Liquid-chromatography isotope-ratio mass spectrometry analysis of halogenated benzoates for characterization of the underlying degradation reaction in *Thauera chlorobenzoica* CB-1^T

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Abstract

RATIONALE: Halogenated benzoic acids occur in the environment due to their widespread agricultural and pharmaceutical use. Compound-specific stable isotope analysis (CSIA) has developed over the last decades for investigation of *in-situ* transformation and reaction mechanisms of environmental pollutants amenable by gas chromatography (GC). As polar compounds are unsuitable for GC analysis we developed a method to perform liquid chromatography (LC)/CSIA for halogenated benzoates.

METHODS: LC/isotope ratio mass spectrometry (IRMS) utilizing a LC-Surveyor Pump coupled to a MAT 253 isotope ratio mass spectrometer via a LC-Isolink interface was applied. For chromatographic separation a YMC-Triart C18 column and a potassium hydrogen phosphate buffer (150 mM, pH 7.0, 40°C, 200 $\mu L \cdot mL^{-1}$) were used, followed by wet oxidation deploying 1.5 mol L^{-1} *ortho*-phosphoric acid and 200 $g \cdot L^{-1}$ sodium peroxodisulfate at 75 $\mu L \cdot mL^{-1}$.

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RESULTS: Separation of benzoate and halogenated benzoates could be achieved in less than 40 min over a concentration range of 2 orders of magnitude. Under these conditions the dehalogenation reaction of *Thauera chlorobenzoica* 3CB-1^T using 3-chloro-, 3-bromo- and 4-chloro-benzoic acid was investigated resulting in inverse carbon isotope fractionation for *meta*-substituted benzoic acids and minor normal fractionation for *para*-substituted benzoic acids. Together with the respective growth rates this led to the assumption that dehalogenation of *para*-halobenzoic acids follows a different mechanism from that of *meta*-halobenzoic acids.

CONCLUSIONS: A new LC/IRMS method for the quantitative determination of halogenated benzoates was developed and used to investigate the *in-vivo* transformation pathways of these compounds, shedding some insights into degradation and removal of these widespread compounds by *T. chlorobenzoica* 3CB-1^T.

Keywords: Compound-specific stable isotope analysis, chloro, bromo, *Thauera chlorobenzoica*, reductive dehalogenation, liquid chromatography

Introduction

Compound-specific isotope analysis (CSIA) has facilitated the assessment of sources and transformation processes of organic pollutants in the environment. Thus far, gas-chromatography isotope ratio mass spectrometry (GC/IRMS) is mainly used within CSIA as multi-element approach [1, 2]. However, many biologically important compounds are unamenable to GC analysis due to their high polarity and therefore non-volatile nature. Thus, GC analysis of compounds with those chemical properties is not possible or requires derivatization prior to analysis [1]. The latter approach leads through additional carbon

insertion to dilution of the original isotope composition and increases the uncertainty as quantitative conversion is hard to achieve. Partial or incomplete derivatization may result in non-systematic isotopic fractionation and an altered the isotopic composition of the analyte [1]. In 2014 quantitative analysis of underivatized polar compounds by liquid chromatography/isotope ratio mass spectrometry (LC/IRMS) became available [3], where the compounds are separated by liquid chromatography using exclusively carbon-free eluents, such as potassium phosphate. After LC separation, the dissolved compounds are oxidized to CO_2 via wet oxidation utilizing sodium persulfate and phosphoric acid [1, 3]. This step is followed by separation with a Nafion™ membrane-based in-line gas drying unit and water traps to separate CO_2 from water vapor. The system was modified by the insertion of a copper-filled reduction reactor for the removal of excess oxygen formed within the wet oxidation step that might damage the filament of the ion source [4]. Since then new applications for CSIA have involved the analysis of nonvolatile, aqueous soluble compounds from more complex mixtures in the fields of pharmaceuticals, nutrition and food authentication for e.g. amino acids, carbohydrates as well as organic acids [5-10].

Nevertheless, a limited number of applications for LC/IRMS is available in comparison with those for GC/IRMS, mainly as a result of restrictions in the aqueous mobile phase compositions and flow rates that can be employed. As solely inorganic and therefore pure aqueous mobile phases and low flow rates can be used, chromatographic separation is challenging and column stability is becoming a major issue. Furthermore, persulfate oxidation has only limited efficiency [11]. In particular, the presence of halogen atoms is expected to decrease the rate of persulfate oxidation due to the high strength of C-halogen-bonds [11, 12]. These highly oxidized carbon atoms result from a permanent bond polarization induced by the high electronegativity of the halogen substituent. Thus, the higher the electronegativity of the halogen, the more resistant the compound is to oxidation [11]. As a

