

# 1,1,2,2-TETRACHLOROETHANE AEROBIC COMETABOLIC BIODEGRADATION BY PROPANE- AND METHANE-UTILIZING CONSORTIA IN SLURRY BIOREACTORS

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**ABSTRACT.** This slurry microcosm study was aimed at investigating the process of 1,1,2,2-tetrachloroethane (TeCA) aerobic cometabolic biodegradation by the indigenous biomasses of 5 aquifers, and the efficacy of different microbial inocula in enhancing the TeCA degradation process. Methane and propane were tested as growth substrates. 4 of the 5 tested indigenous biomasses were capable to start growing on propane and degrading TeCA within acceptable lag-times, indicating that aerobic cometabolism represents a potentially effective approach for the bioremediation of TeCA-contaminated aquifers. The introduction of the tested microbial inocula led to a rapid onset of TeCA aerobic biodegradation in the aquifer material that had proved unable to deplete TeCA in the absence of inoculation. In the microcosms containing the other 4 aquifer materials, the inocula induced marked decreases of the lag-time for the onset of the cometabolic process. Besides, in all the experimental conditions the TeCA biodegradation rates obtained in the inoculated tests were equal or higher than the corresponding rates obtained in the absence of inoculation. This study represents to the best of our knowledge the first investigation entirely dedicated to aerobic TeCA cometabolism.

## 1. INTRODUCTION

Chlorinated Aliphatic Hydrocarbons (CAHs) represent one of the most common causes of subsurface contamination both in Europe and in the United States (US EPA, 2000), as a consequence of the widespread application they find in several areas and of the incorrect disposal techniques often utilized. The toxicity of chlorinated solvents, together with the demonstrated carcinogenicity of some of them, makes the present level of subsurface contamination particularly serious and dangerous. The chlorinated solvents more frequently found in the subsurface are tetrachloroethylene (PCE), trichloroethylene (TCE), cis- and trans-1,2-dichloroethylene (cis- and trans-DCE), vinyl chloride (VC), 1,1,2,2-tetrachloroethane (TeCA), 1,1,1- and 1,1,2-trichloroethane (1,1,1- and 1,1,2-TCA), 1,2-dichloroethane (1,2-DCA), carbon tetrachloride (CT) and chloroform (CF) (Chang and Alvarez-Cohen, 1996; McCarty and Semprini, 1994; US EPA, 1997).

Since the 1980s, numerous literature studies documented the successful biodegradation of CAHs by means of both aerobic and anaerobic processes. Anaerobic degradation processes are of great interest for their high potential for the transformation of widespread high-chlorinated compounds such as PCE and TeCA, but they are also characterized by the risk of accumulation of highly toxic and in some cases carcinogenic low-chlorinated compounds, such as VC and cis-DCE (Bouwer, 1994; Semprini, 1997; Yu *et al.*, 2005).

Aerobic degradation processes, on the other hand, are characterized by the technical difficulties connected with the supply of oxygen in the subsurface and by the risk of an excessive biomass growth in the proximity of the injection wells (McCarty *et al.*, 1998), but at the same time they present the advantage of a rapid and usually complete dechlorination of the low- and medium-chlorinated hydrocarbons (Frascari *et al.*, 2005 and 2006a; Kim *et al.*, 1997). Research on aerobic CAH cometabolism tested primarily the utilization of methane (Anderson and McCarty, 1996; Chang and Alvarez-Cohen, 1996), toluene, phenol (Hopkins and McCarty, 1995; McCarty *et al.*, 1998), propane (Chang and Alvarez-Cohen, 1995; Wilcox *et al.*, 1995) and ammonia (Ely *et al.*, 1997) as growth substrates. Recent studies indicate that some aerobic microorganisms can also perform the cometabolic degradation of CAHs with 4 chlorine atoms, such as PCE (Marco-Urrea *et al.*, 2006) and TeCA (Chang and Alvarez-Cohen, 1996). In particular, our research group recently proved the feasibility of a long-term TeCA biodegradation by methane- and propane-utilizing biomasses up to about 700 µg/L (Frascari *et al.*, 2006b; Gualandi *et al.*, 2007).

Despite the encouraging results of the studies of aerobic cometabolism, practitioners have so far been hesitant to utilize this technology for the full-scale remediation of CAH-contaminated aquifers (Semprini, 2001). This fact can partly be ascribed to the long lag-time frequently required for the onset of the aerobic cometabolic process (Frasconi *et al.*, 2006b; Jitnuyanont *et al.*, 2001; Martin, 1999). The microbial lag-time relative to a given substrate can be defined as the time interval during which no biodegradation is detected. It can vary from a few minutes to years (Frasconi *et al.*, 2006b; van der Meer *et al.*, 1992; Wiggins and Alexander, 1998). A long acclimation time may cause a pollutant to cross the treatment zone and arrive at sensitive targets. Numerous explanations can be found in the literature for the microbial lag-time (Martin, 1999; Wiggins and Alexander, 1998). For example, it can be interpreted as the time required by an initially small number of microorganisms able to metabolize the substrate to grow until their level is sufficient to make the biodegradation detectable. Otherwise, it can represent the time required for the cell to induce the production of specific enzymes, as a consequence of the exposition to the substrate. Cometabolic processes may be characterized by the existence of a double lag-time (primary substrate + cometabolite), which can further delay the onset of biodegradation (Frasconi *et al.*, 2006b).

