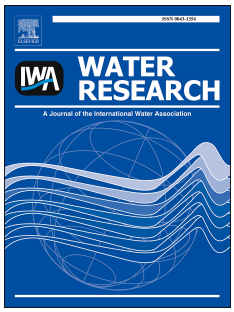
Accepted Manuscript



Evidence of co-metabolic bentazone transformation by methanotrophic enrichment from a groundwater-fed rapid sand filter

Mathilde J. Hedegaard, Hélène Deliniere, Carsten Prasse, Arnaud Dechesne, Barth F. Smets, Hans-Jørgen Albrechtsen

PII: S0043-1354(17)30916-8

DOI: 10.1016/j.watres.2017.10.073

Reference: WR 13330

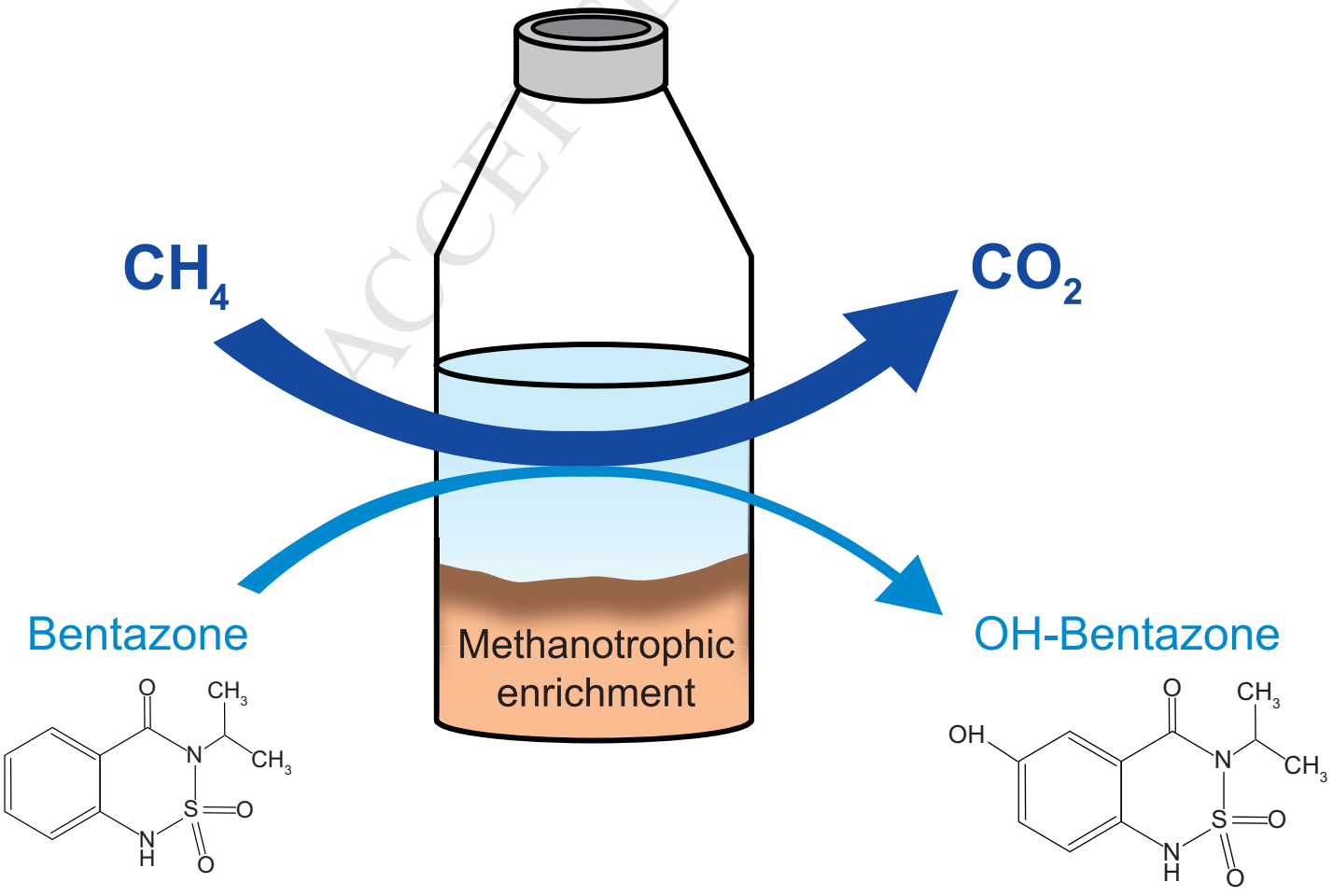
To appear in: *Water Research*

Received Date: 16 June 2017

Revised Date: 27 October 2017

Accepted Date: 31 October 2017

Please cite this article as: Hedegaard, M.J., Deliniere, Héè., Prasse, C., Dechesne, A., Smets, B.F., Albrechtsen, Hans.-Jø., Evidence of co-metabolic bentazone transformation by methanotrophic enrichment from a groundwater-fed rapid sand filter, *Water Research* (2017), doi: 10.1016/j.watres.2017.10.073.



Elsevier Editorial System(tm) for Water Research

Title: Evidence of co-metabolic bentazone transformation by methanotrophic enrichment from a groundwater-fed rapid sand filter

Article Type: Full Paper

Keywords: pesticides; bentazone; degradation; methane oxidation; co-metabolism

Authors: Mathilde J. Hedegaard[1]; Hélène Deliniere[1]; Carsten Prasse[2,3]; Arnaud Dechesne[1]; Barth F. Smets[1]; Hans-Jørgen Albrechtsen[1]

[1] DTU Environment, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; [2] Department of Civil and Environmental, Engineering University of California, Berkeley, CA 94720, United States; [3] Department of Environmental Health and Engineering, Johns Hopkins University, Baltimore, MD 21218, United States

Corresponding Author: Mathilde Hedegaard

Address: DTU Environment**,** Technical University of Denmark**,** Building 113**,** DK-2800 Kgs. Lyngby**,** Denmark

Email-address: mjhe@env.dtu.dk

Telephone: (+45) 4525 1478

Fax: (+45) 4593 2850

Abstract

The herbicide bentazone is recalcitrant in aquifers and is therefore frequently detected in wells used for drinking water production. However, bentazone degradation has been observed in filter sand from a rapid sand filter at a waterworks with methane-rich groundwater. Here, the association between methane oxidation and removal of bentazone was investigated with a methanotrophic enrichment culture derived from methane-fed column reactors inoculated with that filter sand. Several independent lines of evidence obtained from microcosm experiments with the methanotrophic enrichment culture, tap water and bentazone at concentrations below 2 mg/L showed methanotrophic co-metabolic bentazone transformation: The culture removed 53% of the bentazone in 21 days in presence of 5 mg/L of methane, while only 31% was removed in absence of methane. Addition of acetylene inhibited methane oxidation and stopped bentazone removal. The presence of bentazone partly inhibited methane oxidation since the methane consumption rate was significantly lower at high (1 mg/L) than at low (1 µg/L) bentazone concentrations. The transformation yield of methane relative to bentazone normalized by their concentration ratio ranged from 58 to 158, well within the range for methanotrophic co-metabolic degradation of trace contaminants calculated from the literature, with normalized transformation yields varying from 3 to 400. High-resolution mass spectrometry revealed formation of the transformation products (TPs) 6-OH, 8-OH, isopropyl-OH and di-OH-bentazone, with higher abundances of all TPs in the presence of methane. Overall, we found a suite of evidence all showing that bentazone was co-metabolically transformed to hydroxy-bentazone by a methanotrophic culture enriched from a rapid sand filter at a waterworks.

1 Introduction

Pesticides are detected in many fresh water bodies due to their extensive use, environmental mobility and persistence. For example, in Denmark, pesticides such as bentazone, glyphosate, mecoprop (MCPP) and atrazine were detected in 49.5% of the groundwater monitoring wells in the period 1990-2015 (GEUS & Energi-Forsynings og Klimaministeriet, 2016). According to the European Union (EU) 'Water Framework Directive' or 'Groundwater Directive', the concentration of pesticides in drinking water and groundwater should not exceed 0.1 µg/L for a single compound, or 0.5 µg/L for the sum of all pesticides (European Community, 2000; European Union, 2006). It is thus important to identify sustainable methods to remove pesticides at low concentrations (sub µg/L) from polluted water sources.

Trace contaminants can contribute to the growth of degrading bacteria if they are utilized as source of of carbon, energy or potentially nitrogen, phosphorus or sulfur (Alexander, 1994; Benner et al., 2013). However, organic trace contaminants typically occur at too low concentrations (sub µg/L) to support microbial growth and can consequently be difficult to degrade (Alexander, 1994; Benner et al., 2013). In contrast, during co-metabolic degradation the trace contaminants are degraded along with a primary growth substrate without being used as energy or carbon source (Dalton and Stirling, 1982) and thus the degrading populations do neither gain nutrients nor energy from the secondary substrate (Alexander, 1994). This mechanism has gained a lot of attention in bioremediation, since it permits microbial degradation of trace contaminants at low concentrations, by controlling the presence of the primary substrate which can be relatively inexpensive and nontoxic (e.g. CH_4,

NH_4^+) (Iwamoto and Nasu, 2001; Semprini et al., 1990; Semprini and McCarty, 1991; Semrau et al., 2010).