result of the extensive use of halogenated compounds as herbicide, insecticide, fungicide, pharmaceuticals and solvents, there is a considerable impact on the human health and ubiquitous contamination of the environment [13, 14]. Halogenated benzoic acids are probably formed from halogenated phenols, alkylbenzenes and benzenes under anoxic conditions [13]. It was shown that halogenated benzoic acids can be degraded by anaerobic microorganisms (i) under denitrifying conditions as e.g. *Thauera chlorobenzoica* spp. and *Thauera aromatica* [13, 15-19] or (ii) under sulfate-reducing conditions [20], or (iii) by the phototrophic anaerobic microorganism *Rhodopseudomonas palustris* [21]. Furthermore, *T. chlorobenzoica* 3CB-1^T is capable of utilizing 3-bromo-, 2-fluoro-, 4-fluorobenzoic acid [16-18, 22] and 4-chlorobenzoic acid (personal communication: O. Tiedt, University of Freiburg) as substrates for growth. Kuntze *et al* proposed a degradation pathway (Figure 1). starting with the transesterification-product halobenzoyl-CoA, yielded by CoA addition through class I benzoyl CoA-reductase (BcrA), being subsequently dearomatized by the same enzyme [13]. The dehalogenation appears to occur enzymatically or spontaneously by hydrogen chloride (HCl) or hydrogen bromide (HBr) release and re-aromatization of the halobenzoyl-CoA which follows the Benzoyl-CoA degradation pathway [23]. Whereas 4-fluorobenzoic acid can be dehalogenated completely, 3-fluorobenzoic acid forms 3-fluoro-dienoyl-CoA, which is not further converted [19].

The aim of this study, therefore, was to develop a LC/IRMS method for the analysis of halogenated benzoates in order to investigate the microbial dehalogenation reaction within *T. chlorobenzoica* 3CB-1^T and to monitor halogenated benzoic acid degradation *in-vivo*.

Material and Methods

LC-Method development

A high performance liquid chromatography (HPLC) system equipped with a Photodiode Array-detector (Accela 600 and Accela PDA, Thermo Fisher Scientific, Bremen, Germany) was used to develop the method for the determination of halogenated benzoic acids without organic additives in KH_2PO_4 eluent, A YMC-Triart C18 column, 150x2mm inner diameter, 3µm particle size, 12nm pore size (YMC Europe GmbH, Dinslaken, Germany) and an Atlantis T3 column of 150x4.6mm inner diameter, 3µm particle size and 10nm pore size (Waters, Eschborn, Germany) were initially tested. Testing of different column lengths, 150 and 75 mm, buffer strengths of 15 and 150 mM, pHs of 3 to 7.5, flow rates of 100 to 200 µL min^{-1}, and column temperatures of 30 and 40°C was performed using the YMC Triart C18 column (see results for method development). Finally, halogenated benzoates were eluted with KH_2PO_4 at pH 7.0 and a flow rate of 200 µL min^{-1} under isothermal conditions at 40 °C.

Elemental Analyzer (EA)-IRMS Measurements

The carbon isotope compositions of the tested halogenated benzoic acids were determined with a EuroEA3000 elemental analyzer (EuroVector, Pavia, Italy) directly coupled with a ConFlo IV-interface (Thermo Fisher Scientific) to a MAT 253 isotope ratio mass spectrometer (Thermo Fisher Scientific). Normalization of the $\delta^{13}C$ raw data was carried out by a two-point calibration employing reference materials from the International Atomic Energy Agency (Vienna, Austria; IAEA-CH6 = 10.45 ± 0.04‰ and IAEA-CH7 = 32.15 ± 0.05‰, for confirmation IAEA-C3 = 24.72 ± 0.03‰). The precision was always better than ±0.1‰.

LC/IRMS analysis

For determination of the carbon isotope composition of halobenzoates a LC/IRMS system (Thermo Fisher Scientific) consisting of a LC-Surveyor pump coupled via a LC-Isolink

interface to the MAT 253 isotope ratio mass spectrometer. Analytes were injected (10-100 µL) via a HTC PAL autosampler (CTC Analytics, Zwingen, Swizerland) and transferred through a 100-mL sample loop to the YMC Triart C18 column (75mm x 2mm (inner diameter), 3 µm particle size, 12 nm pore size) maintained at 40 °C in a HT HPLC 200 column oven (SIM GmbH, Oberhausen, Germany). The eluent was processed by wet chemical oxidation achieved by online mixing with phosphoric acid (1.5 mol L^{-1}) and sodium peroxodisulfate (200 g L^{-1}) prior to entering the oxidation reactor maintained at 99.9 °C. The two reagents were pumped towards a T-piece by separate pumps, each at a flow rate of 75 µL min^{-1}. The eluent, 150 mmol L^{-1} KH_2PO_4 (pH 7.0), was pumped by a Surveyor MS Pump Plus (Thermo Electron, Bremen, Germany) at a flow rate of 200 µL min^{-1}. To avoid blocking of capillaries, in-line filters with a pore size of 0.5µm (Vici, Schenkon, Swizerland), were placed prior to the HPLC column and in front of the oxidation reactor of the LC-IsoLink interface. The formed CO_2 was removed from the liquid phase by a separation unit and subsequently transferred into a helium carrier gas flow of 1.5 mL min^{-1} in the separation unit. Afterwards, the CO_2 was dried by a Nafion™ membrane-based in-line gas drying unit and finally admitted to the mass spectrometer [3]. Two reduction reactors, consisting of ceramic tubes filled with copper wires kept at 600 °C (controlled by a JUMO iTRON microprocessor controller, JUMO, Fulda, Germany) are located prior to the open split [4, 11]. One reactor was used for removing the exceeding O_2 contained in the He flow while the other was regenerated by flushing with a gas mixture of hydrogen and nitrogen (1:99) for copper reduction. Switching between the two reactors was performed manually when the oxygen background increased.

