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

Mathilde J. Hedegaard^{1}; H el ene Deliniere¹; Carsten Prasse^{2,3}; Arnaud Dechesne¹; Barth F. Smets¹; Hans-J orgen Albrechtsen¹*

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: telephone (+45) 4525 1478, Email: mjhe@env.dtu.dk

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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 (MCP) 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 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. CH₄, NH₄⁺) (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 MOB's or the soluble, cytoplasmic MMO (sMMO) which can only be expressed by some MOB's. 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 mole_{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-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide) and 8-OH-bentazone (IUPAC: 8-Hydroxy-3-Isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one-2,2-dioxide) and 2-amino-*N*-isopropyl-benzamide (AIBA, IUPAC: 2-amino-*N*-propan-2-ylbenzamide) (Figure 1). Hydroxylation of the phenyl ring is the primary biodegradation pathway (Huber and Otto, 1994), where e.g. 65-85% of the added bentazone is transformed to 8-OH-bentazone (Knauber et al., 2000). Generally, bentazone is assumed to be hydroxylated by several fungal species (Huber and Otto, 1994), but both fungi and bacteria may contribute to biotransformation (Knauber et al., 2000). Formed transformation products are all very reactive and are thus rapidly incorporated in the soil organic soil matter (Huber and Otto, 1994).

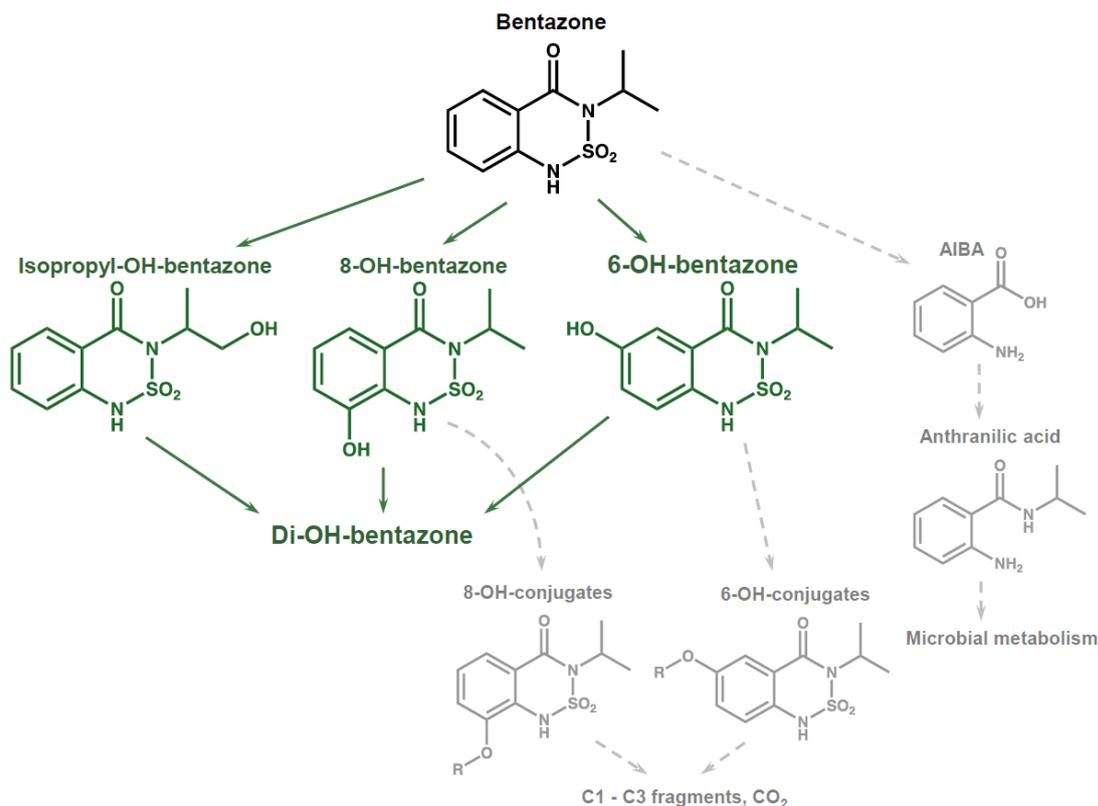


Fig. 1 Degradation pathway of bentazone. In contact with methanotrophic culture bentazone degradation led to accumulation of four transformation products (green). Isopropyl-OH-bentazone and di-OH-bentazone have, to the authors' knowledge, not previously been reported as transformation products in soil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

