was enough growth substrates present to sustain the cell concentration ratio of 1:1, i.e. the inhibitory effect to methanotrophs were to be limited, then the cometabolic activity by AkMO and MMO would both be reduced.

Overall, we learned that when the bottles contained methane (170 μM), ethene (500 μM), and VC (50 μM), the highest rate of VC degradation was about 12 μmol/day. When bacterial were fed with 17 μM of methane, 40 μM of ethene and 170 μM of VC, the highest rate of VC degradation was about 13μmol/day. Finally, when there were 170 μM of methane, 30 μM of ethene and 60 μM of VC dissolved into the liquid cultures, the highest VC degradation rate was still about 12 μmol/day. These results suggested that VC degradation capacity by AkMO is similar in these three different gas concentration ratios.

CHAPTER 6

ENGINEERING SIGNIFICANCE ANE FUTURE RESEARCH

The purpose of this study was to further investigate how known ratios of pure methanotrophs and etheneotrophs cultures behave when cometabolizing VC using the gas volume ratio of methane: ethene: VC= 20:20:1 observed in a groundwater system at the Carver site. The behaviors of pure JS622, pure ATCC49242, and JS622:ATCC49242= 1:1 cometabolize VC were also examined to evaluate how the interactions of the two microbial groups affect VC degradation in a scenario where equal volume of methane, ethene, and VC were provided to the microorganisms.

In a scenario where methane, ethene, and VC arrive together from an upgradient anaerobic source and interact with groundwater containing oxygen, the results of this study suggested that VC had the highest potential to be fully degraded when methane and ethene are both present. When methane is more than 3 fold higher than ethene and VC, and ethene is 3 fold higher than methane, 10 fold higher than VC, the highest VC degradation rates (8-10 μmol/day) was observed in the bottles that contained 75% etheneotrophs. However, VC oxidation rate decreased as the percentage of etheneotrophs decreased under three different concentration ratios of VC, ethene, and methane.

We therefore concluded that the concentration ratios of methanotrophs and etheneotrophs are crucial for natural attenuation of dilute VC plumes. By using quantitative PCR technique in conjunction with geochemical data, we can evaluate the relative abundance of indigenous methantrophs and etheneotrophs at contaminated site, and thereby improve decision making for VC *in situ* bioremediation.

Furthermore, the fact that etheneotrpohs dominated the cometabolism of VC even when methane concentration was 3 fold higher than ethene suggested that etheneotrophs do have higher advantage over methanotrophs. Therefore, by enhancing cometabolism by etheneotrophs, the toxic inhibition of VC and epoxyethane to methanotrophs is also reduced so that the cometabolism by methantrophs is also enhanced.

Overall, I believe the addition of ethene and oxygen to stimulate the growth of etheneotrophic bacteria can help reaching maximum VC degradation as long as the concentration of ethene does not completely inhibit MMO activity. Therefore, the critical biostimulation treating point is to find an optimal methane and ethene concentration ratios that can allow both microbial groups to reach its highest potential of VC cometabolic activity.

Even more promising than cometabolism, future research can be focusing on investigating how VC-assimilators, bacteria that can directly degrade VC as a growth substrate, behave under vary aqueous concentrations of methane, ethene and VC.

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APPENDIX A

STANDARD CURVE SUPPORTING DOCUMENTS

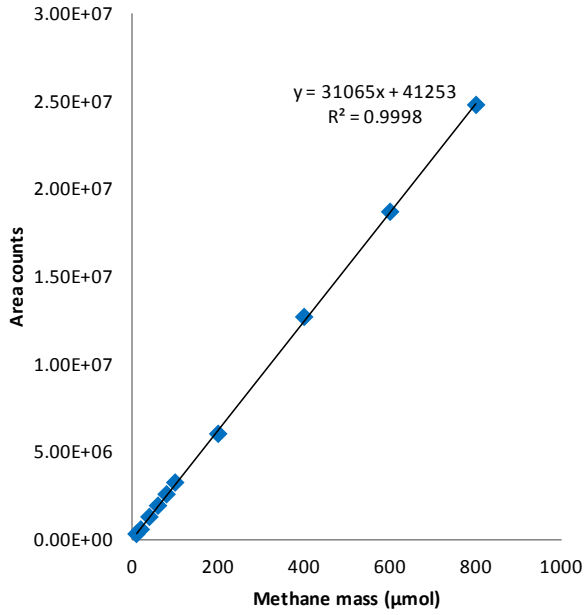

Figure A 1. Standard curve of methane

Table A 1. Raw data for methane mass conversion

methane mass (µmol)	Area counts
10	3.17E+05
20	5.75E+05
40	1.29E+06
60	1.93E+06
80	2.58E+06
100	3.25E+06
200	6.03E+06
400	1.27E+07
600	1.87E+07
800	2.48E+07