Examples include ammonium-oxidizing bacteria and manganese oxidizing bacteria degrading 17α-ethinylestradiol in wastewater treatment effluent (Forrez et al., 2009), and ammonium oxidizing bacteria degrading the pharmaceuticals ibuprofen, ketoprofen, carbamazepine, dexamethasone, and iopromide in water treatment systems (Dawas-Massalha et al., 2014; Xu et al., 2017). However, direct evidence for co-metabolic degradation can be difficult to establish. For example, biotransformation of some trace contaminants has been shown not to be directly associated with ammonia monooxygenase activity although it was linked to ammonia removal (Helbling et al., 2012).

Methane oxidizing bacteria (MOB) can co-metabolically degrade several different trace contaminants; trichloroethylene (TCE) and other chlorinated aliphatic hydrocarbons are especially well studied (e.g. Alvarez-Cohen et al., 1992; Alvarez-Cohen and McCarty, 1991; DiSpirito et al., 1991; Oldenhuis et al., 1989). The methane monooxygenase (MMO) is the key enzyme in methane oxidation and can oxidize trace contaminants co-metabolically (Dalton and Stirling, 1982; Semrau et al., 2010). MMO can either be the particulate, membrane bound enzyme (pMMO), which is expressed by nearly all known MOBs or the soluble, cytoplasmic MMO (sMMO) which can only be expressed by some MOBs. sMMO is expressed at low copper to biomass ratios, whereas pMMO increases when this ratio increases (Semrau et al., 2013; Sirajuddin and Rosenzweig, 2015). Generally, oxidation by pMMO is limited to alkanes up to five carbon-atoms, while sMMO is less specific and able to oxidize alkanes up to eight carbon-atoms, esters, cyclic alkanes and

aromatic compounds (Burrows et al., 1984; Semrau et al., 2010; Trotsenko and Murrell, 2008). Thus, methanotrophic bioremediation can be challenging due to difficulty in microbial consortia design and control (Jiang et al., 2010), but has been shown to work at field scale (Hazen et al., 2009; Strong et al., 2015).

The transformation yield (T_y, in moles of trace contaminant (TC) per mole of methane) expresses the relative rates between consumption of the primary and secondary substrate. Since the organisms do not gain anything from co-metabolic degradation, there is a theoretical upper limit to T_y governed by the availability of reducing energy. This theoretical upper limit is governed by the gap between total produced energy from oxidation of primary substrate (2.64 $moles_{NADH}/mole_{CH4}$ in case of methane oxidation) and the energy used to oxidize the primary substrate, in this case methane (1 $mole_{NADH}/mole_{CH4}$). The remaining energy defines the theoretical limit for oxidation of the secondary substrate, and for methane oxidation $T_{y,max}$ is thus 1.64 $moles_{TC}/mole_{CH4}$ (Anderson and Mccarty, 1997a). A degradation of the primary substrate is therefore essential to obtain co-metabolic removal of the trace contaminant in biopurification systems.

The herbicide bentazone (IUPAC: 3-Isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide) is legally used in EU (Commission, 2017). It is recalcitrant (Albrechtsen et al., 2001; Broholm et al., 2001) and mobile (Boivin et al., 2004) in aquifers and is therefore frequently detected in wells used for drinking water production (detected in 3.3% of the active waterworks wells in Denmark during 1992-2015) (GEUS & Energi- Forsynings og Klimaministeriet, 2016). In soils under aerobic conditions bentazone is biodegraded to 6-OH-bentazone (IUPAC: 6-Hydroxy-3-Isopropyl-1H-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide) and 8-OH-bentazone (IUPAC: 8-

115 Hydroxy-3-Isopropyl-1H-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide) and 2-
116 amino-*N*-isopropyl-benzamide (AIBA, IUPAC: 2-amino-N-propan-2-ylbenzamide)
117 (Figure 1). Hydroxylation of the phenyl ring is the primary biodegradation pathway
118 (Huber and Otto, 1994), where e.g. 65-85% of the added bentazone is transformed to
119 8-OH-bentazone (Knauber et al., 2000). Generally, bentazone is assumed to be
120 hydroxylated by several fungal species (Huber and Otto, 1994), but both fungi and
121 bacteria may contribute to biotransformation (Knauber et al., 2000). Formed
122 transformation products are all very reactive and are thus rapidly incorporated in the
123 soil organic soil matter (Huber and Otto, 1994).

124 Biological degradation of bentazone in treatment systems can be challenging:
125 bentazone was the least degradable of four investigated pesticides (linuron,
126 metalaxyl, isoproturon and bentazone) in an on-farm biopurification system (De
127 Wilde et al., 2009). Therefore bentazone removal was surprising when observed in
128 filter material from rapid sand filters from a groundwater-based waterworks
129 (Hedegaard and Albrechtsen, 2014) receiving raw water with high methane
130 concentrations (1.1-9.2 mg/L before aeration) (Sjælsø waterworks plant II). Ring-
131 hydroxylation is a common initial step in the bentazone transformation (Figure 1)
132 (Huber and Otto, 1994) and since MMO oxidizes aromatic rings co-metabolically
133 (Semrau et al., 2010), we hypothesized that methanotrophs would be essential for the
134 rapid transformation of bentazone in filter sand. However, co-metabolic removal
135 assays with four pure MOB cultures reported no bentazone degradation (Benner et
136 al., 2015). Therefore, co-metabolic bentazone degradation has, to our knowledge, not
137 yet been documented and the aim of this study was to establish direct evidence of co-
138 metabolic degradation of bentazone by methanotrophs and determining

transformation rates, yields and specificity towards methane over bentazone. Therefore, we enriched methanotrophs from a rapid sand filter showing bentazone degradation activity to investigate the interaction between methane oxidation and bentazone removal.

2 Materials and methods

2.1 Growth of methanotrophic biomass in column reactors

Methanotrophic enrichments were cultivated in four replicate continuous flow column reactors (radius 2 cm; height 8 cm), filled with expanded clay (Filtralite NC 0.8-1.6, Saint-Gobain Weber, Norway) and initially augmented (2% v/v) with filter material from Sjælsø waterworks. The column reactors were fed with drinking water with an average methane concentration of 0.6-1.4 mg/L and the average methane consumption in the reactors were 0.14-0.56 µg CH_4/min/g carrier material (Papadopoulou et al., n.d.).

Fresh methanotrophic biomass was collected from the column reactors for each experiment, with a growth period of more than eight weeks between collections.

2.2 Experiment overview

Four experiments were conducted with the methanotrophic culture in batch experiments (Table 1):

Presence of methane (PM). We investigated bentazone removal by the methanotrophic consortium and the influence of presence/absence of methane. We also examined whether bentazone transformation resulted in a production/accumulation of transformation products.

Inhibition of MMO (IMMO). Allylthiourea (ATU) (Bédard and Knowles, 1989) and acetylene (Bédard and Knowles, 1989; Benner et al., 2015) were investigated for their ability to inhibit methane oxidation.

Partial MMO inhibition and bentazone removal (PIB). We investigated how partial inhibition of methane oxidation by acetylene affected bentazone removal, comparing removal in partially inhibited and active microcosms.

Complete MMO inhibition and bentazone removal (CIB). We investigated how complete inhibition of MMO by acetylene affected bentazone removal, comparing removal in completely inhibited and active microcosms. Concurrently, we studied how different bentazone concentrations affected the methane oxidation.

To distinguish removal of bentazone from removal of potentially formed transformation products (OH-bentazone), we primarily used high concentrations (1 mg/L) of bentazone, measured by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD). However, due to the sensitivity of this instrument it was not possible to detect low concentrations (1 µg/L) and experiments with ^{14}C-bentazone were included to investigate removal at low concentrations. Also, experiments were conducted in microcosms and lasted up to 21 days.

2.3 Microcosms and sampling

We collected the methanotrophic culture from the methane-fed column reactors with an autoclaved spoon and homogenized the sample. Within two hours, we transferred 10-20 g biomass and carrier material ($g_{b\&c}$) and 100 mL of the non-chlorinated tap water that fed the column reactors to 300 mL serum bottles (microcosms) which had been acid-washed and heated to 555°C for at least 12 hours. Microcosms were closed

with acid-washed and autoclaved Teflon coated rubber stoppers and aluminium lids and 51 mL of the headspace were replaced by methane with a syringe with a needle through the rubber stopper. We incubated the microcosms at room temperature (18-23ºC) in an orbital shaker (120-140 rpm) and pH remained at 7.5-8 during the experiments. Methane oxidation was monitored in all microcosms during the initial 3-4 days (except in experiment PM) to verify similar methanotrophic activity before addition of inhibitor and bentazone (see concentrations in Table 1).

In order to identify an appropriate inhibitor that stops the methane oxidation completely, methane, allylthiourea (ATU) or acetylene were added to microcosms that oxidized methane at a similar rate for the first 3.7 days (Figure S1). Acetylene successfully inhibited methane oxidation for more than eight days, while ATU only inhibited the methane oxidation for approx. three days (Figure S1). Thus, acetylene (26 mg/L) was chosen as inhibitor in all subsequent experiments.

