Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D2.8

Carbon and hydrogen isotopic enrichment factors for different PAHs as a basis to monitor their degradation

This project is supported by the European Union under the Food, Agriculture and Fisheries and Biotechnology theme of the 7th Framework Programme for Research and Technological Development under GA no. 312139

Work package	WP2 Development of biosensors and *in-situ* monitoring tools to determine biodegradation efficiency
Deliverable no	D2.8
Deliverable title	Carbon and hydrogen isotopic enrichment factors for different PACs as a basis to monitor their degradation
Due date:	Month 36 (2016-01-30)
Actual submission date:	Month 43 (2016-07-08)
Start date of project:	2013-01-01
Deliverable Lead Beneficiary (Organisation name)	HMGU
Participant(s) (Partner short names)	HMGU
Author(s) in alphabetic order:	M. Elsner, S. Marozava, R. Meckenstock, A. Meyer
Contact for queries:	Martin Elsner, HMGU Helmholtz Zentrum München Ingolstädter Landstr. 1 85764 Neuherberg Ph.: +49-89-3187-2565 Fax: +49-89-3187-3361 e-Mail: martin.elsner@helmholtz-muenchen.de
Dissemination Level: (PUblic, Restricted to other Programmes Participants, REstricted to a group specified by the consortium, COnfidential only for members of the consortium)	PU
Deliverable Status:	final

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1 About this Deliverable

Task of WP2 was to develop monitoring tools for polyaromatic hydrocarbons biodegradation efficiency in the field. These tools include monitoring of the presence of polyromantic hydrocarbons-degrading bacteria as well as quantitative evaluation of biodegradation via diagnostic isomer ratios and compound-specific carbon and hydrogen isotope analysis. This deliverable introduces compound-specific isotope analyses as an independent tool for monitoring biodegradation of low molecular weight substituted and non-substituted polyaromatic hydrocarbons. So far carbon isotopic fractionation was investigated for aerobic and anaerobic bacteria cultivated in batch experiments.

2 Introduction

Compound Specific Isotope Analysis (CSIA) is an innovative analytical approach that has been widely used to monitor the biodegradation of organic pollutants like chlorinated and monoaromatic hydrocarbons **(1)**. Within the KILL SPILL project we aimed at expanding the use of CSIA to substituted and non-substituted Polycyclic Aromatic Hydrocarbons (PAHs). Previous studies with aromatic hydrocarbons has shown that C and H isotope fractionation is observable under anaerobic conditions as well as under aerobic conditions ((2-4). Thus our analytical procedures are focused on the study of C and H isotopes during natural and stimulated degradation of PAHs by environmentally relevant aerobic and anaerobic bacteria.

When organic compounds are degraded, bonds containing a light isotope (e.g. ^{12}C-H) typically react faster than those containing a heavy isotope (e.g.^{13}C-H). This phenomenon is known as kinetic isotope effect **(5,6).** As a consequence, the molecules, which are left behind in the pool of unreacted substrate, contain increasingly more ^{13}C as the reaction progresses**.** Non-degradative processes (sorption, transport, dilution), in contrast, cause smaller isotope effects **(7,8).** On a most fundamental level, changes in the isotope ratio of a compound compared to its original isotopic composition can therefore be a strong qualitative indicator of degradation.

In order to use CSIA for the qualitative and/or semi-quantitative assessment of biodegradation of PAHs in the field (WP8), the so called isotope enrichment factors for specific compounds need to be inferred from degradation studies with pure or mixed cultures in the laboratory, under controlled conditions. Isotope enrichment factors link changes in stable isotope ratios given by the δ-notation **(9)** with changes in substrate concentrations during biodegradation.

Since remaining substrate molecules contain increasingly more ^{13}C as the reaction progresses, changes in the isotope composition provide the opportunity to make use of CSIA: these isotope values are indicative of the extent of degradation and can be described by the Rayleigh equation (Eq. 1):

$$\frac{R_t}{R_0} = \frac{1 + \delta^{13}C_t}{1 + \delta^{13}C_o} = f^{\varepsilon} \qquad (1)$$

where R_t and R_0 (or $\delta^{13}C_t$ and $\delta^{13}C_0$) describe the average isotope composition of the heavy isotope to the light isotope in a specific compound at a given time and at the beginning of the reaction,