If a polluted site is characterized by the presence of a long lag phase, the introduction of a microbial inoculum into the treatment system (a technology known as bioaugmentation) can lead to a rapid onset of the biodegradation process (Gentry, 2004). Several studies show that bioaugmentation can be effectively applied also to sites contaminated by CAHs (Jitnuyanont *et al.*, 2001; Major *et al.*, 2005; Munakata-Marr *et al.*, 1996).

This study focused on the aerobic cometabolic biodegradation of TeCA in slurry conditions by methane- and propane-utilizing biomasses. The goals of the work were: a) to evaluate the capacity of the indigenous biomasses of 5 different aquifers to biodegrade TeCA via aerobic cometabolism on methane or propane; b) to investigate the effect of different operational parameters (growth substrate type, TeCA concentration, presence of other CAHs) on the lag-time for the onset of TeCA biodegradation and on the long-term depletion rates achievable in slurry conditions; c) to test the capacity of different microbial inocula to enhance the TeCA biodegradation performance of the indigenous biomasses of the studied aquifers.

## 2. MATERIALS AND METHODS

### 2.1 Microcosm preparation and operation

The experiments were conducted in 41 119-mL slurry bioreactors (indicated in the following as microcosms), closed with Teflon-lined rubber septa and containing 20 g (wet weight) of soil, 50 mL of groundwater and 60 mL of headspace air. To avoid bacterial contamination, bottles, caps and all tools used for preparing the microcosms were autoclaved (121°C, 20 minutes). The experimental scheme, reported in the left-hand part of Table 1, was designed to investigate the effect of the following parameters on TeCA biodegradation:

- type of growth substrate: methane or propane;
- initial TeCA aqueous phase concentration (25 - 420 µg/L);
- type of aquifer material: soil and groundwater from 5 aquifers were utilized; of these, two (aquifers A and B) were historically contaminated by CAH mixtures including TeCA and are constituted primarily by sandy/silty soils; the remaining three (C, D and E) were not contaminated: C contains a sandy soil, whereas D and E contain humic soils characterized by a high fraction of organic carbon, equal to about 1.5%;
- presence or not of other chlorinated solvents: to test the effect of the presence of other CAHs on TeCA biodegradation, some tests were operated in the presence of the following mixture: VC (initial aqueous phase concentration: 1560 µg/L); trans-DCE (330 µg/L); cis-DCE (300 µg/L); TCE (250 µg/L); 1,1,2-TCA (40 µg/L); this CAH mixture is representative of the contamination of aquifer A, and its biodegradation by the methane- and propane-utilizing biomasses indigenous of site A was described in a previous study (Frasconi *et al.*, 2006b);
- addition of a microbial inoculum: 14 microcosms were not bioaugmented, while the remaining 27 were augmented with 3 types of inocula, consisting of biomass/soil suspensions sampled from different TeCA-degrading microcosms included in this study. In particular, inocula type 1 (M1 and P1) were sampled respectively from microcosms MC-NI-A<sub>1,2</sub> and PC-NI-A<sub>1,2</sub> after a long-term cometabolic biodegradation of the above-listed 5-CAH mixture (in addition to TeCA) by the indigenous biomass of aquifer A; inocula M1 and P1 were utilized to bioaugment A-type

microcosms containing only TeCA (MT-I1-A<sub>1,9</sub>, PT-I1-A<sub>1,9</sub>) or TeCA plus the 5 CAH-mixture (MC-I1-A<sub>1,2</sub>, PC-I1-A<sub>1,2</sub>). Conversely, inoculum I2 was sampled from 3 microcosms selected from group PT-I1-A, after a long term cometabolic biodegradation of TeCA. Inoculum I2 was utilized to bioaugment microcosms set up with the 5 aquifer materials and additioned with only TeCA (PT-I2-A, -B, -C, -D and -E). The second part of the study (utilization of a type-2 inoculum to bioaugment microcosms containing the 5 aquifer materials) was limited to propane as the growth substrate, as a result of the higher TeCA degradation performances observed during the first part of the study in the propane-fed tests, with respect with those fed with methane. The following procedure was followed for each inoculation: i) sampling with a sterile syringe 2 mL of soil/biomass suspension from an active TeCA-degrading microcosm after 30 seconds of intense agitation; ii) introduction of the suspension in a newly set-up microcosm; iii) supply of the inoculated microcosm with growth substrate (methane or propane).