To monitor aqueous bentazone concentration over time, water samples were collected with a syringe using a needle that was inserted through the rubber stopper. The collected water samples (5 mL) were replaced by 5 mL pure oxygen. The water samples were filtered through a 0.22 µm Nylon GF filter (Frisenette Aps, Q-max® GPF Syringe Filters, diameter 25 mm) and samples (2-3 mL) were analysed for ^{14}C-bentazone while the remaining fraction was measured for bentazone concentration by HPLC-DAD or high-resolution mass spectrometry (HRMS) (see below).

Methane and pure oxygen were added at least every fourth day in PM and PIB experiments by replacing a volume of the headspace (typically 60 mL) by a mixture

206 of 2:1 v/v of pure oxygen and methane (as stoichiometrically required for the
207 oxidation of methane).

208 When methane concentration in water phase got below 4 mg/L in the CIB
209 experiment, methane was either added directly or microcosms were opened and
210 flushed with air for at least one hour, subsequently closed and methane and pure
211 oxygen (2:1 v/v of pure oxygen and methane) were added to the headspace.

212 Neither methane nor oxygen was added to the inhibited microcosms after acetylene
213 was added.

214 *2.4 Methane and oxygen measurements*

215 A sample of 50 µL was collected from headspace of the microcosms and analysed
216 immediately on GC-FID (see SI for details).

217 Aqueous oxygen saturation was monitored during the experiment by Oxygen-
218 Sensitive Minisensors and a fiber optic oxygen meter (Fibox 3, Loligo Systems
219 ApS). The measurement was based on a two point calibration and the limit of
220 detection was 0.01%, and the objective of the measurement was primarily to
221 document aerobic conditions during the experiment. Thus, in some cases oxygen was
222 measured before addition of oxygen.

223 *2.5 Bentazone measurements by High-Performance Liquid Chromatography with*
224 *Diode-Array Detection (HPLC-DAD)*

225 Water samples were either immediately frozen or preserved with acetic acid (final
226 concentration of 0.1 M) until analysis within a few days; investigations showed no
227 difference between the two different ways of storage. The samples which had been
228 stored frozen were acidified by acetic acid prior to injection on an Ultimate 3000

HPLC-DAD system (Thermo Scientific) (see SI). Presented graphs and removal rates are based on either frozen samples or samples preserved in acetic acid.

2.6 ^{14}C-Carbonyl-bentazone measurements

The ^{14}C-activity of bentazone in the water phase and the produced $^{14}CO_2$ from bentazone mineralization were quantified by a double vial system, where produced $^{14}CO_2$ in the water was stripped off by acidification and captured by a base trap (1 mL 2 M NaOH) (Janniche et al., 2010). The ^{14}C-activity was quantified using a liquid scintillation counter (Hidex 300 SL, 1414 Liquid Scintillation Counter, MikroWin 2000 software). The concentration at a given sampling time was expressed as a fraction of the initial concentration and corrected for the removed mass during sampling (given as $^{14}C/^{14}C_0$ (%)).

2.7 Analysis of bentazone transformation products by high-resolution mass spectrometry

Water samples were analysed for transformation products by high-resolution mass spectrometry (HRMS) using an Orbitrap Velos Fourier Transform Mass Spectrometer coupled to an Accela HPLC system (all Thermo Scientific, Bremen, Germany) with an electrospray ionization (ESI) interface (see SI).

2.8 Estimation of methanotroph abundance using Real-time quantitative PCR (qPCR)

After two weeks and again after one year of operation the methanotrophic enrichment culture was collected from three column reactors and manually blended. The material was drained and stored at -20^{o}C. At the end of the CIB experiments, 2 mL samples containing both water and biomass were taken from the microcosms and

252 after centrifugation the water phase was discharged and the biomass was stored
253 concentrated as a pellet at -80°C until analysis. All bacteria (Eubacteria – targeting
254 the 16S rRNA gene) and methanotrophs (targeting *pmoA*) were quantified by real-
255 time quantitative PCR (qPCR) (see SI). Microbial abundances were calculated under
256 the assumption of an average of two copies of 16S rRNA (Klappenbach, 2001; Lee et
257 al., 2006) or *pmoA* (Semrau et al., 1995; Stolyar et al., 1999) genes per genome (cell)
258 and were subsequently converted to cell densities per mass of filter material (drained
259 wet weight).

260 *2.9 Chemicals*

261 Allylthiourea (ATU) and cold bentazone were dissolved in sterile MilliQ water.
262 Mineralization and removal at very low concentrations (1-2 µg/L) were investigated
263 by [carbonyl-^{14}C]-bentazone (Izotop, Institute of Isotopes Co., Ltd., Hungary) in two
264 experiments (Table 1). The radiochemical purity of [carbonyl-^{14}C]-bentazone was
265 100% (chemical purity 99.77%) according to the manufacturer and a stock solution
266 was prepared in sterile MilliQ water. Bentazone (chemical purity 99.1%, Dr.
267 Ehrenstorfer GmbH), 6-OH-bentazone (chemical purity 97%, TRC, Toronto
268 Research Chemicals Inc., Ontario, Canada) and 8-OH-bentazone (chemical purity
269 97%, TRC, Toronto Research Chemicals Inc., Ontario, Canada) were all dissolved in
270 sterile ultrapure water at least one day prior to the experiment. The concentration was
271 verified by HPLC-DAD immediately before the experiment. Acetylene was added
272 from a gas flask (see SI).

273 *2.10 Statistics*

274 We used the statistical software GraphPad Prism 5 for data treatment.

3 Results and discussion

3.1 Growth of methanotrophic culture

We successfully enriched methanotrophs in the column reactors: the abundance of methanotrophs increased from 1.04×10^4 cells/g carrier material after two weeks growth to 2.55×10^7 cells/g carrier material after more than one year of enrichment. The fraction of methanotrophs compared to the total number of bacteria increased from 1.7 % after two weeks to 12% after one year (Figure S2) and was larger in column reactors and microcosms than in the full-scale rapid sand filters (8.5×10^5 cells/g carrier material, 1.3%).

3.2 Effect of methane on bentazone removal (PM)

The methanotrophic enrichment demonstrated a bentazone removal rate of 42-75 ρmoles/h/$g_{b\&c}$ during the experiment *'Presence of methane'* (PM) (Figure 2). Hence, up to 53% of the initial mass of bentazone was removed after 21 days in microcosms with 5 mg/L methane, while only 31% was removed in microcosms without methane (Figure 2). This was confirmed in a replicate experiment showing a bentazone removal rate of 116 ρmoles/h/$g_{b\&c}$ in presence of methane and 35 ρmoles/h/$g_{b\&c}$ in absence of methane during the first seven days (data not shown).

3.3 Inhibition of methane oxidation and its effect on bentazone removal (CIB)

To investigate how bentazone removal depended on methane oxidation, we inhibited methane oxidation with acetylene. Acetylene functions as a suicide substrate towards MMO and causes a rapid and irreversible self-inactivation by formation of reactive intermediates which binds to the active site of the hydroxylase subunit (component A) (Prior and Dalton, 1985; Sullivan and Chase, 1996).

Before acetylene and bentazone were added, all microcosms demonstrated similar methane consumption rates: 1.3-2.0 µmole methane/h/$g_{b\&c}$ (Figure 3B, time period: -5 to -1 days), in CIB experiments. Acetylene addition (time: -1 day) successfully stopped methane consumption in the inhibited microcosms (Figure 3B). Addition of acetylene also stopped oxygen consumption in the inhibited microcosms, while the active microcosms continuously consumed oxygen (Figure 3D). Other oxygen-consuming metabolic activity was therefore negligible.

At day 0 bentazone was added to all microcosms (experiment CIB). The methane consumption rate clearly followed a linear trend (Figure 3B and Table 2), and, assuming that bentazone removal also depended on the activity of the MMO, a simple linear regression model (removed mass versus time) was applied to describe bentazone removal (Figure 3A and Table 2). In active microcosms the bentazone removal rate was 37 ± 5.0 ρmole/h/$g_{b\&c}$ (r^2 = 0.77), and was thus significantly larger than in the inhibited microcosms ($P<0.0001$, including both samples preserved frozen and in acetic acid; even when the outlier in the inhibited microcosms at day 15 is included P=0.00084) in which the removal rate, 4.3 ± 4.2 ρmole/h/$g_{b\&c}$ (r^2 = 0.06), was not significantly different from zero (Figure 3A).

A similar abundance of *pmoA* genes in all microcosms at the end of the experiment CIB confirmed that the difference in methane consumption and bentazone removal between active and inhibited microcosms was not caused by a difference in the abundance of methanotrophs (Figure S2). Hence, addition of acetylene inhibited *both* methane oxidation and bentazone removal.