Biological degradation of bentazone in treatment systems can be challenging: bentazone was the least degradable of four investigated pesticides (linuron, metalaxyl, isoproturon and bentazone) in an on-farm biopurification system (De Wilde et al., 2009). Therefore bentazone removal was surprising when observed in filter material from rapid sand filters from a groundwater-based waterworks (Hedegaard and Albrechtsen, 2014) receiving raw water with high methane concentrations (1.1-9.2 mg/L before aeration) (Sjælsø waterworks plant II). Ring-hydroxylation is a common initial step in the bentazone transformation (Figure 1) (Huber and Otto, 1994) and since MMO oxidizes aromatic rings co-metabolically (Semrau et al., 2010), we hypothesized that methanotrophs would be essential for the rapid transformation of bentazone in filter sand. However, co-metabolic removal assays with four pure MOB cultures reported no bentazone degradation (Benner et al., 2015). Therefore, co-metabolic bentazone degradation has, to our knowledge, not yet been documented and the aim of this study was to establish direct evidence of co-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 $\mu\text{g CH}_4/\text{min/g}$ 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.

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

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 ¹⁴C-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 (18-23°C) 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 ¹⁴C-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 of 2:1 v/v of pure oxygen and methane (as stoichiometrically required for the oxidation of methane).

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

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

2.4 Methane and oxygen measurements

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

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

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

Water samples were either immediately frozen or preserved with acetic acid (final concentration of 0.1 M) until analysis within a few days; investigations showed no difference between the two different ways of storage. The samples which had been 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 ¹⁴C-Carbonyl-bentazone measurements

The ¹⁴C-activity of bentazone in the water phase and the produced ¹⁴CO₂ from bentazone mineralization were quantified by a double vial system, where produced ¹⁴CO₂ in the water was stripped off by acidification and captured by a base trap (1 mL 2 M NaOH) (Janniche et al., 2010). The ¹⁴C-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 ¹⁴C/¹⁴C₀ (%)).

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°C. At the end of the CIB experiments, 2 mL samples containing both water and biomass were taken from the microcosms and after centrifugation the water phase was discharged and the biomass was stored concentrated as a pellet at -80°C until analysis. All bacteria (Eubacteria – targeting the 16S rRNA gene) and methanotrophs (targeting *pmoA*) were quantified by real-time quantitative PCR (qPCR) (see SI). Microbial abundances were calculated under the assumption of an average of two copies of 16S rRNA (Klappenbach, 2001; Lee et al., 2006) or *pmoA* (Semrau et al., 1995; Stolyar et al., 1999) genes per genome (cell) and were subsequently converted to cell densities per mass of filter material (drained wet weight).

2.9 Chemicals

Allylthiourea (ATU) and cold bentazone were dissolved in sterile MilliQ water. Mineralization and removal at very low concentrations (1-2 µg/L) were investigated by [carbonyl-¹⁴C]-bentazone (Izotop, Institute of Isotopes Co., Ltd., Hungary) in two experiments (Table 1). The radiochemical purity of [carbonyl-¹⁴C]-bentazone was 100% (chemical purity 99.77%) according to the manufacturer and a stock solution was prepared in sterile MilliQ water. Bentazone (chemical purity 99.1%, Dr. Ehrenstorfer GmbH), 6-OH-bentazone (chemical purity 97%, TRC, Toronto Research Chemicals Inc., Ontario, Canada) and 8-OH-bentazone (chemical purity 97%, TRC, Toronto Research Chemicals Inc., Ontario, Canada) were all dissolved in sterile ultrapure water at least one day prior to the experiment. The concentration was verified

by HPLC-DAD immediately before the experiment. Acetylene was added from a gas flask (see SI).

2.10 Statistics

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 $\mu\text{moles/h/g}_{\text{b\&c}}$ 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 $\mu\text{moles/h/g}_{\text{b\&c}}$ in presence of methane and 35 $\mu\text{moles/h/g}_{\text{b\&c}}$ in absence of methane during the first seven days (data not shown).