Each microcosm was operated by supplying a new pulse of primary substrate (2 mg/L in the aqueous phase) and oxygen (8 mg/L in the aqueous phase) each time the previous substrate pulse was completely consumed. Similarly, a new CAH pulse (only TeCA, or TeCA + the 5-CAH mixture) was supplied upon complete biodegradation of the previous pulse. In order to investigate the effect of TeCA concentration on the biodegradation rate of this compound, in each microcosm the TeCA initial concentration in the subsequent pulses was progressively raised; the overall ranges of variation of TeCA concentration tested in each experimental condition are reported in the right-hand part of Table 1. As examples, Figures 1, 2 and 3 show the initial period of operation, respectively, of a non-inoculated microcosm fed with propane and TeCA (PT-NI-A<sub>1</sub>), of an inoculated microcosm fed with propane and TeCA (PT-I1-A<sub>6</sub>), and of an inoculated microcosm fed with methane and with the 5-CAH mixture in addition to TeCA (MC-I1-A<sub>1</sub>).

The desired CAH concentrations in the aqueous phase were obtained by spiking an aqueous stock solution with a 25- $\mu$ L or 100- $\mu$ L syringe into the sealed microcosms, whereas (only in the "C"-type microcosms) VC was spiked as pure gas sampled from a flask. Prior to each primary substrate supply, oxygen (7.5–9 mL) was supplied with a frictionless glass syringe, so as to maintain aerobic conditions and to compensate the pressure decrease due to the headspace samplings for GC analysis and to substrate oxidation to CO<sub>2</sub>. The frictionless syringe allowed the introduction of oxygen until reaching atmospheric pressure in the microcosms. Following oxygen addition, the supply of methane or propane led to a small overpressure in the microcosm headspace. As a result of this operational procedure, the headspace pressure in the microcosms followed within each pulse of primary substrate consumption a cyclic trend with variations between 1.10 and 0.90 atm (1.03-0.90 atm in the propane-fed tests). During the phases of headspace pressure higher than 1 atm, the CAH analytical procedure resulted, at the most, in a 9% underestimation of the actual headspace concentration (3% in the propane-fed tests). Microcosms were periodically opened (in the absence of CAHs and growth substrate) and stripped with 0.22-filtered air for 5 minutes to eliminate dissolved carbon dioxide and any possible volatile product of CAH degradation. Macronutrients (N and P) were provided every time the nitrate concentration was lower than 0.16 mM, by adding an aqueous solution of 1.6 mM nitrate (as KNO<sub>3</sub>) and 0.1 mM phosphate (as KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> at 0.65:1 weight ratio). The operation and the results relative to the microcosms containing the 5-CAH mixture in addition to TeCA (MC-NI-A<sub>1,2</sub>, MC-I1-A<sub>1,2</sub>, PC-NI-A<sub>1,2</sub>, PC-I1-A<sub>1,2</sub>) are described in more detail in a previous work of our research group (Frascari *et al.*, 2006b), where the aerobic biodegradation of TeCA by methane- and propane-growing consortia was described for the first time.

In addition to the microcosms reported in Table 1, 12 sterile microcosms were set up to monitor abiotic TeCA reactions and losses through the caps. As shown in the left-hand part of Table 2, in order to investigate the possible effect of the type of soil and groundwater on the abiotic TeCA depletion, 6 sterile controls contained A-type aquifer material, whereas the remaining 6 were made with E-type material. Within each sub-group, duplicate sterile tests were set up at three different initial TeCA concentrations. These tests, which were sterilized by the addition of 4 g/L of NaN<sub>3</sub>, did not contain the 5-CAH mixture. All the microcosms (viable and sterile) were kept in continuous agitation in rollers operated at 3.3 rpm and maintained at 25  $\pm$  0.5 °C.

## 2.2 Analysis

The CAHs and propane were purchased from Aldrich (Gillingham, UK), whereas methane was taken from the distribution network of the local gas company. Purities were: propane, VC and TCE, 99.5%; 1,1,2-TCA, 99%; t-DCE, TeCA and methane, 98%; c-DCE, 97%. The gas-phase concentrations of methane, propane and CAHs were measured with a HP6890 gaschromatograph equipped with a capillary HP-VOC column (30 m x 0.32 mm) connected to a flame ionization detector

Table 1. Experimental scheme relative to the viable microcosms, lag-times and first-order TeCA degradation constants <sup>a</sup>