Cultivation and isotope fractionation experiments

T. chlorobenzoica 3CB-1^T was cultivated under anoxic, slightly modified conditions as described previously [24]. It was cultivated using a phosphate-based mineral medium

consisting of 0.7 g·L^{-1} $NaH_2PO_4 \cdot 2H_2O$, 5.6 g·L^{-1} K_2HPO_4 and 0.5g·L^{-1} NH_4Cl with 1.5 mmol·L^{-1} 3-Cl-, 4-Cl- or 3-Br-benzoic acid as carbon and 6.3 mmol·L^{-1} sodium nitrate as energy sources, respectively. The medium was amended with 0.1 mol·L^{-1} $CaCl_2$, 0.8 mol·L^{-1} $MgSO_4$, 1 mL of a 1000-fold VL7 [25] and 1 mL of a 1000-fold SL9 trace element solution [26]. Cultivation of the pre-cultures was carried out in 50-mL volume in 100-mL serum bottles, whereas for the fractionation experiment 250-mL cultures in 500-mL serum bottles were used. After distribution of the medium into serum bottles the bottles were flushed with N_2, crimp closed with grey butyl rubber stoppers, and sterilized for 40 min at 120°C. Afterwards the medium was amended with the components described above under anoxic and sterile conditions. The prepared bottles were inoculated with 5 % (v/v) of a culture grown on the respective substrate. One non-inoculated bottle per set of two inoculated bottles was prepared with substrate as a negative control. The cultures were incubated without shaking in the dark at 20°C. Dehalogenation was checked by HPLC) (see below. For isotope fractionation experiments 0.5-mL aliquots were taken out of the cultures every 2 h, filtered through 0.2 µm PES-filters (WICOM, Heppenheim. Germany) and stored at -4 °C until measurements were conducted.

Evaluation of isotopic data

The isotopic composition of compounds was determined in $\delta^{13}C$-notation (‰) relative to the international standard VPDB [27]. The carbon isotope enrichment factor ($\varepsilon^{13}C$) was calculated using the linearized and simplified Rayleigh equation (1). Therefore, the concentrations at different time points (t) of the reaction and the starting concentration (0), represented as C_t and C_0, respectively, as well as $R = 1 + \delta^{13}C$ were used [28].

$$\ln\left(\frac{R_t}{R_0}\right) = \varepsilon^{13}C \times \ln\left(\frac{C_t}{C_0}\right) \quad (1)$$

Based on the slope of the regression a two-tailed T-test was used to calculate the 95 % confidence interval (data evaluation in Micosoft EXCEL).

Results and Discussion

Method development for halogenated benzoic acids

As LC/IRMS analysis requires solely aqueous buffer systems without organic additives, only a minor selection of HPLC columns is suitable for this approach. A YMC-Triart C18 column, which was finally chosen for the method development and an Atlantis T3© column were tested. However, as halogenated benzoic acids were eluted under neutral to alkaline conditions, the Atlantis T3© column was not suitable as it was reported to have a reduced lifetime with phosphate buffers of approximately neutral pH (Atlantis T3 column care and use manual, Waters, 2007). Based on literature reports [11, 29] a potassium phosphate buffer was utilized. Testing of buffer strength of 15 and 150 mmol L^{-1} and pH values from 3.0 to 7.5 revealed 150 mmol L^{-1} and pH 7.0 as optimal eluent properties. To reduce the analysis time the flow rate was set to the highest possible rate suitable for the connection of the LC-IsoLink Interface and the LC-pump, viz. 200 µL min^{-1}. The column oven temperature was adjusted to 40 °C to maintain column stability at neutral/alkaline pH (YMC-Triart C18 column maintenance manual). With this method halogenated the benzoates could be separated from benzoic acid with retention times of 17 min for benzoate, 43 min for 3-Cl-benzoate, 45 min for 4-Cl-benzoate and 63 min for 3-Br-benzoate. Although separation was achieved, the analysis time per sample was still quite high. Therefore, the column length was reduced to 75 mm resulting in a decrease of the retention times to 6 min for benzoate, 25 min for both 3-Cl- and 4-Cl-benzoate, and 37 min for 3-Br-benzoate (Figure S1, supporting information). As for the application only one halogenated benzoate was used for each setup

and benzoate was expected to be one of the products, no further chromatographic separation of the halogenated benzoates from each other was targeted.