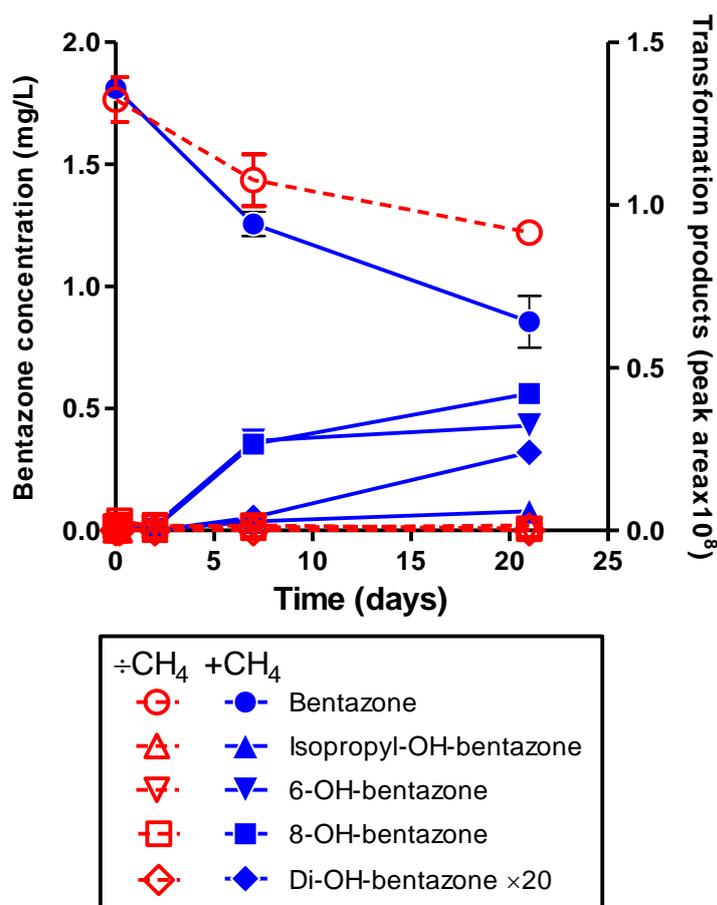


Fig. 2 Effect of methane on removal of bentazone and formation of bentazone transformation products by the methanotrophic enrichment culture. Bentazone measured by HPLC-DAD (duplicates) and isopropyl-OH-, 6-OH-, 8-OH-bentazone and di-OH-bentazone measured by High-res-MS as peak areas (single microcosm).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

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 $\mu\text{mole methane/h/g}_{\text{b\&c}}$ (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.