| EXPERIMENTAL SCHEME |               |                  |  |                              |                      |   | RESULTS: onset of biodegradation        |                                     | RESULTS: elaboration of the TeCA biodegradation rates |   |
|---------------------|---------------|------------------|--|------------------------------|----------------------|---|---|-------------------------------------|---|---|
| Growth substrate    | Inoculum type | Aquifer material | Presence of the CAH mixture in addition to TeCA <sup>b</sup> | Microcosm label <sup>c</sup> | Number of replicates | Initial TeCA aq. phase concentration (µg/L) | Growth substrate average lag-time (day) | TeCA average further lag-time (day) | TeCA studied concentration range (µg/L)               | TeCA pseudo first-order biodegradation constant, $k^*$ (day <sup>-1</sup> ) |
| Methane             | No inoculum   | A                | Yes  | MC-NI-A <sub>1,2</sub>       | 2                    | 25  | 236                                     | 0.0                                 | 0 - 110   | 0.28 <sup>d</sup>   |
|                     | M1            | A                | Yes  | MC-I1-A <sub>1,2</sub>       | 2                    | 25  | 0.0                                     | 6.0                                 | 0 - 110   | 0.32 <sup>d</sup>   |
|                     | M1            | A                | No   | MT-I1-A <sub>1,2,3</sub>     | 3                    | 40  | 0.0                                     | 5.0                                 | 0 - 1000  | 0.66 <sup>d</sup>   |
|                     | M1            | A                | No   | MT-I1-A <sub>4,5,6</sub>     | 3                    | 90  | 0.0                                     | 5.3                                 |   |   |
|                     | M1            | A                | No   | MT-I1-A <sub>7,8,9</sub>     | 3                    | 420   | 0.0                                     | 5.6                                 |   |   |
| Propane             | No inoculum   | A                | Yes  | PC-NI-A <sub>1,2</sub>       | 2                    | 25  | 121                                     | 3.5                                 | 0 - 110   | 1.1 <sup>d</sup>  |
|                     |               | A                | No   | PT-NI-A <sub>1,2</sub>       | 2                    | 160   | 9.8                                     | 2.1                                 | 0 - 220   | 0.74 <sup>e</sup>   |
|                     |               | B                | No   | PT-NI-B <sub>1,2</sub>       | 2                    | 160   | 7.6                                     | 0.6                                 |   |   |
|                     |               | C                | No   | PT-NI-C <sub>1,2</sub>       | 2                    | 160   | 4.3                                     | 1.1                                 |   |   |
|                     |               | D                | No   | PT-NI-D <sub>1,2</sub>       | 2                    | 160   | 6.8                                     | 4.9                                 |   |   |
|                     |               | E                | No   | PT-NI-E <sub>1,2</sub>       | 2                    | 160   | > 150                                   | -                                   | -   | -   |
|                     | P1            | A                | Yes  | PC-I1-A <sub>1,2</sub>       | 2                    | 25  | 10.0                                    | 2.5                                 | 0 - 110   | 1.1 <sup>d</sup>  |
|                     | P1            | A                | No   | PT-I1-A <sub>1,2,3</sub>     | 3                    | 40  | 0.0                                     | 1.8                                 | 0 - 2660  | 2.4 <sup>d</sup>  |
|                     | P1            | A                | No   | PT-I1-A <sub>4,5,6</sub>     | 3                    | 90  | 0.0                                     | 0.9                                 |   |   |
|                     | P1            | A                | No   | PT-I1-A <sub>7,8,9</sub>     | 3                    | 420   | 0.0                                     | 0.3                                 |   |   |
|                     | P2            | A                | No   | PT-I2-A                      | 1                    | 160   | 2.8                                     | 0.3                                 | 0 - 140   | 3.5 <sup>e</sup>  |
|                     | P2            | B                | No   | PT-I2-B                      | 1                    | 160   | 0.3                                     | 0.3                                 |   |   |
|                     | P2            | C                | No   | PT-I2-C                      | 1                    | 160   | 0.3                                     | 0.8                                 | 0 - 180   | 1.4 <sup>e</sup>  |
|                     | P2            | D                | No   | PT-I2-D                      | 1                    | 160   | 0.3                                     | 2.3                                 |   |   |
| P2                  | E             | No               | PT-I2-E  | 1                            | 160                  | 0.5   | 2.0                                     |                                     |   |   |

<sup>a</sup> Methane and propane aqueous phase concentrations were equal to 2 mg/L in all the tests and in all the subsequent pulses. <sup>b</sup> The composition of the CAH mixture is reported in section 2.1 of the Materials and Methods. <sup>c</sup> 1<sup>st</sup> letter: M = methane, P = propane; 2<sup>nd</sup> letter: C = with the 5-CAH mixture; T = only TeCA (in addition to the growth substrate); 3<sup>rd</sup> and 4<sup>th</sup> character: NI = no inoculation; I1 = inoculum type M1 (for M-type tests) or P1 (for P-type tests); I2 = inoculum type P2; last letter: type of aquifer material. The superscript numbers identify the replicate microcosms. <sup>d</sup> Estimated from long-term degradation rates (after the attainment of a pseudo-stationary situation). <sup>e</sup> Estimated from degradation rates obtained in the 2<sup>nd</sup> or 3<sup>rd</sup> TeCA pulse (before the attainment of a pseudo-stationary situation).

Table 2. Experimental scheme and first-order TeCA depletion constants relative to the sterile microcosms

| Aquifer material | Microcosm label       | Initial TeCA aq. phase concentration ( $\mu\text{g/L}$ ) | TeCA average first-order biodegradation constant, $k_{1\text{-ctr}}$ ( $\text{day}^{-1}$ ) | $k_{\text{st}} / k_{\text{viable microcosms}}^{\text{a}}$ |      |
|------------------|-----------------------|--|--|---|------|
|                  |                       |  |  | min   | max  |
| A                | CTR-A <sub>-1,2</sub> | 40   | 0.009  | 3.2%  | 0.3% |
|                  | CTR-A <sub>-3,4</sub> | 90   |  |   |      |
|                  | CTR-A <sub>-5,6</sub> | 420  |  |   |      |
| E                | CTR-E <sub>-1,2</sub> | 40   | 0.020  | 1.4%  | 1.4% |
|                  | CTR-E <sub>-3,4</sub> | 90   |  |   |      |
|                  | CTR-E <sub>-5,6</sub> | 420  |  |   |      |

<sup>a</sup> Ratio of  $k_{\text{st}}$  to the minimum or maximum pseudo first-order constant  $k^*$  estimated in the viable microcosms containing the same type of aquifer material.