The isotopic composition of the halogenated benzoic acids were determined by an elemental analyzer prior to LC/IRMS analysis, resulting in values of $\delta^{13}C_{3\text{-Cl-BA}}$ = -26.2 ‰, $\delta^{13}C_{4\text{-Cl-BA}}$ = -28.0 ‰ and $\delta^{13}C_{3\text{-Br-BA}}$ = -24.3 ‰ (Table 1). In order to obtain complete conversion of the investigated halobenzoates to CO_2, the flow rates and concentrations of *o*-phosphoric acid and sodium peroxodisulfate were optimised (Table 1): 75 µL min^{-1} of each, 200 g L^{-1} $Na_2S_2O_8$ and 1.5 mol L^{-1} *o*-H_3PO_4 were determined as appropriate conditions.

Method validation was performed by determining the relationship of the measured delta values and amplitudes depending on the injected C on column. The results are shown in Figures 2A-C. A stable delta value was obtained in the range of 0.03 mmol L^{-1} to 3 mmol L^{-1} (0.13 ng C up to 1.4 ng C on column). The amplitudes showed a linear dependency on the determined concentration. Hence, the analyzable concentration range was determined to be greater than 2 orders of magnitude.

Carbon isotope fractionation of halobenzoates during degradation by *T. chlorobenzoica*

Kuntze et al [13] proposed a dehalogenation mechanism starting with an energy dependent dearomatization step through a hydrogen insertion, followed by rearomatization and subsequent dehalogenation yielding benzoyl-CoA. However, throughout the whole fractionation experiment no benzoic acid was detected within the LC/IRMS analysis pointing towards a fast ongoing degradation of the benzoyl-CoA [13]. Thus, only substrate isotopic changes could be determined.

During the course of degradation by *T. chlorobenzoica* 3CB-1^T the carbon isotope ratios of 3-Cl-benzoate changed from initially -25.8 ± 0.1 ‰ to -27.4 ± 0.4 ‰ (98 % degradation) and of 3-Br-benzoate from -24.0± 0.1 ‰ to -28.7± 0.3 ‰ (98 % degradation), whereas the 4-Cl-benzoate $\delta^{13}C$ values did not change significantly, from -27.6± 0.1 ‰ to -27.1± 0.1 ‰ (73 %

degradation). The determined isotopic fractionation were: $\varepsilon^{13}C_{3\text{-Cl-benzoate}} = 0.3 \pm 0.1$ ‰ (R^2=0.93), $\varepsilon^{13}C_{3\text{-Br-benzoate}} = 0.9 \pm 0.1$ ‰ (R^2=0.97) and $\varepsilon^{13}C_{4\text{-Cl-benzoate}}$ = not significant (Fig.3). Thus, a slightly inverse isotope effect (viz. enrichment of the light isotopes in the remaining substrate) was observed for the degradation of 3-Cl and 3-Br-benzoic acid. Inverse isotopic effects for carbon may result from strengthening or formation of bonds within the rate determining step [30]. According to the mechanism (Figure 1) proposed by Kuntze et al [13], the dehalogenation step is expected to be very fast and therefore not rate determining. This results from the strong impetus of the re-aromatization, as delocalization of the π-electron system is energetically more favorable than formation of a 1,3-cyclohexadiene [31]. Thus, CoA-transesterification by benzoyl-CoA-ligase and BcrA class I-mediated de-aromatization are potential rate determining steps [13]. Whereas a carbon-sulfur bond is formed within transesterification, two carbon-hydrogen bonds are formed when halobenzoyl-CoA is de-aromatized. As the isotope effects were determined on non-CoA activated benzoates, and as the benzoyl-CoA ligase reaction is not reported to be reversible nor is spontaneous hydrolysis energetically favorable for the organism, transesterification is assumed to yield the isotopic effects determined here. Furthermore, we can assume that once the halo-dienoyl-CoA is formed, re-aromatization is the impetus to halogen-elimination and formation of benzoyl-CoA instead of a reaction back towards the halobenzoyl-CoA. Furthermore, the isotopic effects seem to correspond to (i) the position of the substitution, as *meta*-halobenzoates (3-Cl- and 3-Br-benzoate) display slight inverse isotopic effects in comparison with the *para*-halobenzoate 4-Cl-benzoate, possibly due to mesomeric stabilization effect differences and (ii) the electronegativity of the substituent, as 3-Br-benzoate leads to a threefold higher inverse enrichment factor than 3-Cl-benzoate. As bromo-substituents have a lower electronegativity than chloro-substituents, the electron withdrawing properties and resulting positive mesomeric effects of bromine are lower than those of chlorine. Thus, the electron

density within the ring system is higher and this also affects the remaining substituent, probably resulting in a slightly stronger C-S-bond with Br-substitution than with Cl-substitution. This corresponds to the statement of Aeppli et al [30] analyzing polychlorinated phenols, who attriibutes inverse isotopic effects to bond strengthening and/or formation in the rate determining step.