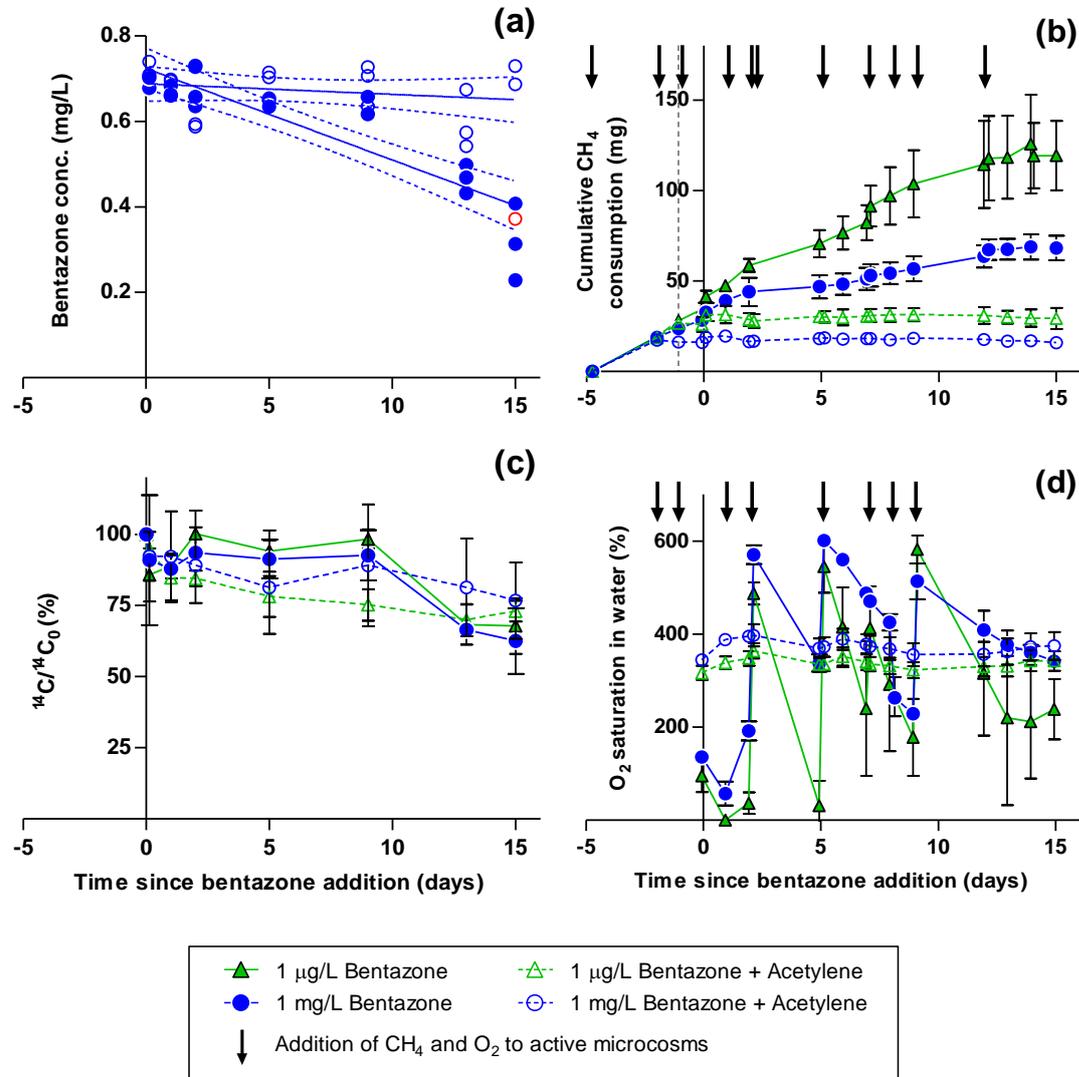


Fig. 3 Bentazone removal, methane and oxygen consumption by the methanotrophic enrichment culture. Removal in active microcosms and microcosms with acetylene (both in triplicates) with 10 g biomass and carrier material, 100 mL tap water, approx. 5 mg/L methane and bentazone at high (1 mg/L) and low (1 µg/L) concentrations. **A)** Bentazone concentration measured by HPLC-DAD, linear regression curves (full lines) with 95% confidence intervals (dotted lines). The red dot marks an outlier (not included in the regression). **B)** Methane consumption. **C)** ¹⁴C-bentazone concentration given as percentage of initial concentration. **D)** % oxygen saturation in the water phase. Acetylene (26 mg/L) was added to inhibited microcosms (grey dotted line at time -1 day) prior to bentazone addition (time 0). When $C_{w,CH_4} < 4$ mg/L methane was either added directly or microcosms were flushed with air, and methane and oxygen were added subsequently (2:1 v_{O_2}/v_{CH_4}).

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 pmole/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 pmole/h/g_{b&c} ($r^2 = 0.06$), was not significantly different from zero (Figure 3A).

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, $T_{y,bentazone/CH_4}$, expresses the removal rate of bentazone over methane.

Exp.	Inhibition	Time (days)	Bentazone consumption,	Methane consumption,	Transformation yield,
			$\Gamma_{Bentazone}$ (pmole/h/g _{b&c})	Γ_{CH_4} (nmole/h/g _{b&c})	T_y ($\Gamma_{Bentazone}/\Gamma_{CH_4}$) (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	∞

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, $T_{y,BTZ/CH_4}$, 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, $T_{y,BTZ/CH_4}$, 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/CH_4} , 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, $T_{y,CH_4/TC}^*$:

$$T_{y,CH_4/TC}^* = T_{y,TC/CH_4} / C_{TC/CH_4} = T_{y,CH_4/TC} / C_{CH_4/TC}$$

Where $T_{y,CH_4/TC}^*$ is the CH₄/TC-normalized transformation yield of MMO for oxidizing methane over the trace contaminant, $T_{y,TC/CH_4}$ is the transformation yield of the trace contaminant relative to methane and C_{TC/CH_4} is the concentration ratio between secondary and primary substrates. From reported data we calculated the CH₄/TC-normalized transformation yields, $T_{y,CH_4/TC}^*$, ranging from 3 to 400, and $T_{y,CH_4/BTZ}^*$ in our study (58-158) was thus within this range (Table 3). Hence, the CH₄/TC normalized transformation yields show that, in a situation with an even presence of bentazone and CH₄-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.

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, $C_{\text{TC}/\text{C}_{\text{CH}_4}}$.