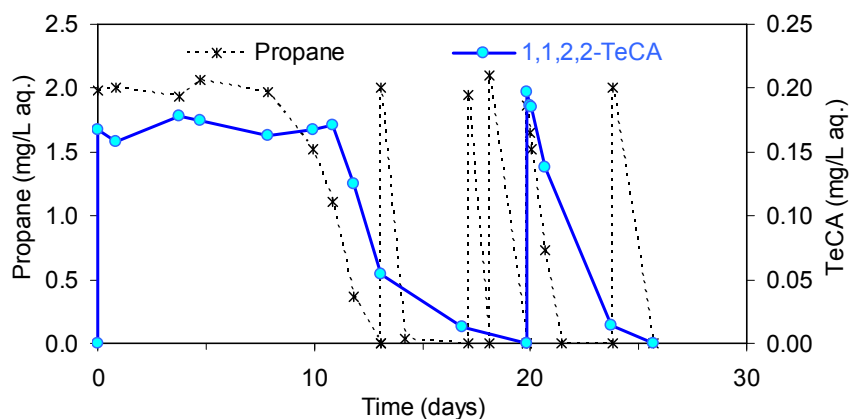


Figure 1. Propane and TeCA aqueous phase concentrations versus time during the initial 30 days of operation of a non-inoculated, propane-fed microcosm (PT-NI-A).

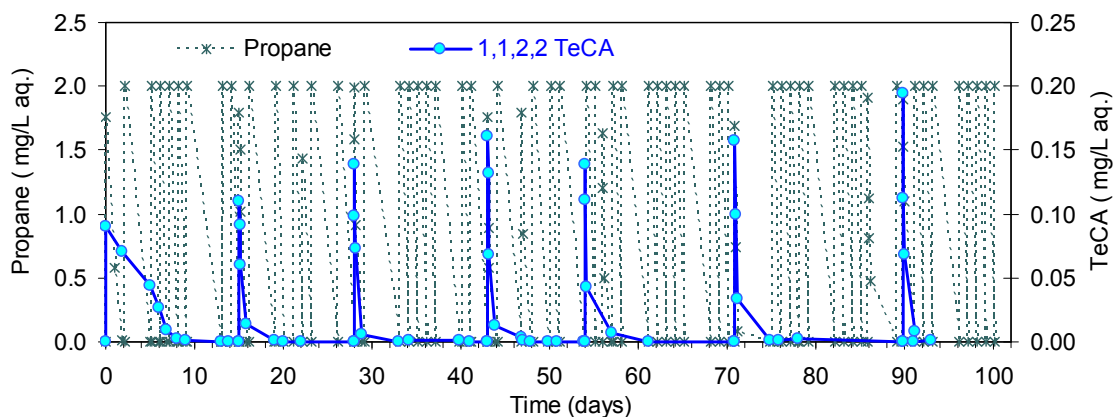


Figure 2. Propane and TeCA aqueous phase concentrations versus time during the initial 100 days of operation of an inoculated, propane-fed microcosm (PT-I1-A<sub>6</sub>).