When considering the benzoyl-CoA-reduction and dehalogenation reaction Tiedt et al [19] showed differences in elimination reactions between *meta*- and *para*-substitution of CoA-halobenzoic acids. Whereas *meta*-substitution leads to spontaneous halogen abstraction by E2-elimination reaction, protonation of the anionic intermediate after hydride addition would lead to an unfavorable halo-dienoyl-CoA following an E1cB elimination of the *para*-substituent. It was shown that 3-F-benzoyl-CoA is protonated yielding 3-fluoro-dienoyl-CoA, which is not further converted, whereas 4-F-benzoyl-CoA can be slowly dehalogenated. This results from the strength of the C-F-bond [19]. In contrast, as C-Cl- and C-Br-bonds are weaker reaction is more feasible but we also find differences in reaction rates according to the position of the substituent. Whereas 3-Cl-benzoic acid and 3-Br-benzoic acid were completely degraded after 46 h and 55 h, respectively, 4-Cl-benzoic acid was only completely degraded after 98 h. This points towards differences in the *para*-substituent removal reaction from *meta*-substituent removal confirming results by Tiedt et al. [19]

Conclusion

In this study we developed a LC/IRMS method for determining the stable carbon isotopic composition of halogenated benzoates. The method is suitable for the separation of halogenated benzoates from non-halogenated benzoates within 37 min per run using only inorganic buffer conditions. The concentration range in which stable delta values are obtained was found to be from 0.13 ng carbon to 1.4 ng carbon on column. Consequently, this method

facilitates the LC/IRMS measurement of the carbon isotope fractionation of halogenated benzoic acids in a reliable way and in a suitable concentration range without the need for derivatization, as is necessary with GC/IRMS. Moreover, 1 nmol C has to be injected for halogenated benzoates to yield stable isotope ratios by this LC/IRMS method, whereas the sensitivity limits for GC/IRMS are described as 0.1 – 5 nmol [32]. Consequently, analysis in LC/IRMS is possible in a similar concentration range to GC/IRMS.

Fractionation experiments with *T. chlorobenzoica* 3CB-1^T resulted in slightly inverse isotope effects for 3-Cl- and 3-Br-benzoic acids and no significant effects for 4-Cl-benzoic acid. Those values pointed towards different reaction mechanisms depending on the substitution positions and support the findings of Tiedt et al [19] that *para*-substitution leads to an elimination reaction of the halogen. Whereas an E2-elimination is favorable for *meta*-substitution, *para*-substituted halogens follow E1cB-elimination without involving the halo-dienoyl-CoA intermediate. To further evaluate the protonation and elimination reactions, future experiments should include isotopic determination of the halobenzoate-CoA instead of halobenzoate. Thus, a different rate-determining step would be investigated, possibly giving further mechanistic insights.

LC/IRMS is a powerful tool for the investigation of polar, water-soluble compounds. However, at the moment this method is only applicable for single isotope analysis. The isotope composition of other elements such as chlorine, bromine or hydrogen, which may reveal interesting insights in reaction mechanisms, can at the moment only be analyzed after derivatization by GC/Cr/HTC-IRMS [33] or GC/ICP-MS [34, 35].

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References

1. Elsner M, Jochmann MA, Hofstetter TB, et al. Current challenges in compound-specific stable isotope analysis of environmental organic contaminants. *Anal Bioanal Chem.* 2012;403(9):2471-2491. doi: 10.1007/s00216-011-5683-y.

2. Nijenhuis I, Renpenning J, Kümmel S, Richnow HH, Gehre M. Recent advances in multi-element compound-specific stable isotope analysis of organohalides: Achievements, challenges and prospects for assessing environmental sources and transformation. *Trends Environ Anal Chem.* 2016;11:1-8. doi: 10.1016/j.teac.2016.04.001.

3. Krummen M, Hilkert AW, Juchelka D, et al. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom.* 2004;18(19):2260-2266. doi: 10.1002/rcm.1620.

4. Hettmann E, Brand WA, Gleixne, G. Improved isotope ratio measurement performance in liquid chromatography/isotope ratio mass spectrometry by removing excess oxygen. *Rapid Commun Mass Spectrom.* 2007;21(24):4135-4141. doi: 10.1002/rcm.3304.

5. Karabagias IK, Badeka A, Kontako, S, Karabournioti S, Kontominas MG. Characterisation and classification of Greek pine honeys according to their geographical origin based on volatiles, physicochemical parameters and chemometrics. *Food Chem.* 2014;146:548-557. doi: 10.1016/j.foodchem.2013.09.105.

6. Godin JP, McCullagh JS. Review: Current applications and challenges for liquid chromatography coupled to isotope ratio mass spectrometry (LC/IRMS). *Rapid Commun Mass Spectrom.* 2011;25(20):3019-3028. doi: 10.1002/rcm.5167.

7. Smith CI, Fuller BT, Choy K, Richards MP. A three-phase liquid chromatographic method for delta13C analysis of amino acids from biological protein hydrolysates using liquid chromatography-isotope ratio mass spectrometry. *Anal Biochem.* 2009;390(2):165-172. doi: 10.1016/j.ab.2009.04.014.

8. Godin JP, Stellingwerff T, Actis-Goretta L, et al. The role of liquid chromatography and flow injection analyses coupled to isotope ratio mass spectrometry for studying human in vivo glucose metabolism. *Rapid Commun Mass Spectrom.* 2011;25(20):2989-2994. doi: 10.1002/rcm.5179.