3.4 Transformation yield of bentazone versus methane removal (PIB and CIB)

The transformation yield, $T_{y,BTZ/CH4}$, expresses the bentazone (BTZ) removal rate over the methane removal rate. Removal rates were estimated by linear regression models (removed mass of bentazone or methane per time) at three different methane consumption rates and in two independent experiments (Table 2). At similar concentrations of bentazone (0.7-0.9 mg/L) and methane (approx. 5 mg/L) the consumption rate of bentazone followed the consumption rate of methane, and e.g. in the experiment *'Partial MMO inhibition and bentazone removal'* (PIB) the lower methane consumption rate in the partially inhibited control (compared to active microcosms) was accompanied by a correspondingly slow bentazone removal (Table 2). Thus, the transformation yield, $T_{y,BTZ/CH4}$, varied between 0.6×10^{-4} and 1.7×10^{-4} $mole_{BTZ}/mole_{CH4}$ for active and partially inhibited microcosms in two independent experiments (Table 2). The transformation yields were thus within a factor three across our experiments (Table 2), which strongly indicates an association between methane monooxygenase activity and bentazone removal.

This measured transformation yield was in the low range of values reported for the trace pollutants (TC) chlorinated aliphatic hydrocarbons (2.2×10^{-4} to 6.3×10^{-1} $mole_{TC}/mole_{CH4}$) (Table 3). However, the ratio $C_{BTZ/CH4}$, between the secondary substrate, bentazone, and the primary substrate, methane, was close to environmentally-relevant conditions in the present study (9.6×10^{-3} $mole_{BTZ}/mole_{CH4}$) but low compared to previous studies: 1.1×10^{-2} to 6.5 $mole_{TC}/mole_{CH4}$ (Table 3). The large difference in the relative abundance of primary and secondary substrates can make the comparison of the transformation yields between different studies of no relevance.

Assuming a constant number of active enzymes in our experiments (as indicated by linear consumption of methane with time), we expect that an increased abundance of a secondary substrate relative to the primary would result in an increased transformation yield of secondary compared to primary substrate. To establish a metric independent of the substrates relative concentrations, we suggest to normalize the transformation yield with respect to the concentration ratio between secondary and primary substrate, $T_y^*{}_{,CH4/TC}$:

$$T_y^*{}_{,CH4/TC} = T_{y,TC/CH4}{}^{-1} / C_{TC/CH4}{}^{-1} = T_{y,CH4/TC} / C_{CH4/TC}$$

Where $T_y^*{}_{,CH4/TC}$ is the CH_4/TC-normalized transformation yield of MMO for oxidizing methane over the trace contaminant, $T_{y,TC/CH4}$ is the transformation yield of the trace contaminant relative to methane and $C_{TC/CH4}$ is the concentration ratio between secondary and primary substrates. From reported data we calculated the CH_4/TC-normalized transformation yields, $T_y^*{}_{,CH4/TC}$, ranging from 3 to 400, and $T_y^*{}_{,CH4/BTZ}$ in our study (58-158) was thus within this range (Table 3). Hence, the CH_4/TC normalized transformation yields show that, in a situation with an even presence of bentazone and CH_4-molecules, bentazone would at maximum be oxidized in 1 out of 58 incidences. Similar magnitude in the preference of MMO for methane over other trace contaminants, indicates that the removal mechanism of bentazone is similar to co-metabolic degradation of other trace contaminants by MMO.

3.5 Effect of bentazone on the methane oxidation (CIB)

After bentazone addition at day 0 in the CIB experiment, the methane consumption was 1.5 $\mu mole_{CH4}/h/g_{b\&c}$ in active microcosms at low (1 µg/L) bentazone

concentrations, which was similar to before bentazone addition (1.3-2.0 $\mu mole_{CH4}/h/g_{b\&c}$ in all microcosms and inhibited controls) (Figure 3B). In contrast, in active microcosms at high bentazone concentration (1 mg/L), methane consumption decreased to 0.6 $\mu mole_{CH4}/h/g_{b\&c}$ (Figure 3B). Thus, a high bentazone concentration led to a significantly lower ($P<0.0001$) methane consumption rate than a low bentazone concentration, at similar conditions (oxygen and methane concentration, number of methanotrophs and methanotrophs/total bacteria (Figure S2)).

Oxidation of trace pollutants can negatively affect the methane oxidation due to 1) competition for binding to MMO; 2) consumption of reducing equivalents; and 3) toxic effects (Alvarez-Cohen and McCarty, 1991; Semrau et al., 2010). The theoretical upper limit for transformation yields on 1.64 $mole_{TC}/mole_{CH4}$ (Anderson and Mccarty, 1997a) and transformation yields found in literature (Table 3) are much higher than the measured bentazone transformation yields ($T_{y,BTZ/CH4}$ = 0.6×10^{-4} to 1.7×10^{-4} $mole_{BTZ}/mole_{CH4}$). Therefore the reduced methane oxidation rate at high bentazone concentrations was unlikely only the result of excessive consumption of reducing equivalents. No toxic effects have been reported for bentazone in soil microbial community toxicity tests or in Microtox tests at 2 mg /L, the maximum concentration applied in our study (Allievi et al., 1996; Ruiz et al., 1997). Also the bentazone degradation products 6-OH-bentazone and 8-OH-bentazone are less acute toxic than the parent compound (Kanungo et al., 2012). We posit that the reduced methane consumption at high bentazone concentrations was, in part, due to competitive inhibition of methane oxidation by bentazone. Yet, the decrease in methane consumption was disproportionally high and not consistent with simple competitive inhibition. We speculate additional MMO inactivation caused by

accumulation of a toxic bentazone transformation products, as shown for MMO-driven co-metabolic transformation of TCE in previous studies (Semprini et al., 1990; Suttinun et al., 2013). At environmentally relevant bentazone concentrations and bentazone/methane ratios (1 µg/L, 1.4×10^{-5} $mole_{BTZ}/mole_{CH4}$), methane oxidation was not affected.

3.6 Formation of bentazone transformation products (CIB, PIB and PM)

Quantification of bentazone removal by ^{14}C-carbonyl-bentazone only allowed determination of complete removal from the water phase, and, accordingly, a transformation from bentazone to hydroxy-bentazone would not be detected, since the ^{14}C-carbonyl-group would still be present in the transformation products (Figure 1). During the experiments there was no significantly different loss of ^{14}C from the water phase in inhibited and active microcosms (Figure 3B), and no $^{14}CO_2$ from bentazone mineralization was detected (measured in PIB experiment - data not shown). Indicating that bentazone was only transformed and not mineralized by the methanotrophic culture.

Measurements by HRMS confirmed an accumulation of four bentazone transformation products (6-OH-bentazone, 8-OH-bentazone, isopropyl-OH-bentazone and dihydroxy-bentazone) in the water phase during bentazone degradation by the methanotrophic enrichment culture (Figure 2; Figure 1; Table S1 in SI). The chemical structures of 6-OH-bentazone and 8-OH-bentazone were confirmed by comparison with commercially available reference standards. Even though no reference standard was available for isopropyl-OH-bentazone (IUPAC: 3-(1-hydroxypropan-2-yl)-1*H*-benzo[c][2,1,3]thiadiazin-4(3*H*)-one-2,2-dioxide), hydroxylation of the isopropyl moiety was clearly indicated by cleavage of C_3H_6O

415 instead of C_3H_6 as in bentazone and the ring hydroxylated TPs (Table S1 in SI). A
416 dihydroxylated bentazone TP (di-OH-bentazone) was also detected based on the
417 exact mass determinations, however, concentrations were too low to obtain
418 fragmentation data from MS^2 experiments and thus, it was not possible to determine
419 the exact position of the hydroxylation. After 21 days, four times more isopropyl-
420 OH-, 132 times more 6-OH- and 85 times more 8-OH-bentazone were observed in
421 presence versus absence of methane. In addition, formation of di-OH-bentazone was
422 only observed in the presence of methane (Figure 2; Figure 1).

423 The two substituents on the aromatic ring of bentazone are a strongly electron
424 donating secondary amine and an electron withdrawing ketone-group (McMurry and
425 Simanek, 2007), both substituents are directing oxidation towards the 6-OH- (para-
426 position with respect to the donating amine group) and 8-OH- (ortho-) position of
427 bentazone, which were both formed during degradation of bentazone in the
428 methanotrophic culture. Methanotrophic oxidation is typically regioselective towards
429 the para-position of monosubstituted aromatic compounds (Anthony, 1986; Dalton
430 and Leak, 1985; Lindner et al., 2000). Our results indicate a similar formation of 6-
431 OH- and 8-OH-bentazone by the methanotrophic culture (Figure 1), though presence
432 of methane had the largest impact (concentration increased 132 times) on the
433 formation of 6-OH-bentazone (para-position). This is in contrast to field soils where
434 primary 8-OH-bentazone was formed (Knauber et al., 2000), indicating the
435 involvement of an additional transformation process. This was also indicated by the
436 formation of isopropyl-OH- and di-OH-bentazone in our study, which so far has not
437 been reported as a transformation product (Huber and Otto, 1994).

In fresh field soils, 6-OH- and 8-OH-bentazone were further metabolised faster than they were formed from degradation of bentazone (Huber and Otto, 1994; Knauber et al., 2000). The accumulation of OH-transformation products in the water phase illustrated that the methanotrophic enrichment culture only performed the primary transformation step and that other metabolic pathways capable of metabolizing 6-OH- and 8-OH-bentazone were not sufficiently abundant to substantially degrade these transformation products.