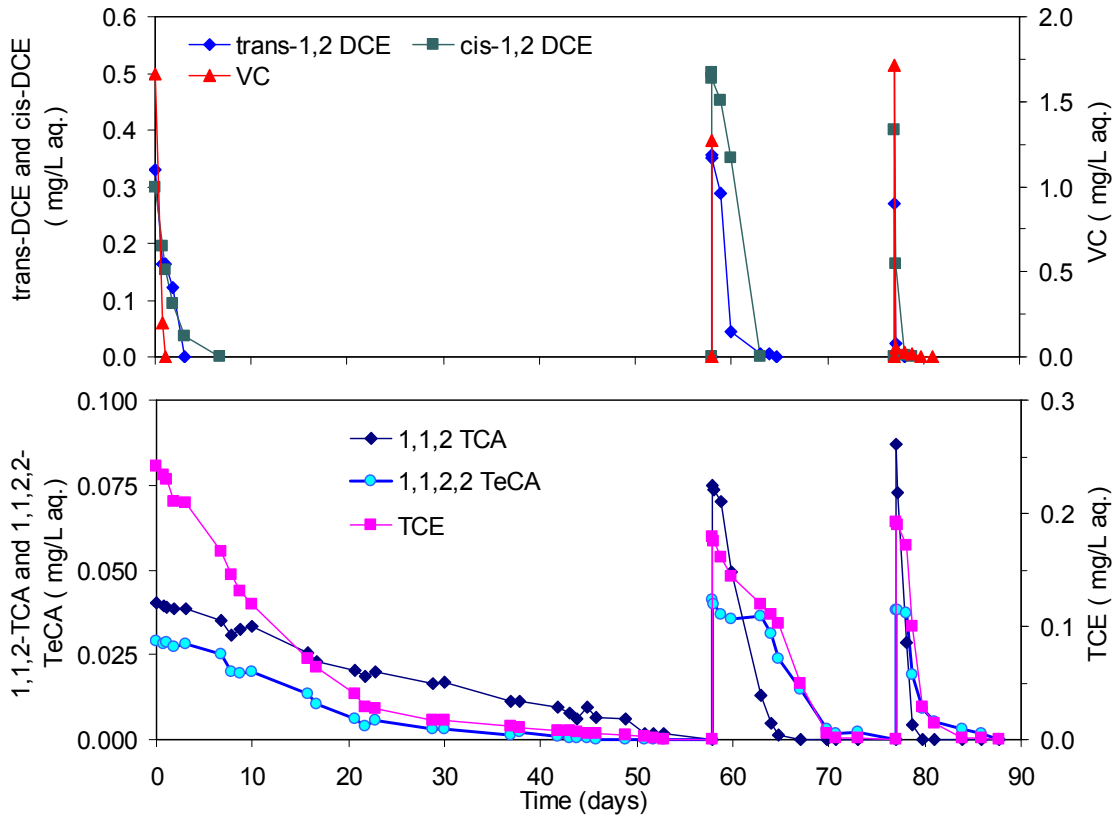


Figure 3. CAH aqueous phase concentrations versus time during the initial 3 pulses of the CAH mixture in a non-inoculated, methane-fed microcosm (MC-I1-A<sub>1</sub>). To allow a good readability, the daily methane pulses are not represented.

(250 °C) for the analysis of methane, propane and VC and to an electron capture detector (250 °C) for the analysis of the remaining CAHs (injector temperature 250 °C; injection volume 500- $\mu$ L; split ratio 10:1; oven temperature 3 min at 60 °C, ramp to 230 °C at 20 °C/min, 5 min at 230 °C; carrier gas He at 0.9 mL/min). Detection limits were ( $\mu$ g/L in the aq. phase): VC, 20; trans-DCE, 8; cis-DCE, 15; TCE, 0.0005; 1,1,2-TCA, 1.3; TeCA, 3.4. All the methods were calibrated with external standards. Total masses and aqueous phase concentrations were calculated utilizing the gas/liquid and solid/liquid equilibrium constants estimated by Sander (1999) and Delle Site (2001). Bacterial plate counts were performed as described by Frascari *et al.* (2005).

### 2.3 Estimation of lag-times and maximum degradation rates

In each microcosm, the lag-time for the onset of growth substrate utilization was evaluated as the time elapsed between growth substrate addition and the time when a decrease in substrate concentration could be detected. The (further) lag-time for the onset of TeCA biodegradation was evaluated as the time elapsed between the end of the growth substrate lag-period and the time when a sharp decrease in TeCA concentration could be detected. In fact, as evidenced by the sterile controls, TeCA is subject to a slow abiotic transformation since microcosm set up; however, in all the tests included in this study, the instant of the onset of TeCA biodegradation was clearly identifiable thanks to a sharp increase of the overall TeCA depletion rate. For example, Figure 1 refers to a non-inoculated microcosm clearly characterized by the presence of a propane lag-time and by a further TeCA lag-time. Each pulse of growth substrate or TeCA was characterized by the initial biodegradation rate, calculated from the graph of total mass versus time by dividing the initial slope by the volume of the liquid phase. Equilibrium between the gaseous, liquid and solid phase was assumed.

### 3. RESULTS AND DISCUSSION

#### 3.1. TeCA depletion in the sterilized controls

The data of TeCA depletion relative to the sterile controls were elaborated by determining the average initial disappearance rate of each duplicate test and by constructing, for each type of aquifer material (A or E), a rate-concentration plot. Both plots were satisfactorily interpolated with a linear model ( $r^2 = 0.995$  and  $0.991$ ), indicating that the overall abiotic phenomena of TeCA depletion in these slurry tests can be described with a first-order model. The best-fitting first-order constants ( $k_{st}$ ) are reported in Table 2. All the TeCA depletion rates relative to the viable microcosms were therefore evaluated as net biodegradation rates, obtained by subtracting to each initial depletion rate the corresponding initial abiotic rate,  $r_{i,st}$ . The latter was evaluated as  $k_{st} c_{i,TeCA}$ , where  $c_{i,TeCA}$  indicates the initial TeCA concentration in the pulse. For the B-, C- and D-type viable microcosms, in the absence of sterile tests set up with the same aquifer materials,  $r_{i,st}$  was conservatively evaluated by utilizing the estimate of  $k_{st}$  obtained in the E-type controls (the highest of the two available estimates).