respectively (i.e., when nothing has been degraded so far). The remaining fraction *f* of the compound is given by the ratio C_t/C_0, where C_t is the concentration of this compound at a given time and C_0 at the beginning of the reaction. The enrichment factor ε links the shift in isotope ratios to the extent of degradation and can be derived from the linearized form of the Rayleigh equation (Figure 1A). In good approximation the isotope shift after 50% of compound degradation is $\Delta\delta^{13}C \approx 0.7 \bullet \varepsilon$ (Figure 1B). Further, $\Delta\delta^{13}C \approx 2 \bullet 0.7 * \varepsilon$ for $f = 0.25$ (75 % degraded), $\Delta\delta^{13}C \approx 3 \bullet 0.7 * \varepsilon$ for $f = 0.125$ (87.5 %) degraded and so on.

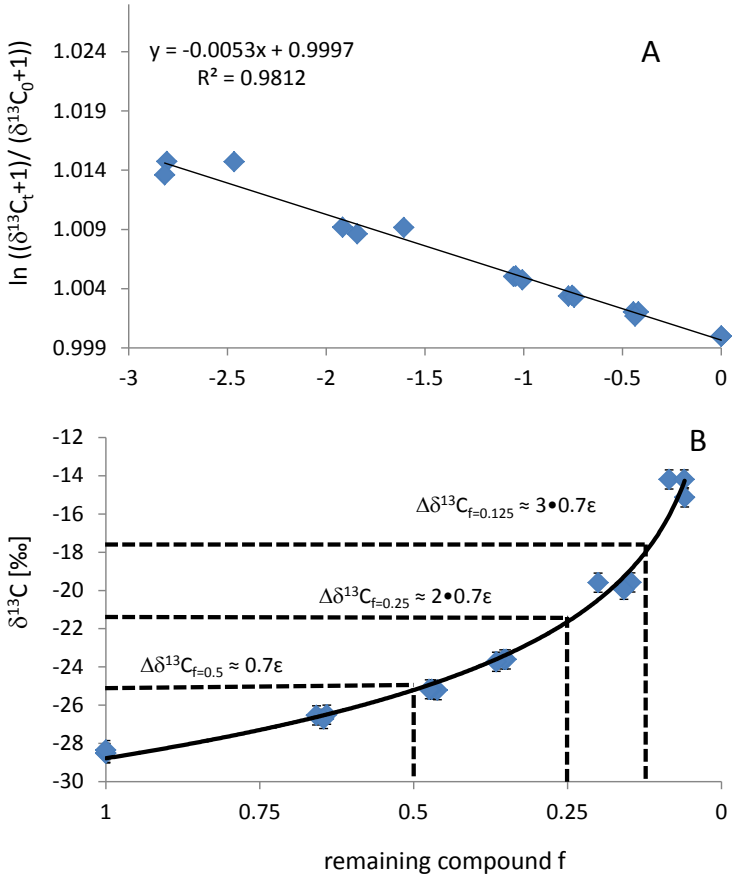

Figure 1 **A) Logarithmic plot of carbon isotope ratios according to the Rayleigh equation for an exemplary compound. From the slope of the regression the enrichment factor ε is derived. For practicability ε is given in ‰. B) Measured (blue rhombi) and modelled (black line) isotope fractionation according to Equation (2) with an enrichment factor ε of -5.3 ‰. (see also deliverable 8.7 (HMGU))**

These enrichment factors ε are informative because they have the virtue to allow quantifying degradation in the field. Once values of ε have been established for a certain degradation of a specific compound, as they have been determined in controlled batch or microcosm experiments mimicking field condition – the extent of degradation can be estimated from changes in isotope values according to Eq. 2:

$$B = (1 - f) \cdot 100\ [\%] = 1 - \left(\frac{1+\delta^{13}C_t}{1+\delta^{13}C_o}\right)^{\frac{1}{\epsilon}} \cdot\ 100[\%] \qquad (2)$$

where B is the extent of its degradation in %. In contrast to conventional methods, which are usually based on the measurement of compound concentrations, this concentration-independent approach makes it possible to estimate degradation of an organic contaminant in natural systems without the need of identifying metabolites and of establishing closed mass balances (**1**). Further explanation about isotope analyses and its procedure can be found in the report of deliverable 8.7.