Note: Each sample was run in triplicate and average quantifications were used to calculate the conversion

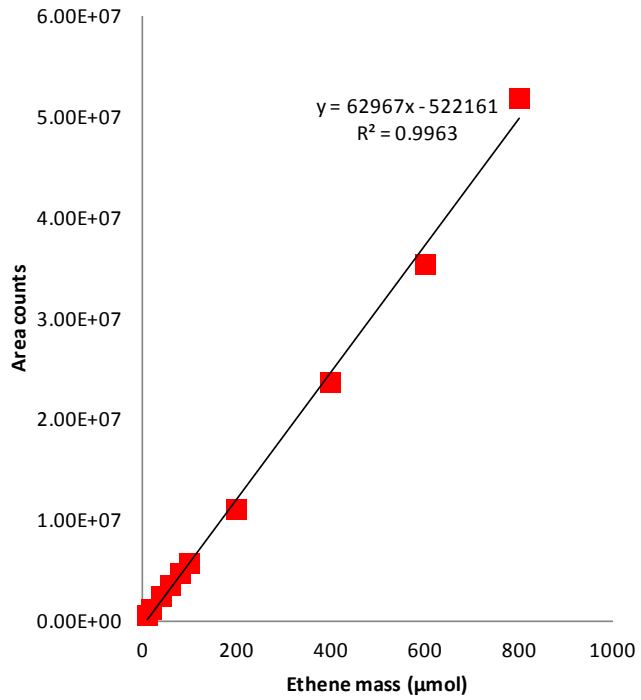

Figure A 2. Standard curve for ethane

Table A 2. Raw data for ethene mass conversion

ethene mass (µmol)	Area counts
10	5.72E+05
20	1.18E+06
40	2.42E+06
60	3.54E+06
80	4.75E+06
100	5.67E+06
200	1.11E+07
400	2.37E+07
600	3.54E+07
800	5.19E+07

Note: Each sample was run in triplicate and average quantifications were used to calculate the conversion

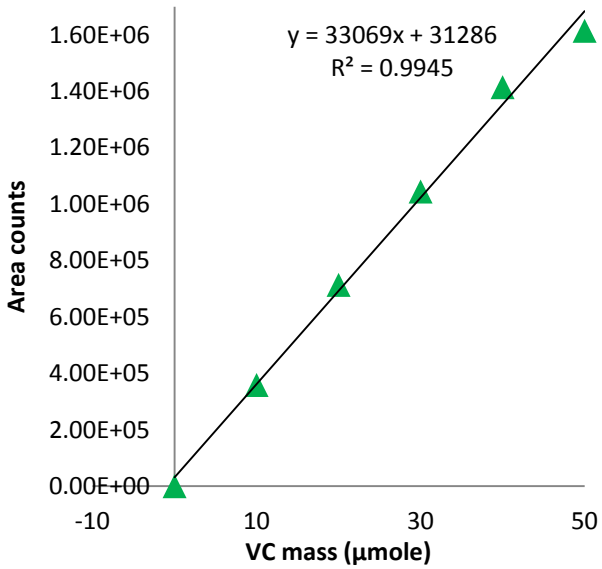

Figure A 3. Standard curve for VC

Table A 3. Raw data for VC mass conversion

VC mass (µmol)	Area counts
0	0
10	3.59E+05
20	7.14E+05
30	1.05E+06
40	1.42E+06
50	1.62E+06

Note: Each sample was run in triplicate and average quantifications were used to calculate the conversion