The average first-order constants reported in the literature for the two main abiotic reactions of TeCA transformation, evaluated at 25 °C and pH 7, are equal to  $0.004 \text{ day}^{-1}$  for hydrolysis (resulting in the formation of trichloroethanol), and to  $0.006 \text{ day}^{-1}$  for the elimination reaction (resulting in the formation of TCE) (Jeffers *et al.*, 1989; Joens *et al.*, 1995). Therefore, the overall first-order constant relative to these two abiotic reactions accounts for the entire sterile depletion rate observed in the A-type controls and to about 50% of the abiotic disappearance obtained in the E-type controls. This results suggests that losses through caps gave a minor contribution to the abiotic TeCA depletion we observed in the sterile controls. Besides, the fact that the first-order abiotic constant observed in the E-type tests is about twice the value obtained in the A-type tests indicates that the rate of TeCA abiotic depletion is affected by the chemical-physical conditions of the slurry test.

#### 3.2 Lag-times for the onset of TeCA cometabolic biodegradation

The average growth substrate and TeCA lag-times obtained in each experimental condition are reported in the right-hand part of Table 1.

In the non-inoculated tests, 4 out of the 5 indigenous biomasses proved capable to grow on propane and to cometabolise TeCA under aerobic conditions, whereas aquifer material E did not show any activity on propane within a 5-month monitoring time. A-type indigenous biomass proved also capable to degrade TeCA via cometabolism on methane, whereas the remaining 4 aquifer materials were not tested in the presence of methane. In these non-bioaugmented tests, the observed lag-times for the onset of growth substrate utilization varied between 4 days and 8 months, whereas TeCA biodegradation began very soon (0 – 5 days) after the onset of a detectable biomass growth on the primary substrate. The longest primary substrate lag-times were observed in A-type microcosms characterized by the presence of the 5-CAH mixture (VC, cis- and trans-DCE, TCE, 1,1,2-TCA).

In the bioaugmented tests the lag-times for the onset of a detectable primary substrate consumption varied between 0 and 10 days, with an average 98% reduction with respect to the lags obtained in the corresponding non-inoculated tests. This result indicates that the inoculated biomasses, sampled from active TeCA degrading slurry microcosms, were able to rapidly take root and start growing on the supplied primary substrate, even in the case of aquifer material E, whose indigenous biomass did not show (within the 5-month monitoring time) any capacity to start growing on propane. The bioaugmentation treatments were also characterized by an average reduction of the further TeCA lag-time equal to about 50%.

#### 3.3 Kinetic analysis of cometabolic biodegradation

All the methane-fed microcosms, and the A-type propane-fed microcosms characterized by the absence of inoculation (NI) or by the addition of inoculum I1, were subjected to a long-term operation characterized by a progressive increase of the TeCA concentration in the subsequent pulses, for a total number of TeCA pulses ranging between 6 and 25. In these microcosms, we generally observed a sharp increase of TeCA biodegradation rate during the initial 2-3 pulses of this compound, followed by the attainment of a pseudo-stationary situation characterized (in each microcosm group) by the attainment of a roughly constant growth substrate utilization rate, and by a TeCA biodegradation rate depending only on the initial TeCA concentration in each pulse.

The net TeCA biodegradation rates relative to this pseudo-stationary condition were elaborated by constructing, for each microcosm group characterized by uniform experimental conditions (e.g., MC-

NI-A, MC-I1-A, PC-I1-A, etc.) a rate-concentration plot. All the rate-concentration plots were satisfactorily interpolated with a linear model ( $r^2 \geq 0.90$ ). The slopes of these linear interpolations, indicated in the following as  $k^*$ , can be considered pseudo first-order constants, where "pseudo" refers to a constant that includes biomass concentration. The estimates of  $k^*$  relative to the different microcosm groups are reported in the last column of Table 1. As an example, the plots relative to microcosm groups MT-I1-A and PT-I1-A are shown in Figure 4.

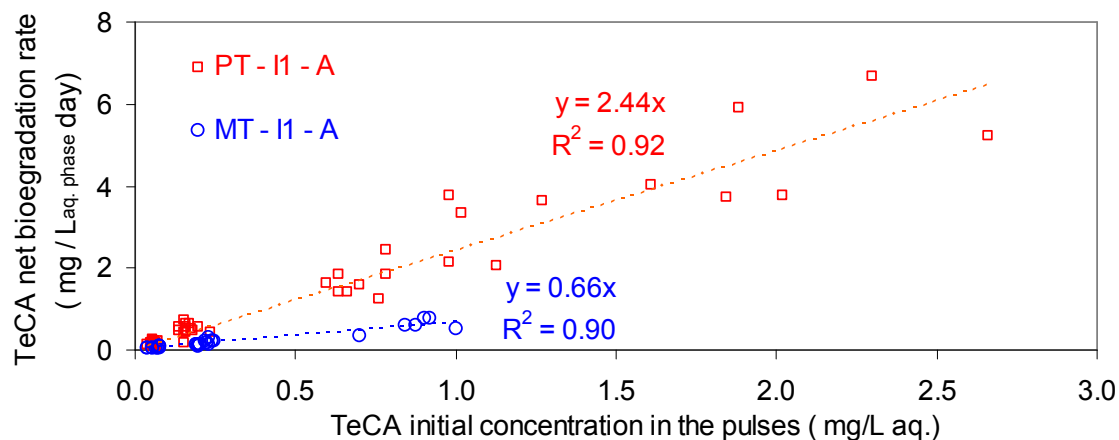


Figure 4. Plot of TeCA net biodegradation rate vs. concentration relative the A-type, inoculated microcosms fed with methane (MT-I1-A) or propane (PT-I1-A), and best-fitting linear interpolation.