9. Birkigt J, Gilevska T, Ricken B, et al. Carbon Stable Isotope Fractionation of Sulfamethoxazole during Biodegradation by Microbacterium sp. Strain BR1 and upon Direct Photolysis. *Environ Sci Technol.* 2015;49(10):6029-6036. doi: 10.1021/acs.est.5b00367.

10. Kujawinski DM, Zhang L, Schmidt TC, Jochmann MA. When other separation techniques fail: compound-specific carbon isotope ratio analysis of sulfonamide containing pharmaceuticals by high-temperature-liquid chromatography-isotope ratio mass spectrometry. *Anal Chem.* 2012;84(18):7656-7663. doi: 10.1021/ac300116w.

11. Gilevska T, Gehre M, Richnow HH. Performance of the wet oxidation unit of the HPLC isotope ratio mass spectrometry system for halogenated compounds. *Anal Chem.* 2014;86(15):7252-7257. doi: 10.1021/ac501174d.

12. Huang KC, Zhao Z, Hoag GE, Dahmani A, Block PA. Degradation of volatile organic compounds with thermally activated persulfate oxidation. *Chemosphere.* 2005;61(4):551-560. doi: 10.1016/j.chemosphere.2005.02.032.

13. Kuntze K, Kiefer P, Baumann S, et al. Enzymes involved in the anaerobic degradation of meta-substituted halobenzoates. *Mol Microbiol.* 2011;82(3):758-769. doi: 10.1111/j.1365-2958.2011.07856.x.

14. Szabo DT, Loccisano AE., POPs and Human Health Risk Assessment, In: *POPs and Human Health Risk Assessment.* ed. Schecter A. 3rd ed. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2012:579-618. doi: 10.1002/9781118184141.ch19.

15. Song B, Kerkhof LJ, Haggblom MM. Characterization of bacterial consortia capable of degrading 4-chlorobenzoate and 4-bromobenzoate under denitrifying conditions. *FEMS Microbiology Letters.* 2002;213(2):183-188. doi: 10.1111/j.1574-6968.2002.tb11303.x.

16. Song B, Palleroni NJ, Haggblom MM. Description of strain 3CB-1, a genomovar of Thauera aromatica, capable of degrading 3-chlorobenzoate coupled to nitrate reduction. *Int J Syst Evol Microbiol.* 2000;50(2):551-558. doi: 10.1099/00207713-50-2-551.

17. Song B, Palleroni NJ, Haggblom MM. Isolation and characterization of diverse halobenzoate-degrading denitrifying bacteria from soils and sediments. *Appl Environ Microbiol.* 2000;66(8):3446-3453. doi: 10.1128/AEM.66.8.3446-3453.2000

18. Song B, Palleroni NJ, Kerkhof LJ, Haggblom MM. Characterization of halobenzoate-degrading, denitrifying Azoarcus and Thauera isolates and description of Thauera chlorobenzoica sp. nov. *Int J Syst Evol Microbiol.* 2001;51(2):589-602. doi: 10.1099/00207713-51-2-589.

19. Tiedt O, Mergelsberg M, Boll K, et al. ATP-Dependent C-F Bond Cleavage Allows the Complete Degradation of 4-Fluoroaromatics without Oxygen. *MBio.* 2016;7(4):1-9. doi: 10.1128/mBio.00990-16.

20. Drzyzga O, Jannsen S, Blotevogel K. Mineralization of monofluorobenzoate by a diculture under sulfate-reducing conditions. *FEMS Microbiol Lett.* 1994;116(2):215-219. doi: 10.1111/j.1574-6968.1994.tb06703.x.

21. Egland PG, Gibson J, Harwood CS. Reductive, coenzyme A-mediated pathway for 3-chlorobenzoate degradation in the phototrophic bacterium Rhodopseudomonas palustris. *Appl Environ Microbiol.* 2001;67(3):1396-1399. doi: 10.1128/AEM.67.3.1396-1399.2001.

22. Häggblom MM, Young LY. Anaerobic degradation of 3-halobenzoates by a denitrifying bacterium. *Archives of Microbiology.* 1999;171(4):230-236. doi: 10.1007/s002030050704.

23. Harwood CS, Burchhardt G, Herrmann H, Fuchs G. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiology Reviews* 1998;22(5):439-458. doi: 10.1111/j.1574-6976.1998.tb00380.x.

24. Tschech A, Fuchs G. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. *Arch Microbiol.* 1987;148(3):213-217. doi: https://doi.org/10.1007/BF00414814.

25. Pfennig N. Rhodocyclus purpureus gen. nov. and sp. nov., a Ring-Shaped, Vitamin B12-Requiring Member of the Family Rhodospirillaceae. *International Journal of Systematic Bacteriology.* 1978;28(2):283-288. doi: 10.1099/00207713-28-2-283.

26. Tschech A, Pfennig N. Growth yield increase linked to caffeate reduction in Acetobacterium woodii. *Archives of Microbiology.* 1984;137(2):163-167. doi: 10.1007/bf00414460.