A major challenge for the development of CSIA approaches for PAHs is their low solubility in water and large hydrophobicity, particularly for three- and higher-ringed PAHs. As a consequence, large volumes of water are required for the detection of the analytes which may cause experiments in the laboratory impracticable. By contrast, concentrations of PAHs in sediments, where they accumulate, or in oil phases, from which they may originate, can be large enough so that small volumes or a sample mass is required for analyte detection. As microbes cannot access contaminants in these phases directly and mass transfer limitation may occur, it remains unclear whether significant isotope fractionation can be recorded in sediments or oil samples. Thus, to develop sound CSIA methodologies for monitoring PAHs following approach was proposed: i) obtain isotope enrichment factors for both C and H in laboratory experiments with pure or mixed cultures in aqueous medium, ii) validate CSIA in a model system mimicking conditions similar to those encountered in natural systems, that is, a two-phase system with a carrier phase (e.g. an organic solvent or adsorbent resin beads), where the PAH is concentrated, and an aqueous phase into which the PAH is slowly delivered here, the compound is present at very low concentrations compared to the carrier phase; and iii):determination of isotope fractionation of PAHs in the presence of biosurfactants, which are to be applied by project partners for stimulated bioremediation in the field as these surfactants increase the water solubility of PAHs ,

3 Experimental set up for the determination of isotope enrichment factors of PAHs

To address the goals of the deliverable a set of experiments was conducted in batch mode. In one approach we were using 2-methylnaphathalene (2-MN) as a model PAH and two sulfate-reducing cultures capable of anaerobic biodegradation of 2-MN: the marine pure culture NaphS2 (DSMZ collection). Cultures were grown in two types of systems: i) one-phase system, consisting of 2-MN dissolved in aqueous medium and a target concentration of 0.115 mM, and ii) two-phase system, with 2-MN dissolved in a carrier hexadecane phase and a target concentration of either 100 mM or 10 mM. Changes in both isotope ratios and substrate concentrations were assessed in the aqueous solution for the one-phase system experiments and in the hexadecane phase for the two-phase systems.

In the second approach, we were using 2-MN with and without (bio)surfactants and again the sulfate-reducer NaphS2 (DSMZ collection) and the aerobic marine bacteria *Cycloclasticus* sp. (provided by CNR-IAMS), which is also capable to degrade 2-MN. The following surfactants were used: synthetic Triton X-100 (1 g/l) (Merck, Darmstadt), biosurfactants rhamnolipids (0.5 - 1 g/L) and sophorolipids (1.5 – 2.5 g/L) provided by Kill Spill partners from ACTY (unpure surfactants in fermentation broth) and Ulster (purified surfactants from fermentation broth). The concentrations of

2-MN were above water solubility in the range of 60-120 mg/L to simulate likely concentrations during an oil-spill.

To demonstrate the applicability of CSIA, to detect degradation also for different PAHs, we to our knowledge investigated for the first time, hydrogen isotope fractionation associated with the degradation of 1-methylnaphthalene under iron reducing condition. Degradation experiments were carried out by the enrichment culture 1MN (1MN enrichment culture, groundwater iron-reducer consisting of two key members Desulfobulboceae and Firmicutes) in aqueous solution.

4 Results

4.1 Determination of carbon isotope enrichment factors for 2-MN degradation by anaerobic bacteria in an one and two phase system

In the one phase system (aqueous media), 88 % degradation of 2-MN by NaphS2 was associated with a carbon isotope enrichment of 4.3 ‰ ($\delta^{13}C_{2\text{-MN, initially}}$ = -23.1 ‰ , $\delta^{13}C_{2\text{-MN, 88\% degradation}}$ = -18.8 ‰), resulting in a carbon isotope enrichment factor $\varepsilon_{carbon/aqueous\ phase}$ of -2.1 $\pm$ 0.1 ‰ (uncertainty of 95 % confidence interval; Figure 2A).