It can be observed, in the first place, that in both the M-type and P-type tests containing A-type materials and added with inoculum M1 or P1, the microcosms with the 5-CAH mixture (MC-I1-A, PC-I1-A) are characterized by estimates of  $k^*$  about 50% lower than the corresponding estimates obtained in the microcosms containing only TeCA (MT-I1-A, PT-I1-A). This result indicates that the 5-CAH mixture exerts a significant inhibition on TeCA cometabolic biodegradation by the biomasses investigated in this study. Besides, in both the methane-fed and propane-fed tests containing A-type aquifer materials and characterized by the presence of the 5-CAH mixture, the addition of inoculum M1 or P1 (tests MC-I1-A and PC-I1-A) led to the attainment of the same values of  $k^*$  obtained in the non-inoculated tests (MC-NI-A and PC-NI-A). Therefore, combining the results obtained with inocula M1 and P1 in terms of lag-times and biodegradation rates, it can be stated that, thanks to the drastic reduction of overall lag-time for the onset of TeCA biodegradation, the addition of these consortia led to a strong decrease of the time required to attain the TeCA rates observed in the non-augmented tests. This observation is in agreement with the results obtained by Gualandi *et al.* (2007) in an investigation of the effect of different microbial inocula on the lag-times and degradation rates relative to a 6-CAH mixture.

The non-inoculated P-type microcosms containing only TeCA and set up with the different types of aquifer materials (PT-NI-A, -B, -C, -D, -E), and the corresponding microcosms added with inoculum I2 (PT-I2-A, -B, -C, -D, -E), were characterized by a shorter operation period, corresponding to 2-4 pulses of TeCA biodegradation. Also for these tests, the net TeCA biodegradation rates were elaborated by means of rate-concentration plots. All the rates relative to the non-inoculated tests were satisfactorily interpreted with a single linear interpolation ( $k^* = 0.74 \text{ day}^{-1}$ ). Conversely, the tests added with inoculum I2 were divided into two groups: those containing aquifer materials historically contaminated by CAH mixtures (A and B) yielded a  $k^*$  equal to  $3.5 \text{ day}^{-1}$ , whereas those containing aquifer materials not pre-exposed to CAHs (C, D and E) resulted in a  $k^*$  equal to  $1.4 \text{ day}^{-1}$ . These results indicate that the addition of inoculum I2 led not only to a strong decrease of the overall lag-time for the onset of TeCA biodegradation, but also to a 2 to 5 fold increase of the TeCA biodegradation rate estimated in the initial TeCA pulses of each microcosm. This study therefore provides encouraging indications on the possibility to effectively bioaugment different types of TeCA-contaminated sites with the inocula developed within this experimental work. However, the effective inoculation of slurry microcosms does not necessarily imply the success of an eventual *in-situ* bioaugmentation of the aquifers object of this investigation. In fact, successful bioaugmentation depends also on other conditions, such as the effective delivery of the inoculum to a sufficiently wide portion of the contaminated zone (Gentry *et al.*, 2004). It should be noted that the estimates of  $k^*$  relative to microcosms PT-NI- and PT-I2-A, -B, -C, -D, -E do not derive from TeCA biodegradation

rates obtained in a pseudo stationary condition, as a result of the shorter observation time; therefore, their comparison with the estimates of  $k^*$  obtained in the microcosms characterized by a long-term operation should be performed with caution.

The first-order constants estimated in the A-type and E-type sterile controls ( $k_{st}$ ) are equal to 0.3% - 3.2% of the pseudo first-order constant estimated in the corresponding viable microcosms. This result indicates that abiotic reactions and losses through caps gave a minor contribution to the TeCA depletion rates observed in the viable tests.

## CONCLUSIONS

This study represents to the best of our knowledge the first investigation entirely focused on aerobic TeCA cometabolism. The results show in the first place that 4 of the 5 tested indigenous biomasses were capable to start growing on propane and degrading TeCA within acceptable lag-times. This indicates that the enzymes responsible for TeCA aerobic degradation are relatively widespread in the subsurface, and that consequently aerobic cometabolism represents a potentially effective approach for the bioremediation of TeCA-contaminated aquifers.

The introduction of microbial consortia deriving from the A-type microcosms into the tests containing 4 other types of aquifer materials yielded encouraging results: in fact, we observed the rapid onset of TeCA biodegradation in the E-type tests, which had proved unable to deplete TeCA in the absence of inoculation, and a strong decrease of the lag-time for the onset of TeCA cometabolism in the remaining 3 types of aquifer materials. Besides, in all the experimental conditions the TeCA biodegradation rates obtained in the inoculated tests were equal or higher than the corresponding rates obtained in the absence of inoculation.

This work also confirms that methane and propane represent suitable growth substrates for aerobic cometabolism, as the onset of their consumption was in all cases rapidly followed by the onset of TeCA biodegradation. It constitutes therefore an important advancement on the potential applicability of aerobic cometabolism for the remediation of contaminated sites.

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