27. Coplen TB. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Commun Mass Spectrom.* 2011;25(17):2538-2560. doi: 10.1002/rcm.5129.

28. Coplen TB, Brand WA, Gehre M, et al. After two decades a second anchor for the VPDB delta13C scale. *Rapid Commun Mass Spectrom.* 2006;20(21):3165-3166. doi: 10.1002/rcm.2727.

29. Godin JP, Hopfgartner G, Fay L. Temperature-programmed high-performance liquid chromatography coupled to isotope ratio mass spectrometry. *Anal Chem.* 2008;80(18):7144-7152. doi: 10.1021/ac8004204.

30. Aeppli C, Tysklind M, Holmstrand H, Gustafsson O. Use of Cl and C isotopic fractionation to identify degradation and sources of polychlorinated phenols: mechanistic study and field application. *Environ Sci Technol.* 2013;47(2):790-797. doi: 10.1021/es303343u.

31. Fuchs G, Boll M, Heider J. Microbial degradation of aromatic compounds - from one strategy to four. *Nat Rev Microbiol.* 2011; 9(11):803-816. doi: 10.1038/nrmicro2652.

32. Schmidt TC, Jochmann MA. Origin and fate of organic compounds in water: characterization by compound-specific stable isotope analysis. *Annu Rev Anal Chem.* 2012;5:133-155. doi: 10.1146/annurev-anchem-062011-143143.

33. Renpenning J, Schimmelmann A, Gehre M. Compound-specific hydrogen isotope analysis of fluorine-, chlorine-, bromine- and iodine-bearing organics using gas chromatography-chromium-based high-temperature conversion (Cr/HTC) isotope ratio mass spectrometry. *Rapid Commun Mass Spectrom.* 2017;31(13):1095-1102. doi: 10.1002/rcm.7872.

34. Horst A, Renpenning J, Richnow HH, Gehre M. Compound Specific Stable Chlorine Isotopic Analysis of Volatile Aliphatic Compounds Using Gas Chromatography Hyphenated with Multiple

Collector Inductively Coupled Plasma Mass Spectrometry. *Anal Chem.* 2017;89(17):9131-9138. doi: 10.1021/acs.analchem.7b01875.

35. Renpenning J, Horst A, Schmidt M, Gehre M. Online isotope analysis of 37Cl/35Cl universally applied for semi-volatile organic compounds using GC-MC-ICPMS. *J Anal At Spectrom.* 2018. doi: 10.1039/c7ja00404d.

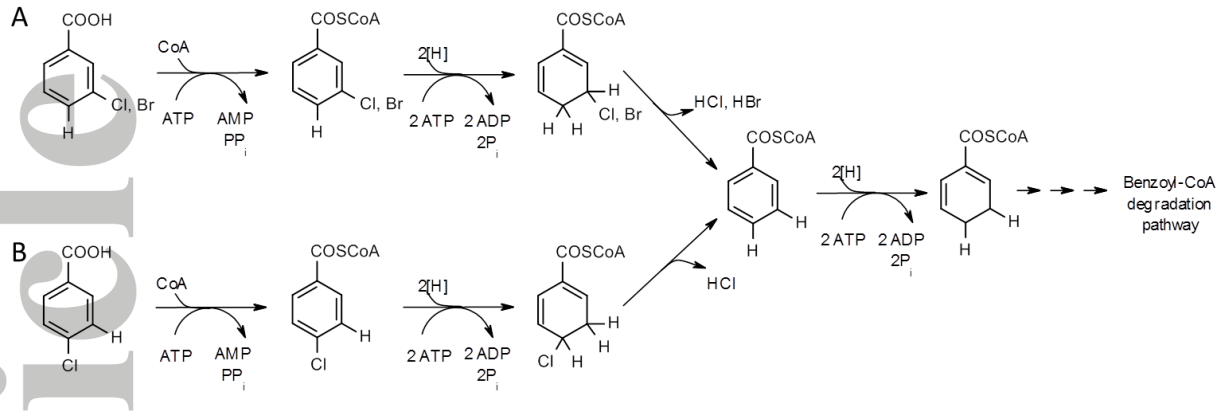

Figure 1: Proposed mechanism for dehalogenation of (A) 3-Cl-, 3-Br- and (B) 4-Cl-benzoate by *T. chlorobenzoica* 3CB-1^T (modified from Kuntze et al [13]). Initially, the halobenzoate is activated by Coenzyme A (CoA) through benzoyl-CoA-ligase transesterification, resulting in the formation of the respective CoA-thioester. The further degradation is proposed to occur via a reductive dearomatization by ATP-dependent BcrA class I. Subsequently, rearomatization takes place accompanied by hydrogen halide removal resulting in benzoate being the subject of further degradation via the benzoyl-CoA-pathway [23].