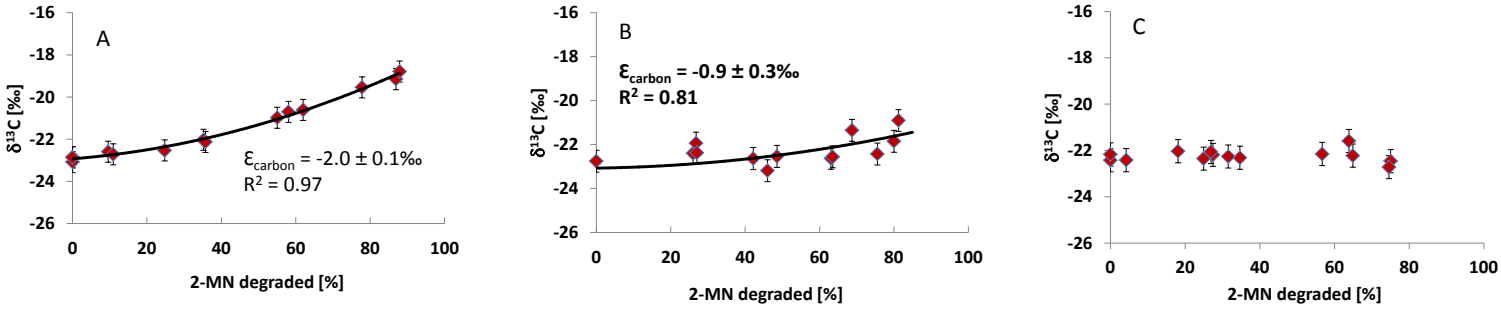

Figure 2 Carbon isotope fractionation associated with the degradation of 2-methylnaphthalene (2-MN) by NaphS2.

A) Degradation of 2-MN in aqueous one-phase system (initial concentration of 2-MN in aqueous solution, 0.115 mM), B) in hexadecane/water two phase system (initial concentration of 2-MN in hexadecane, 100 mM), and C) in hexadecane/water two phase system (initial concentration of 2-MN in hexadecane, 10 mM).

In comparison to the one-phase system, changes in carbon isotope ratios of 2-MN (initial concentration 100 mM; sampled from hexadecane phase) of the two-phase system showed an enrichment factor of -0.9 $\pm$ 0.1 ‰ (uncertainty of 95% confidence interval), less pronounced but still detectable (Figure 2B). However, in the two-phase experiment starting with a lower initial concentration (10 mM) of 2-MN in the hexadecane phase, degradation was not associated with an observable carbon isotope effect (Figure 2C).

Similiar picture was observed for the hydrogen isotope effects. Also here in the pure aqueous phase degradation of 2-MN was related to the greatest observable isotope effects in comparison to the two-phase system (Figure 3). The enrichment of $\Delta\delta^2H$ in the remaining 2-MN was 78 ‰ after 88 % degradation ($\varepsilon_{hydrogen/aqueous}$= -40 ± 7 ‰). In the two phase system with the hexadecane phase, the

observable hydrogen isotope shifts in the remaining 2-MN of the hexadecane phase were only 8 ‰ after 80% of degradation in the experiments with initial concetration of 100 mM (Figure 3 B) or even not observable for the experiments with initial concetration of 10 mM (Figure 3 C).