It is commonly accepted that sMMO oxidizes aromatic rings, while pMMO cannot attack these structures (Burrows et al., 1984; Semrau et al., 2010). The acetylene concentrations applied in our investigations, 16 mg/L (614 µM) and 26 mg/L (998 µM), are reported not to completely inhibit pMMO, but are reported to inhibit sMMO (Lontoh et al., 2000). Hence, the complete inhibition of the methane oxidation at both 16 mg/L (614 µM) and 26 mg/L (998 µM) (Figure S1) supports the involvement of sMMO in bentazone degradation.

4 Conclusion

We investigated the first step in the transformation of bentazone – the biological hydroxylation - and provided a suite of evidence supporting that bentazone can be co-metabolically transformed to hydroxy-bentazone transformation products by a methanotrophic culture. This conclusion is based on the following lines of evidence:

- Bentazone was removed from the water phase in contact with methanotrophic culture enriched from a rapid sand filter.
- The presence of methane stimulated the removal rate of bentazone.

- Inhibiting the methane oxidation by acetylene also halted bentazone removal.
- The CH_4/TC-normalized transformation yield, $T_y^*{}_{,CH4/BTZ}$, for bentazone ranged from 58 to 158 which is comparable to CH_4/TC-normalized transformation yields of methanotrophic co-metabolism calculated from the literature (3-400).
- The methane consumption rate was significantly lower at high bentazone concentrations (1 mg/L) than at low concentrations (1 µg/L), which indicated one-way competitive inhibition of bentazone towards methane.
- Presence of methane stimulated formation of hydroxylated bentazone transformation products.

Even though the experiments were conducted with a long term methanotrophic enrichment culture, the enrichment was still a complex community containing many non-methanotrophs. Therefore, obtaining a full enzymatic proof of the hydroxylation of bentazone by MMO would require further studies including pure cultures of methanotrophs.

Acknowledgements

This research was partly financed by the Grundfos Prize. The authors thank the staff at Sjælsø waterworks, Nordvand A/S, for help with filter sand collection, Aikaterini Papadopoulou (MIRESOWA project) for constructing the column reactors and Mikael Emil Olsson for technical assistance. We confirm that there are no known conflicts of interest associated with this publication.

5 References

Albrechtsen, H.J., Mills, M.S., Aamand, J., Bjerg, P.L., 2001. Degradation of herbicides in shallow Danish aquifers: An integrated laboratory and field study, in: Pest Management Science. pp. 341–350. doi:10.1002/ps.305

Alexander, M., 1994. Biodegradation and bioremediation, 2nd ed. Academic Press.

Allievi, L., Gigliotti, C., Salardi, C., Valsecchi, G., Brusa, T., Ferrari, A., 1996. Influence of the herbicide bentazon on soil microbial community. Microbiol. Res. 151, 105–111. doi:10.1016/S0944-5013(96)80064-4

Alvarez-Cohen, L., McCarty, P.L., 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57, 228–235.

Alvarez-Cohen, L., McCarty, P.L., Boulygina, E., Hanson, R.S., Brusseau, G.A., Tsien, H.C., 1992. Characterization of a methane-utilizing bacterium from a bacterial consortium that rapidly degrades trichloroethylene and chloroform. Appl. Environ. Microbiol. 58, 1886–1893.

Anderson, J.E., Mccarty, P.L., 1997a. Transformation yields of chlorinated ethenes by a methanotrophic mixed culture expressing particulate methane monooxygenase. Appl. Environ. Microbiol. 63, 687–693.

Anderson, J.E., Mccarty, P.L., 1997b. Effect of chlorinated ethenes on S(min) for a methanotrophic mixed culture. Environ. Sci. Technol. 31, 2204–2210. doi:10.1021/es9606687

501 Anthony, C., 1986. Bacterial Oxidation of Methane and Methanol, Advances in
502 Microbial Physiology. doi:10.1016/S0065-2911(08)60305-7

503 Arvin, E., 1991. Biodegradation kinetics of chlorinated aliphatic hydrocarbons with
504 methane oxidizing bacteria in an aerobic fixed biofilm reactor. Water Res. 25, 873–
505 881. doi:10.1016/0043-1354(91)90168-P

506 Bédard, C., Knowles, R., 1989. Physiology, biochemistry, and specific inhibitors of
507 CH4, NH4+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53,
508 68–84. doi:0146-0749/89/010068-17

509 Benner, J., De Smet, D., Ho, A., Kerckhof, F.M., Vanhaecke, L., Heylen, K., Boon,
510 N., 2015. Exploring methane-oxidizing communities for the co-metabolic
511 degradation of organic micropollutants. Appl. Microbiol. Biotechnol. 99, 3609–3618.
512 doi:10.1007/s00253-014-6226-1

513 Benner, J., Helbling, D.E., Kohler, H.E., Wittebol, J., Kaiser, E., Prasse, C., Ternes,
514 T.A., Albers, C.N., Aamand, J., Horemans, B., Springael, D., Walravens, E., Boon,
515 N., 2013. Is biological treatment a viable alternative for micropollutant removal in
516 drinking water treatment processes ? Water Res. 47, 5955–5976.
517 doi:10.1016/j.watres.2013.07.015

518 Boivin, A., Cherrier, R., Perrin-Ganier, C., Schiavon, M., 2004. Time effect on
519 bentazone sorption and degradation in soil. Pest Manag. Sci. 60, 809–814.
520 doi:10.1002/ps.889

521 Broholm, M.M., Rügge, K., Tuxen, N., Højberg, A.L., Mosbaek, H., Bjerg, P.L.,
522 2001. Fate of herbicides in a shallow aerobic aquifer: A continuous field injection

experiment (Vejen, Denmark). Water Resour. Res. 37, 3163–3176. doi:10.1029/2000WR000002

Burrows, K.J., Cornish, A., Scott, D., Higgins, I.J., 1984. Substrate specificities of the soluble and particulate methane mono-oxygenases of Methylosinus trichosporium OB3b. J. Gen. Microbiol. 130, 3327–3333. doi:10.1099/00221287-130-12-3327

Commission, E., 2017. EU pesticides database [WWW Document]. URL http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN

Dalton, H., Leak, D.J., 1985. Mechanistic studies on the mode of action of methane monooxygenase. Gas Enzymol. 169–186.

Dalton, H., Stirling, D.I., 1982. Co-metabolism. Philos. Trans. R. Soc. London. Ser. B Biol. Sci. 297, 481–496.

Dawas-Massalha, A., Gur-Reznik, S., Lerman, S., Sabbah, I., Dosoretz, C.G., 2014. Co-metabolic oxidation of pharmaceutical compounds by a nitrifying bacterial enrichment. Bioresour. Technol. 167, 336–342. doi:10.1016/j.biortech.2014.06.003

De Wilde, T., Spanoghe, P., Mertens, J., Sniegowksi, K., Ryckeboer, J., Jaeken, P., Springael, D., 2009. Characterizing pesticide sorption and degradation in macro scale biopurification systems using column displacement experiments. Environ. Pollut. 157, 1373–1381. doi:10.1016/j.envpol.2008.11.032

DiSpirito, A.A., Gulledge, J., Shiemke, A.K., Murrell, J.C., Lidstrom, M.E., Krema, C.L., 1991. Trichloroethylene oxidation by the membrane-associated methane

545 monooxygenase in type I, type II and type X methanotrophs. Biodegradation 2, 151–
546 164. doi:10.1007/BF00124489

547 Dolan, M.E., McCarty, P.L., 1995. Small-column microcosm for assessing methane-
548 stimulated vinyl chloride transformation in aquifer samples. Environ. Sci. Technol.
549 29, 1892–1897. doi:10.1021/es00008a005

550 European Community, 2000. Directive 2000/60/EC of the European Parliament and
551 of the Council of 23 October 2000 establishing a framework for Community action
552 in the field of water policy. Off. J. Eur. Parliam. L327, 1–82.
553 doi:10.1039/ap9842100196

554 European Union, 2006. Directive 2006/118/EC of the European Parliament and of
555 the council of 12 December 2006 on the protection of groundwater against pollution
556 and deterioration. Off. J. Eur. Union 19, 19–31. doi:http://eur-lex.europa.eu/legal-
557 content/EN/TXT/?uri=CELEX:32006L0118

558 Fennell, D.E., Nelson, Y.M., Underhill, S.E., White, T.E., Jewell, W.J., 1993. TCE
559 degradation in a methanotrophic attached???film bioreactor. Biotechnol. Bioeng. 42,
560 859–872. doi:10.1002/bit.260420711

561 Forrez, I., Carballa, M., Noppe, H., De Brabander, H., Boon, N., Verstraete, W.,
562 2009. Influence of manganese and ammonium oxidation on the removal of 17??-
563 ethinylestradiol (EE2). Water Res. 43, 77–86. doi:10.1016/j.watres.2008.10.006

564 GEUS & Energi- Forsynings og Klimaministeriet, 2016. Grundvandsovervågningen
565 1989 – 2015.Hazen, T.C., Chakraborty, R., Fleming, J.M., Gregory, I.R., Bowman,
566 J.P., Jimenez, L., Zhang, D., Pfiffner, S.M., Brockman, F.J., Sayler, G.S., 2009. Use

567 of gene probes to assess the impact and effectiveness of aerobic in situ
568 bioremediation of TCE. Arch. Microbiol. 191, 221–232. doi:10.1007/s00203-008-
569 0445-8

570 Hedegaard, M.J., Albrechtsen, H.J., 2014. Microbial pesticide removal in rapid sand
571 filters for drinking water treatment - Potential and kinetics. Water Res. 48, 71–81.
572 doi:10.1016/j.watres.2013.09.024

573 Helbling, D.E., Johnson, D.R., Honti, M., Fenner, K., 2012. Micropollutant
574 biotransformation kinetics associate with WWTP process parameters and microbial
575 community characteristics. Environ. Sci. Technol. 46, 10579–10588.
576 doi:10.1021/es3019012

577 Huber, R., Otto, S., 1994. Environmental behavior of bentazon.pdf. Rev. Environ.
578 Contam. andToxicology 137, 111–134.