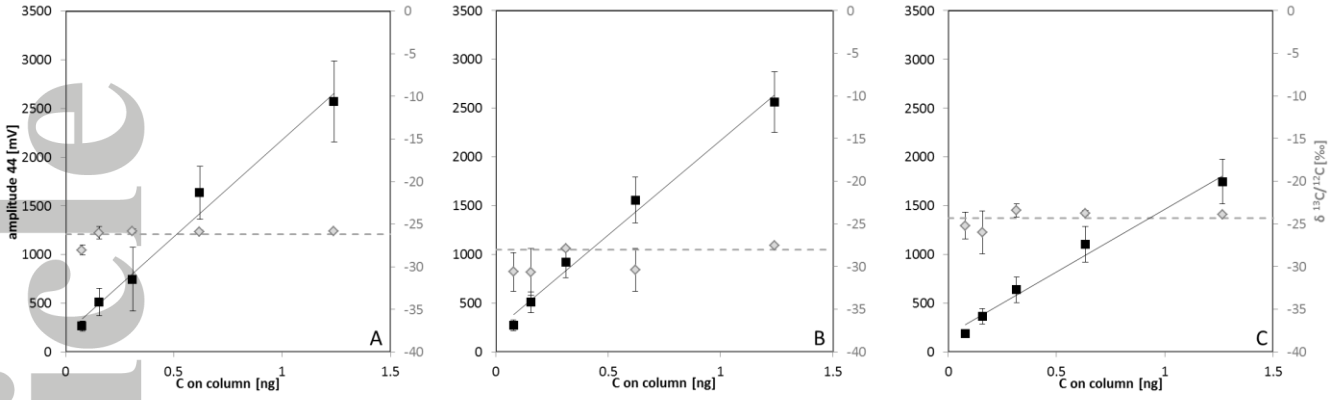

Figure 2: Concentration dependent analysis of amplitude (black squares) and resulting delta value for carbon isotope analysis (grey diamonds) of (A) 3-Cl-benzoate, (B) 4-Cl-benzoate and (C) 3-Br-benzoate. Grey dashed lines indicate determined EA-values.

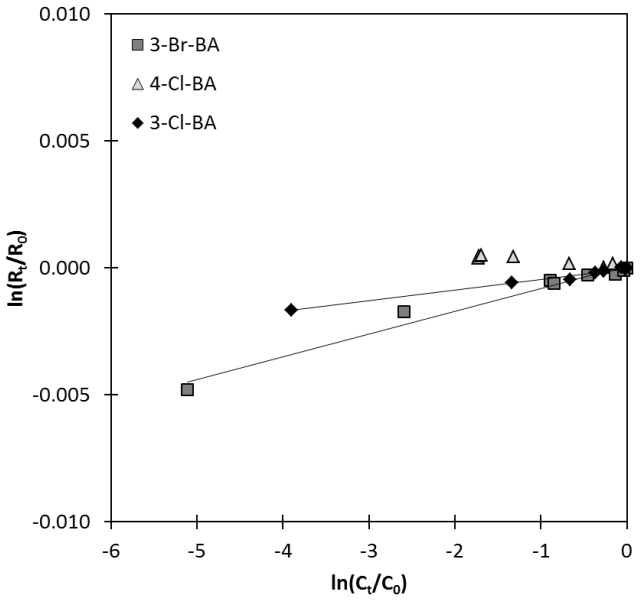

Figure 3: Rayleigh Plot for carbon isotope analysis of 3-Cl-, 4-Cl- and 3-Br-benzoate degradation by *T. chlorobenzoica* 3CB-1^T. Whereas no significant isotopic fraction was visible for 4-Cl-benzoate, 3-Cl-benzoate and 3-Br-benzoate reveal significant inverse carbon isotopic fractionation.

Table 1: Optimization of wet oxidation conditions. Isotopic composition ($\delta^{13}C$ values [‰]) of investigated halogenated benzoates at different *ortho*-phosphoric acid and sodium peroxodisulfate concentrations and flow rates.

conditions			BA	3-Cl-BA	4-Cl-BA	3-Br-BA
o-H_3PO_4 [mol·L^{-1}]	$Na_2S_2O_8$ [g·L^{-1}]	flow rate [µL·min^{-1}]	$\delta^{13}C$ [‰]	$\delta^{13}C$ [‰]	$\delta^{13}C$ [‰]	$\delta^{13}C$ [‰]
Elemental analyzer			-28.6 ± 0.1	-26.1 ± 0.1	-28.0 ± 0.2	-24.3 ± 0.1
0.75	100	50	-28.9 ± 0.1	-23.5 ± 0.1	-28.3 ± 0.1	-24.2 ± 0.0
1.5	100	50	-26.5 ± 0.4	-22.8 ± 0.1	-27.1 ± 0.2	n.d.
1.5	200	50	-28.7 ± 0.1	-25.1± 0.1	-27.1 ± 0.1	n.d.
1.5	200	100	-28.6 ± 0.2	-26.9 ± 0.7	-28.2 ± 0.2	n.d.
1.5	200	75	-28.5 ± 0.1	-26.6 ± 0.5	-28.0 ± 0.1	n.d.

Legend: BA: benzoic acid, n.d.: not determined,