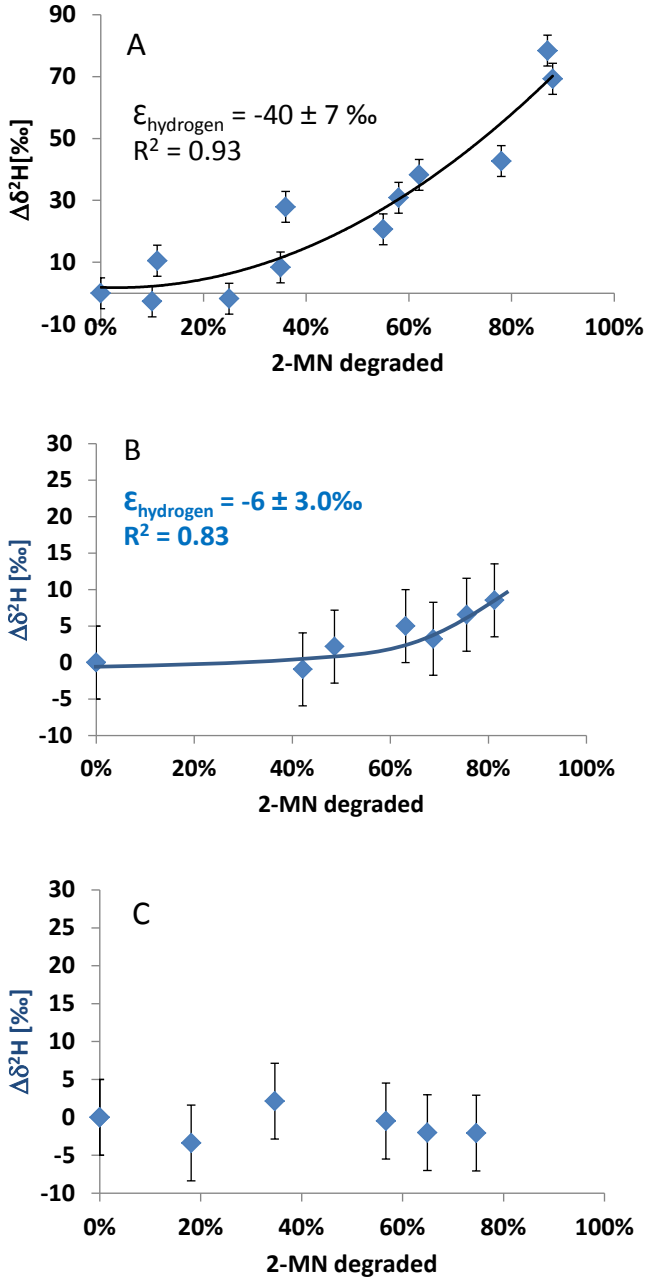

Figure 3 Hydrogen isotope fractionation associated with the degradation of 2-methylnaphthalene (2-MN) by NaphS2.

A) Degradation of 2-MN in aqueous one-phase system (initial concentration of 2-MN in aqueous solution, 0.115 mM), B) in hexadecane/water two phase system (initial concentration of 2-MN in hexadecane, 100 mM), and C) in hexadecane/water two phase system (initial concentration of 2-MN in hexadecane, 10 mM).

The results of the two-phase experiments show clearly that mass transfer of 2-MN between hexadecane and water phase is partly rate-limiting, as indicated by the observation that the carbon isotope effect associated with the degradation of 2-MN is “masked”.

4.2 Compound specific isotope analyses of 2-MN degradation in the presence of surfactants

First, experiments were carried out to test how different concentrations of (bio)surfactants influence the solubility of 2-MN, which is 25 mg/L in pure water. The maximum concentrations of surfactants applied were in the range of their critical miscele concentrations. As expected, addition of surfactants concentrations increased 2-MN solubility (Figure 4). The highest solubility of 2-MN was reached in the presense of 1 g/L of Triton X-100 with around 90 mg/L, while 1g/L of rhamnolipids and 2.5/L g of sophorolipids led to lower solubilities of ca. 40 mg/L. Based on these preliminary results, the surfactant concentrations for biotic degradation experiments have been chosen. In degradation experiments with NaphS2 pure surfactants were used in the following concentrations, respectively: 1.7 g/L for rhamnolipids, 1.45 g/L for sophorolipids, 1.0 g/L for Triton X-100. In degradation experiments with *Cycloclasticus sp*. unpure sophorolipids in the concentration of 0.8 g/L and 1.0 g/L Triton X-100 were used, respectively.