579 Iwamoto, T., Nasu, M., 2001. Current bioremediation practice and perspective. J.
580 Biosci. Bioeng. 92, 1–8. doi:10.1016/S1389-1723(01)80190-0

581 Janniche, G.S., Lindberg, E., Mouvet, C., Albrechtsen, H.J., 2010. Mineralization of
582 isoproturon, mecoprop and acetochlor in a deep unsaturated limestone and sandy
583 aquifer. Chemosphere 81, 823–831. doi:10.1016/j.chemosphere.2010.08.023

584 Janssen, D.B., Grobben, G., Hoekstra, R., Oldenhuis, R., Witholt, B., 1988.
585 Degradation of trans-1,2-dichloroethene by mixed and pure cultures of
586 methanotrophic bacteria. Appl. Microbiol. Biotechnol. 29, 392–399.
587 doi:10.1007/BF00265825

588 Jiang, H., Chen, Y., Jiang, P., Zhang, C., Smith, T.J., Murrell, J.C., Xing, X.-H.,
589 2010. Methanotrophs: Multifunctional bacteria with promising applications in
590 environmental bioengineering. Biochem. Eng. J. 49, 277–288.
591 doi:10.1016/j.bej.2010.01.003

592 Kanungo, D., Dellarco, V., Davies, L., 2012. Bentazone. World Heal. Organ. 4, 31–
593 98.

594 Klappenbach, J.A., 2001. rrndb: the Ribosomal RNA Operon Copy Number
595 Database. Nucleic Acids Res. 29, 181–184. doi:10.1093/nar/29.1.181

596 Knauber, W.R., Krotzky, A.J., Schink, B., 2000. Microbial metabolism and further
597 fate of bentazon in soil. Environ. Sci. Technol. 34, 598–603. doi:10.1021/es990426h

598 Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative QPCR
599 quantification of plasmid copy number in Escherichia coli. J. Biotechnol. 123, 273–
600 280. doi:10.1016/j.jbiotec.2005.11.014

601 Lindner, A., Adriaens, P., Semrau, J., 2000. Transformation of ortho-substituted
602 biphenyls by Methylosinus trichosporium {OB3b:} substituent effects on oxidation
603 kinetics and product formation. Arch. Microbiol. 174, 35–41.
604 doi:10.1007/s002030000170

605 Lontoh, S., Dispirito, A.A., Krema, C.L., Whittaker, M.R., Hooper, A.B., Semrau,
606 J.D., 2000. Differential inhibition in vivo of ammonia monooxygenase, soluble
607 methane monooxygenase and membrane-associated methane monooxygenase by
608 phenylacetylene. Environ. Microbiol. 2, 485–494. doi:10.1046/j.1462-
609 2920.2000.00130.x

610 McMurry, J., Simanek, E., 2007. Fundamentals of Organic Chemistry, sixth edit. ed.
611 Thomson Brooks/Cole.

612 Nelson, Y.M., Jewell, W.J., 1993. Vinyl chloride biodegradation with
613 methanotrophic attached films. J. Environ. Eng. 119, 890–907.

614 Oldenhuis, R., Vink, R.L.J.M., Janssen, D.B., Witholt, B., 1989. Degradation of
615 chlorinated aliphatic hydrocarbons by Methylosinus trichosporium OB3b expressing
616 soluble methane monooxygenase. Appl. Environ. Microbiol. 55, 2819–2826.

617 Papadopoulou, A., Hedegaard, M.J., Dechesne, A., Albrechtsen, H.-J., Musovic, S.,
618 Smets, B.F., n.d. Methanotrophic contribution to phenoxy acids degradation by
619 cultures enriched from a groundwater-fed rapid sand filter. Prep.

620 Phelps, T.J., Niedzielski, J.J., Schram, R.M., Herbes, S.E., White, D.C., 1990.
621 Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors.
622 Appl. Environ. Microbiol. 56, 1702–1709.

623 Prior, S.D., Dalton, H., 1985. Acetylene as a suicide substrate and active site probe
624 for methane monooxygenase from Methylococcus capsulatus (Bath). FEMS
625 Microbiol. Lett. 29, 105–109.

626 Ruiz, M.J., Redondo, M.J., Font, G., 1997. Toxicity Assessment of Pesticides Using
627 the Microtox Test : Application to Environmental Samples. Bull. Environ. Contam.
628 Toxicol. 59, 619–625. doi:10.1007/s001289900524

629 Semprini, L., McCarty, P.L., 1991. Comparison between model stimulations and
630 field results for in-situ biorestoration of chlorinated aliphatics: Part 1. Biostimulation
631 of methanotrophic bacteria. Groundwater 29, 365–374.

632 Semprini, L., Roberts, P. V., Hopkins, G.D., McCarty, P.L., 1990. A Field
633 Evaluation of In-Situ Biodegradation of Chlorinated Ethenes: Part 2, Results of
634 Biostimulation and Biotransformation Experiments. Groundwater.
635 doi:10.1111/j.1745-6584.1990.tb01987.x

636 Semrau, J.D., Chistoserdov, a, Lebron, J., Costello, a, Davagnino, J., Kenna, E.,
637 Holmes, a J., Finch, R., Murrell, J.C., Lidstrom, M.E., 1995. Particulate methane
638 monooxygenase genes in methanotrophs . These include : Particulate Methane
639 Monooxygenase Genes in Methanotrophs 177.

640 Semrau, J.D., Dispirito, A.A., Yoon, S., 2010. Methanotrophs and copper. FEMS
641 Microbiol. Rev. doi:10.1111/j.1574-6976.2010.00212.x

642 Semrau, J.D., Jagadevan, S., Dispirito, A.A., Khalifa, A., Scanlan, J., Bergman, B.H.,
643 Freemeier, B.C., Baral, B.S., Bandow, N.L., Vorobev, A., Haft, D.H., Vuilleumier,
644 S., Murrell, C.J., 2013. Methanobactin and MmoD work in concert to act as the
645 “copper-switch” in methanotrophs. Environ. Microbiol. 15, 3077–3086.
646 doi:10.1111/1462-2920.12150

647 Sirajuddin, S., Rosenzweig, A.C., 2015. Enzymatic oxidation of methane.
648 Biochemistry 54, 2283–2294. doi:10.1021/acs.biochem.5b00198

649 Smith, L.H., McCarty, P.L., 1997. Laboratory evaluation of a two-stage treatment
650 system for TCE cometabolism by a methane-oxidizing mixed culture. Biotechnol.
651 Bioeng. 55, 650–659. doi:10.1002/(SICI)1097-0290(19970820)55:4<650::AID-
652 BIT7>3.0.CO;2-G

653 Stolyar, S., Costello, A.M., Peeples, T.L., Lidstrom, M.E., 1999. Role of multiple
654 gene copies in particulate methane monooxygenase activity in the methane-oxidizing
655 bacterium Methylococcus capsulatus Bath. Microbiology 145, 1235–1244.
656 doi:10.1099/13500872-145-5-1235

657 Strong, P.J., Xie, S., Clarke, W.P., 2015. Methane as a resource: Can the
658 methanotrophs add value? Environ. Sci. Technol. doi:10.1021/es504242n

659 Sullivan, J.P., Chase, H.A., 1996. 1,2,3-Trichlorobenzene transformation by
660 Methylosinus trichosporium OB3b expressing soluble methane monooxygenase.
661 Appl. Microbiol. Biotechnol. 45, 427–433.

662 Suttinun, O., Luepromchai, E., Müller, R., 2013. Cometabolism of trichloroethylene:
663 Concepts, limitations and available strategies for sustained biodegradation. Rev.
664 Environ. Sci. Biotechnol. doi:10.1007/s11157-012-9291-x

665 Trotsenko, Y.A., Murrell, J.C., 2008. Metabolic aspects of aerobic obligate
666 methanotrophy. Adv. Appl. Microbiol. 63, 183–229. doi:10.1016/S0065-
667 2164(07)00005-6

668 Xu, Y., Yuan, Z., Ni, B.J., 2017. Impact of Ammonium Availability on Atenolol
669 Biotransformation during Nitrification. ACS Sustain. Chem. Eng. 5, 7137–7144.
670 doi:10.1021/acssuschemeng.7b01319

671

672 **Fig. 1 Degradation pathway of bentazone**. In contact with methanotrophic culture
673 bentazone degradation led to accumulation of four transformation products (green).
674 Isopropyl-OH-bentazone and di-OH-bentazone have, to the authors' knowledge, not
675 previously been reported as transformation products in soil metabolism pathways
676 (grey – incl. 6-OH- and 8-OH-bentazone) (modified from Huber and Otto, 1994).