APPENDIX B

THE SUMMARY OF METHANE, ETHENE, AND VC DEGRADATION RATES

Table B 1The summary of VC degradation rates

		100%JS622		75%JS622		50%JS622		25%JS622		10%JS622		5%JS622		100%ATCC49242	
MTH:ETH:VC (μM)		Ini.	Max.	Ini.	Max.	Ini.	Max.	Ini.	Max.	Ini.	Max.	Ini.	Max.	Ini.	Max.
170:500:50	MTH	0	0	98		174		222						176	
	ETH	24	197	93	118	34	121	52	113					28	
	VC	13		12		3		10						3	
	MTH	0	0	86		185		210						193	
	VC	11		13		7		10						6	
	ETH	32	204	110	206	51	122	45	113					31	
	VC	0.5	9	0.9	3.3	0.3	1.6	0.6	2.9					0.5	
	MTH	0	0	23		22		35						23	
	ETH	32	179	131	179	67	115	57	68					50	
	MTH	0	0	21		17		7						32	
	ETH	38	209	125	201	48	115	54	78					52	
	VC	0.6	10	3	9	0.6	4	0.7	2					2	
170:40:50	MTH	0	0	34		80	563	128	522	200		274		141	
	ETH	24		22		21		18		15		14		9	
	VC	10		7		6		8		6		7		5	
	MTH	0		67		100	236	95	210	194		187		117	
	VC	8*		11		**8**		8		10		14		2	
	ETH	12*		17		18		15		12		13		8	
	VC	3		4		3		3		2	4	2		2	
	MTH	0		30		52	606	58	600	128		195	128	19	
	ETH	13		14		23		17		24		26		9	
	MTH	0		22		54		61		97		112		40	
	ETH	13*		19		14		15		22		24		9	
	VC	5		8		5		4		5		5		2	
15: 40: 170	MTH	0				69								75	
	ETH	31				30								21	
	VC	6				6								3	
	MTH	0				11								7	
	VC	16				12								7	
	ETH	23				21								16	
	VC	11				4								9	
	MTH	0				13								13	
	ETH	30				28								31	
	MTH	0				3								6	
	ETH	22				16								17	
	VC	11				2								3	

APPENDIX C

THE CONTROL BOTTLE SUPPORTING DATA

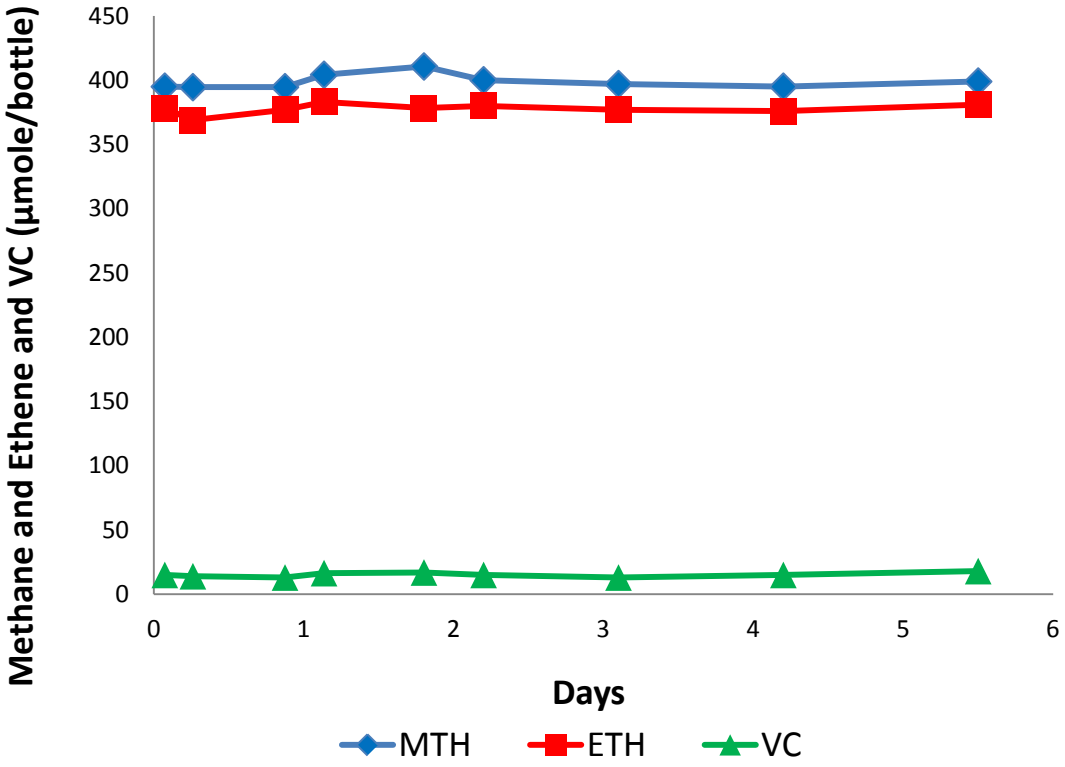

Figure C 1 The control bottle that contained 400 µmole of methane and ethene, and 20 µmole of VC.