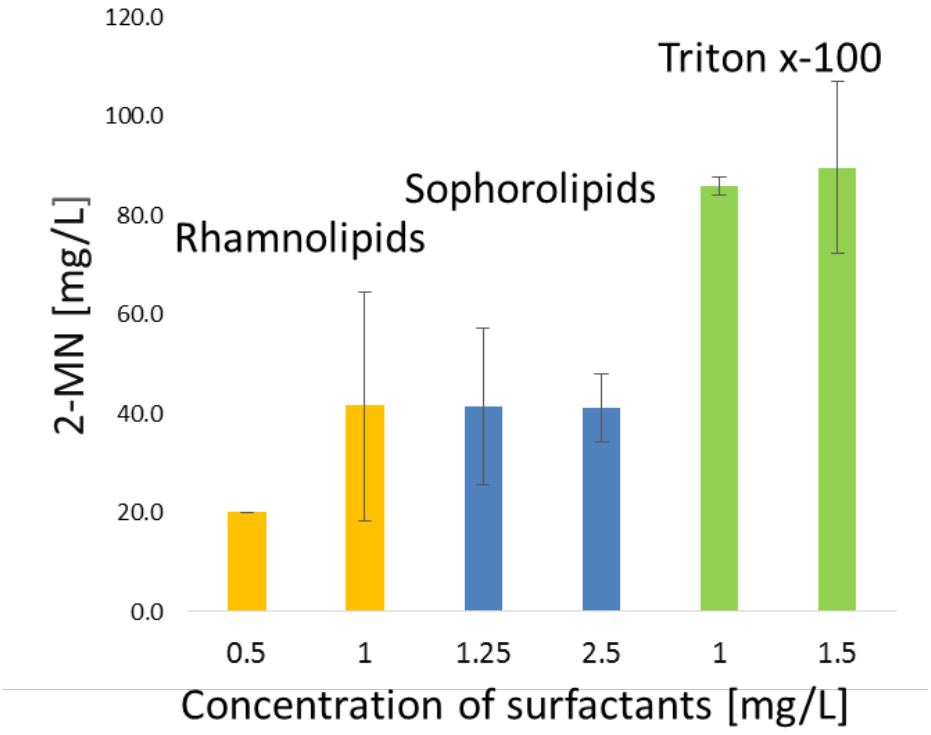

Figure 4 **Effect of pure surfactants on solubility of 2-MN, 0.12 g/L 2-MN was added into the salt medium; error bars represent standard deviation of duplicates.**

NaphS2 did not grow in the presence of 90 mg/L of 2-MN and surfactants. In comparison, control experiments showed growth of NaphS2 in the presence of the same surfactants concentrations and

non-toxic aromatic compound benzoate. To this end, we hypothesize that increased amounts of dissolved 2-MN (90 mg/l) were toxic for the anaerobic sulfate-reducer.

In contrast, the aerobic marine degrader *Cycloclasticus* sp. was able to grow in the presence of the same high concentrations of 2-MN (up to 100 mg/l). However, no degradation of 2-MN was observed in the presence of unpure biosurfactants. The growth of *Cycloclasticus* sp. was probably due to consumption of components of fermentation broth. In contrast to the rhamnolipids and sophorolipids, degradation of 2-MN occurred in the presence of Triton X-100. However, carbon isotope fractionation was not observable (Figure 5). As the absence of isotope carbon fractionation was also observed in the control without surfactant (Figure 5) we assume that either mechanisms of aerobic degradation (catalyzed by monooxygenases) are less carbon isotope sensitive and cannot be detected or transport of 2-MN into the cell or to the degrading enzyme of *Cycloclasticus* sp. is mass transfer limiting. Although, no carbon isotope effect was observed, we expect to see hydrogen isotope effect for aerobic degradation of 2-MN as this approach is more sensitive for isotope effects. These analyses will be performed in the ongoing period.

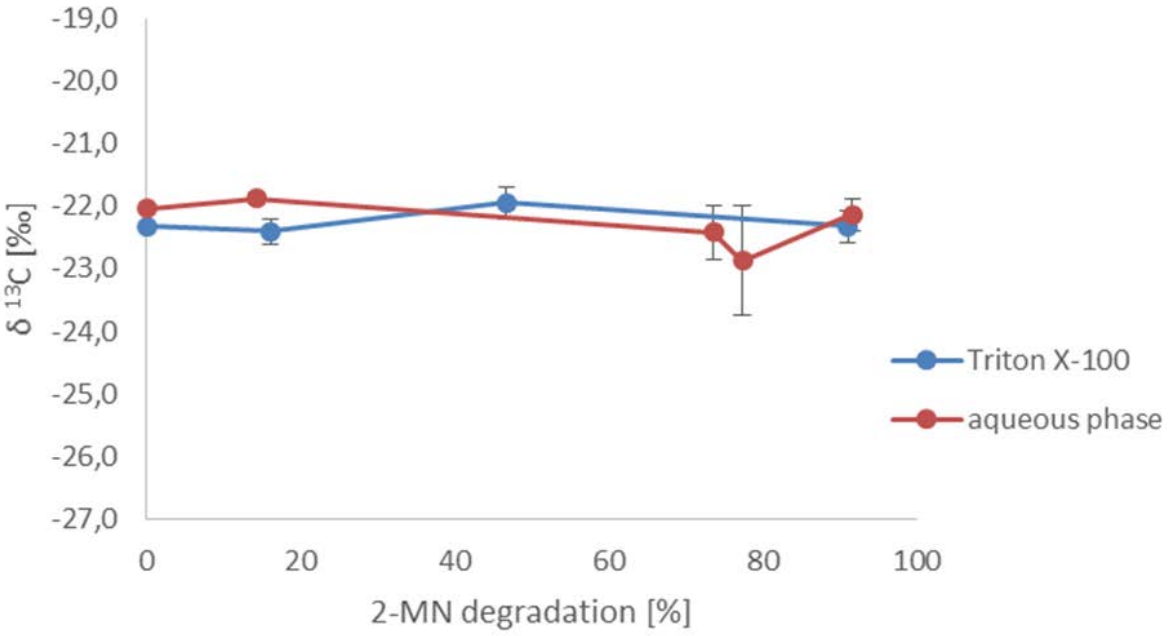

Figure 5 Isotopic fractionation of 2-MN by *Cycloclasticus sp*. in aqueous phase, and in presence of Triton X-100.