677

678 **Fig. 2 Effect of methane on removal of bentazone and formation of bentazone**
679 **transformation products by the methanotrophic enrichment culture.** Bentazone
680 measured by HPLC-DAD (duplicates) and isopropyl-OH-, 6-OH-, 8-OH-bentazone
681 and di-OH-bentazone measured by High-res-MS as peak areas (single microcosm).
682 Microcosms with 10 g methane enriched biomass and carrier material and 100 mL
683 tap water with methane (approx. 5 mg/L in the water) or without methane.

684

685 **Fig. 3 Bentazone removal, methane and oxygen consumption by the**
686 **methanotrophic enrichment culture.** Removal in active microcosms and
687 microcosms with acetylene (both in triplicates) with 10 g biomass and carrier
688 material, 100 mL tap water, approx. 5 mg/L methane and bentazone at high (1 mg/L)
689 and low (1 µg/L) concentrations. **A)** Bentazone concentration measured by HPLC-
690 DAD, linear regression curves (full lines) with 95% confidence intervals (dotted
691 lines). The red dot marks an outlier (not included in the regression). **B)** Methane
692 consumption. **C)** ^{14}C-bentazone concentration given as percentage of initial
693 concentration. **D)** % oxygen saturation in the water phase. Acetylene (26 mg/L) was
694 added to inhibited microcosms (grey dotted line at time -1 day) prior to bentazone

addition (time 0). When $C_{w,CH4}$ < 4 mg/L methane was either added directly or microcosms were flushed with air, and methane and oxygen were added subsequently (2:1 v_{O2}/v_{CH4}).

Table 1 Experimental conditions. Overview of the set-up in the four experiments: Presence of methane (PM), Inhibition of MMO (IMMO), Partial MMO inhibition and bentazone removal (PIB), Complete MMO inhibition and bentazone removal (CIB). Concentrations are all given for the water phase.

Experiment	**PM**	**IMMO**	**PIB**	**CIB**	
				High conc.	Low conc.
Number active microcosms	6*	2	3	3	3
Number inhibited microcosms	-	4	2	3	3
Biomass and carrier material (g)	10	10	20	10	10
Bentazone (mg/L)	1.7-1.8	-	0.8-0.9	0.7	-
^{14}C-bentazone (µg/L)	-	-	1.4	1.6	1.6
Microcosms with CH_4	3 (Maintained at 5 mg CH_4/L)	6 (Initial injection to 5 mg CH_4/L)	5 (initial injection to 5 mg CH_4/L)	12 (Maintained at 5 mg CH_4/L)	
Microcosms w/o CH_4	3	-	-	-	
Inhibitor	-	ATU: 1.2 mg/L and 2.4 mg/L Acetylene: 16 mg/L and 26 mg/L	Acetylene: 16 mg/L and 26 mg/L	Acetylene: 26 mg/L	
Conservation of bentazone samples	Frozen	-	Frozen	Acetic acid	

*Four microcosms analysed by HPLC-DAD – results in Figure 2. Two microcosms analysed by High-res-MS for bentazone and transformation products

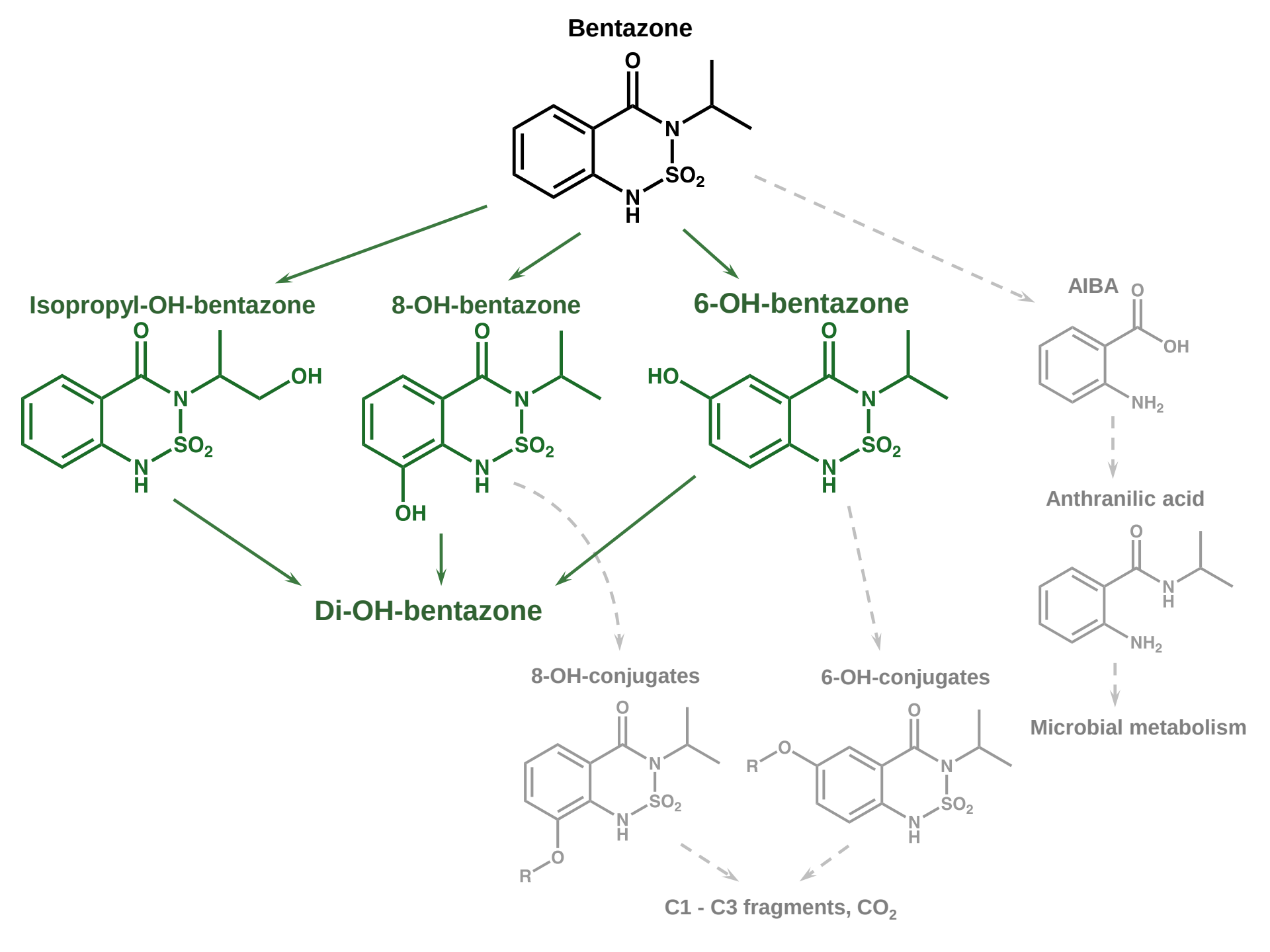
Table 2 Removal rates of bentazone and methane and transformation yield by the methanotrophic culture. The consumption rates are derived from linear regression models (n refer to the number of data points) for two experiments (Partial MMO inhibition and bentazone removal (PIB) and Complete MMO inhibition and bentazone removal (CIB)). The transformation yield, $T_{y,bentazone/CH4}$, expresses the removal rate of bentazone over methane.

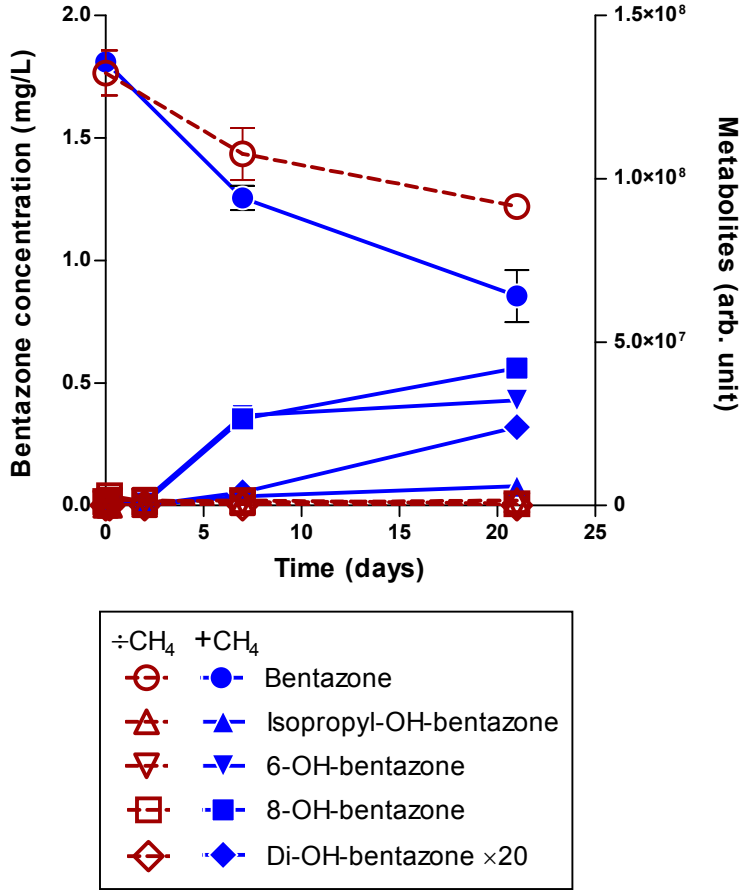
Exp.	Inhibition	Time (days)	Bentazone consumption, $r_{Bentazone}$ (pmole/h/$g_{b\&c}$)	Methane consumption, r_{CH4} (nmole/h/$g_{b\&c}$)	Transformation yield, T_y ($r_{Bentazone}/r_{CH4}$) (mole/mole)
PIB	-	1	270±60, n=12	2000±200, n=12	1.4×10^{-4}
	Partially	1	150±50, n=12	880±280, n=9	1.7×10^{-4}
CIB	-	15	37±5.0, n=21	610±50, n=42	0.6×10^{-4}
	+	15	4.3±4.2, n=20	Not detected, n=45	∞