Culture	Trace contaminant	Max. transformation yield	Max. aqueous conc. of		Conc. ratio	Normalized transformation yield	Reference
		T_y ($r_{\text{TC}}/r_{\text{CH}_4}$) ($\text{mole}_{\text{TC}}/\text{mole}_{\text{CH}_4}$)	CH_4 (μM)	Trace contaminant (μM)	$C_{\text{TC}/\text{CH}_4}^a$ ($\text{mole}_{\text{TC}}/\text{mole}_{\text{CH}_4}$)	$T_y^*, \text{CH}_4/\text{TC}^a$ ($T_y, \text{CH}_4/\text{TC}/C_{\text{CH}_4/\text{TC}}$) -	
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)
	TCE	5.3×10^{-3}	75	150	2.0	377	Fennell <i>et al.</i> (1993)
	TCE	7.5×10^{-3}	60	150	2.5	333	Phelps <i>et al.</i> (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)
Pure cultures	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 <i>et al.</i> (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

3.5 Effect of bentazone on the methane oxidation (CIB)

After bentazone addition at day 0 in the CIB experiment, the methane consumption was $1.5 \mu\text{mole}_{\text{CH}_4}/\text{h}/\text{g}_{\text{b\&c}}$ in active microcosms at low ($1 \mu\text{g}/\text{L}$) bentazone concentrations, which was similar to before bentazone addition ($1.3\text{-}2.0 \mu\text{mole}_{\text{CH}_4}/\text{h}/\text{g}_{\text{b\&c}}$ in all microcosms and inhibited controls) (Figure 3B). In contrast, in active microcosms at high bentazone concentration ($1 \text{mg}/\text{L}$), methane consumption decreased to $0.6 \mu\text{mole}_{\text{CH}_4}/\text{h}/\text{g}_{\text{b\&c}}$ (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 $1.64 \text{mole}_{\text{TC}}/\text{mole}_{\text{CH}_4}$ (Anderson and McCarty, 1997a) and transformation yields found in literature (Table 3) are much higher than the measured bentazone transformation yields ($T_{\text{y,BTZ/CH}_4} = 0.6 \times 10^{-4}$ to $1.7 \times 10^{-4} \text{mole}_{\text{BTZ}}/\text{mole}_{\text{CH}_4}$). 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 $2 \text{mg}/\text{L}$, 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 disproportionately 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 ($1 \mu\text{g}/\text{L}$, $1.4 \times 10^{-5} \text{mole}_{\text{BTZ}}/\text{mole}_{\text{CH}_4}$), 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 $^{14}\text{CO}_2$ 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 C₃H₆O instead of C₃H₆ as in bentazone and the ring hydroxylated TPs (Table S1 in SI). A dihydroxylated bentazone TP (di-OH-bentazone) was also detected based on the exact mass determinations, however, concentrations were too low to obtain fragmentation data from MS² experiments and thus, it was not possible to determine the exact position of the hydroxylation. After 21 days, four times more isopropyl-OH-, 132 times more 6-OH- and 85 times more 8-OH-bentazone were observed in presence versus absence of methane. In addition, formation of di-OH-bentazone was only observed in the presence of methane (Figure 2; Figure 1).

The two substituents on the aromatic ring of bentazone are a strongly electron donating secondary amine and an electron withdrawing ketone-group (McMurry and Simanek, 2007), both substituents are directing oxidation towards the 6-OH- (para-position with respect to the donating amine group) and 8-OH- (ortho-) position of bentazone, which were both formed during degradation of bentazone in the methanotrophic culture. Methanotrophic oxidation is typically regioselective towards the para-position of monosubstituted aromatic compounds (Anthony, 1986; Dalton and Leak, 1985; Lindner et al., 2000). Our results indicate a similar formation of 6-OH- and 8-OH-bentazone by the methanotrophic culture (Figure 1), though presence of methane had the largest impact (concentration increased 132 times) on the formation of 6-OH-bentazone (para-position). This is in contrast to field soils where primary 8-OH-bentazone was formed (Knauber et al., 2000), indicating the involvement of an additional transformation process. This was also indicated by the formation of isopropyl-OH- and di-OH-bentazone in our study, which so far has not 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 μ M) and 26 mg/L (998 μ M), 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 μ M) and 26 mg/L (998 μ M) (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, $T_{y, \text{CH}_4/\text{BTZ}}^*$, 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 μ g/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.

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