4.3 Hydrogen isotope fractionation associated with 1-methylnaphthalene (1-MN) degradation under iron reducing conditions

Degradation of 1-MN by enrichment culture 1MN led to an observable hydrogen isotope shift in the remaining aromatic hydrocarbon of $\Delta\delta^2H$ =50 ± 5 ‰ after 99 % of degradation (Figure 6). Shifts varied between the replicates, indicating that condition changed, which may be caused by changing specifity of the available iron as described in Tobler et al, 2008 **(10)**, where degradation of toluene with Fe (III) ions and solid iron led to different isotope enrichment patterns within the remaining

toluene. However, the analyses of this study demonstrate, considering also the results given in paragraph 4.1, that compound specific isotope analyses of higher molecular mass PAHs can be a successfull approach to determine degradation of PAHs in environmental systems.

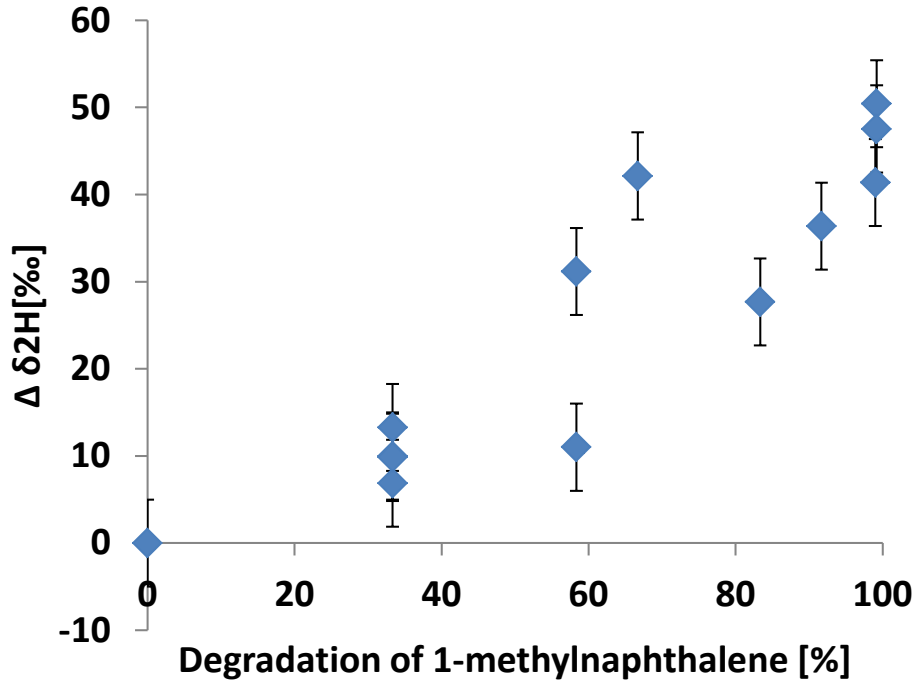

Figure 6 **Hydrogen isotope fractionation of 1-MN under iron reducing conditions by enrichment culture 1MN**

5 Achievements after 36th months and ongoing work

- Successful determination of C and H isotope fractionation factor for 2-MN degradation under anaerobic conditions (NaphS2 culture, marine sulfate-reducer).
- Successful determination of H isotope fractionation factor for 1-MN degradation under anaerobic conditions (1MN enrichment culture, groundwater iron-reducer consisting of two key members Desulfobulboceae and Firmicutes)
- No C isotope fractionation is detecable for aerobic degradation of 2-MN (*Cycloclasticus* sp.).
- Publication of protocol for the compound specific isotope analysis of aromatic hydrocarbons (published in Springer Protocol Handbooks; Meyer, A.H.; Maier, M.P.; Elsner, M.;2015.;doi: 10.1007/8623_2015_174)
- Mesocosm studies with partner of WP8 (CNR-IAMS) have been setup
- C and H isotope analysis for PAHs is planned to be extended to more compounds such as phenanthrene.
- Solubility tests and investigation of isotope fractionation of phenanthrene in the presence of surfactants will be carried out.
- Baseline peak separation for heavy contaminated samples by GCxGC-IRMS will be carried out (recently started project).

6 References

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