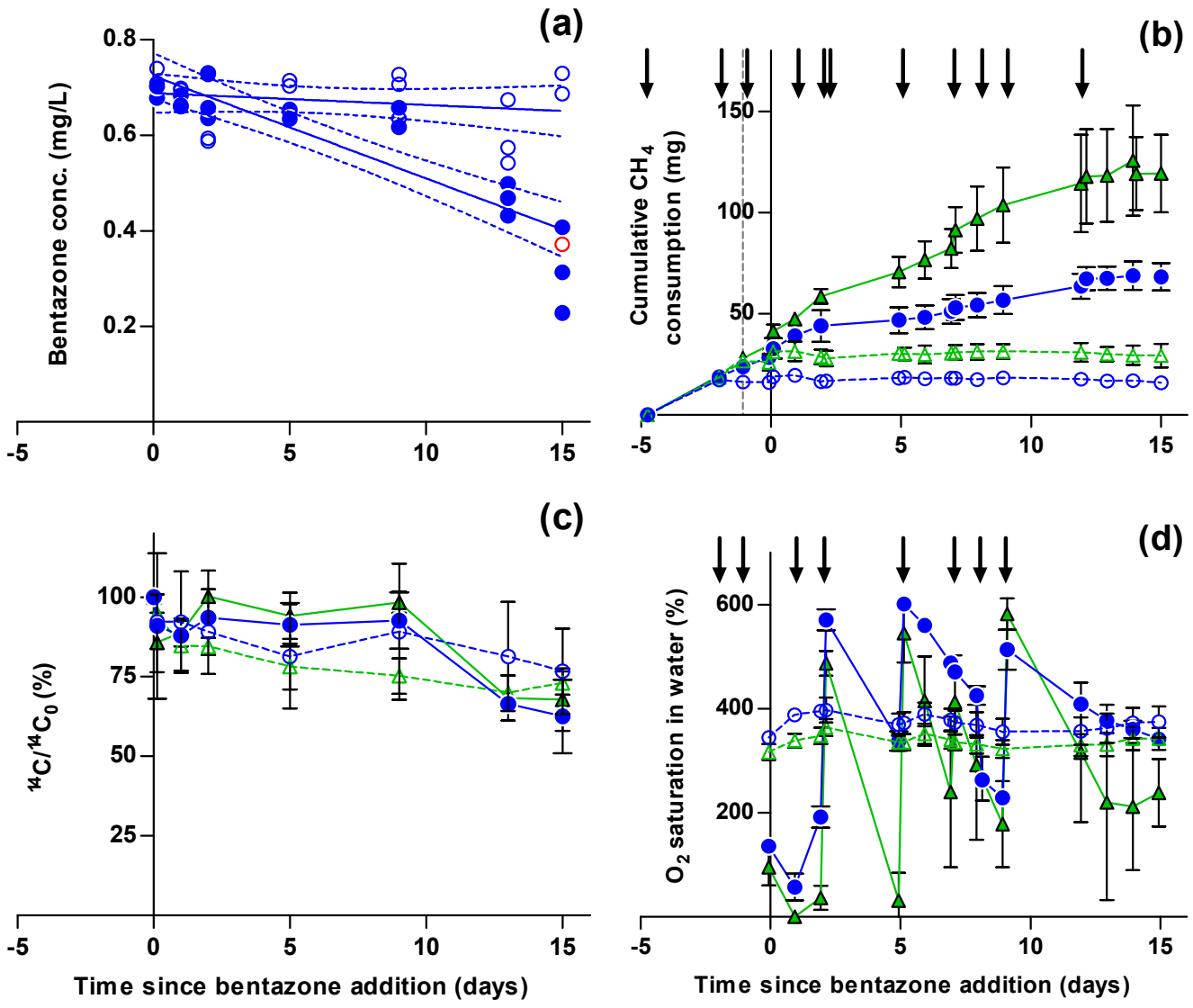
Table 3 Comparison of normalized transformation yields. Data from the present study compared to reported data (see reference). The comparison is based on maximum measured transformation yields, T_y, in absence of formate. T_y, maximum aqueous concentration of methane (CH_4) and trace contaminant (TC) for cultures expressing sMMO and pMMO is given as in Anderson and Mccarty (1997). The normalized transformation yield, T_y^*, is the transformation yield, normalized to the concentration ratio, $C_{TC/c,CH4}$.

Culture	Trace contaminant	Max. transformation yield T_y (r_{TC}/r_{CH4}) ($mole_{TC}/mole_{CH4}$)	Max. aqueous conc. of CH_4 (μM)	Max. aqueous conc. of Trace contaminant (μM)	Conc. ratio $C_{TC/CH4}$[a] ($mole_{TC}/mole_{CH4}$)	Normalized transformation yield $T_y^{*},_{CH4/TC}$[a] ($T_{y,CH4/TC}/C_{CH4/TC}$) -	Reference
Mixed cultures	Bentazone	1.7×10^{-4}	312	3.0	9.6×10^{-3}	**58**	Present study
	TCE	4.9×10^{-3}	349	43	1.2×10^{-1}	**25**	Smith and McCarty (1997)
Pure cultures	TCE	5.3×10^{-3}	75	150	2.0	**377**	Fennell *et al.* (1993)
	TCE	7.5×10^{-3}	60	150	2.5	**333**	Phelps *et al.* (1990)
	VC	6.6×10^{-3}	6.3	2.2	3.5×10^{-1}	**53**	Nelson and Jewell (1993)
	TCE	1.9×10^{-3}	50	13	2.6×10^{-1}	**137**	Anderson and Mccarty (1997b)
	TCE	4.1×10^{-3}	4.7	7	1.5	**363**	Arvin (1991)
	1,1-DCE	2.2×10^{-4}	50	0.56	1.1×10^{-2}	**51**	Anderson and Mccarty (1997b)
	c-DCE	5.8×10^{-2}	30	86	2.9	**49**	Anderson and Mccarty (1997b)
	c-DCE	2.5×10^{-2}	4.7	28	6.0	**238**	Arvin (1991)
	t-DCE	5.7×10^{-1}	30	160	5.3	**9**	Anderson and Mccarty (1997b)
	t-DCE	3.9×10^{-2}	4.7	0.6	1.3×10^{-1}	**3**	Arvin (1991)
	t-DCE	6.3×10^{-2}	40	100	2.5	**40**	Janssen *et al.* (1988)
	t-DCE	6.3×10^{-1}	3.1	20	6.5	**10**	Anderson and Mccarty (1997a)
	VC	2.6×10^{-1}	205	208	1.0	**4**	Dolan and McCarty (1995)
	VC	2.0×10^{-1}	30	17	5.7×10^{-1}	**3**	Anderson and Mccarty (1997b)

[a] Calculated from data in given reference


Bentazone
Isopropyl-OH-bentazone
8-OH-bentazone
6-OH-bentazone
AIBA
Anthranilic acid
Di-OH-bentazone
8-OH-conjugates
6-OH-conjugates
Microbial metabolism
C1 - C3 fragments, CO2


2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time (days)
Bentazone concentration (mg/L)
1.5×10⁸
1.0×10⁸
5.0×10⁷
0
Metabolites (arb. unit)
÷CH4
+CH4
Bentazone
Isopropyl-OH-bentazone
6-OH-bentazone
8-OH-bentazone
Di-OH-bentazone ×20


(a)
Bentazone conc. (mg/L)
0.8
0.6
0.4
0.2
-5
0
5
10
15
(b)
Cumulative CH4 consumption (mg)
150
100
50
(c)
14C/14C0 (%)
100
75
50
25
Time since bentazone addition (days)
(d)
O2 saturation in water (%)
600
400
200
Time since bentazone addition (days)
1 µg/L Bentazone
1 mg/L Bentazone
1 µg/L Bentazone + Acetylene
1 mg/L Bentazone + Acetylene
Addition of CH4 and O2 to active microcosms

Highlights

- Bentazone was co-metabolically transformed by a methanotrophic culture
- Bentazone was transformed to 6-OH, 8-OH, isopropyl-OH and di-OH-Bentazone
- Bentazone removal and formation of TPs was largest in the presence of methane
- Addition of acetylene inhibited methane oxidation and stopped bentazone removal
- Presence of bentazone partly inhibited methane oxidation