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# $^{13}C/^{12}C$ Isotope Fractionation during Aerobic and Anaerobic Biodegradation of Naphthalene

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## ABSTRACT

**Microcosm experiments were conducted to determine the fractionation of stable carbon isotopes during biodegradation of naphthalene. The microcosms were performed under aerobic conditions, anaerobic (amended with sulfate, amended with nitrate and with no amendments) and sterile controls. The liquid phase was analyzed to determine naphthalene concentration and stable carbon isotope signature. Aerobic microcosm showed that naphthalene degraded aerobically within 60 hours. The $\delta^{13}C$ increased from −25.5‰ to −25.1‰ (enrichment of 0.4‰ ± 0.08‰) in a single sample in which 95% of the naphthalene was biodegraded. Anaerobic microcosms show that after 288 days, the microcosms with no amendments, amended with nitrate and amended with sulfate had consumed respectively 30%, 50% and 60% of naphthalene on average, compared to control microcosms. Under the denitrifying conditions, the $\delta^{13}C$ of naphthalene increased from −25.2‰ to −23.9‰ (enrichment of 1.3‰ ± 0.3‰) after a 95% of naphthalene biodegradation. For the unamended microcosms, a slight enrichment on $\delta^{13}C$ napththalene was detected, from −25.2‰ to −24.5‰ (enrichment of 0.7‰ ± 0.3‰) after a biodegradation of approximately 65% of naphthalene (after 288 days). For sulfate reducing microcosms, no significant changes were detected on the $\delta^{13}C$ during naphthalene biodegradation.**

## KEYWORDS

**Naphthalene; Isotope; Fractionation; Enrichment; Aerobic; Anaerobic; Biodegradation**

## 1. Introduction

Contamination of groundwater by hydrocarbons has been widely documented in literature [1,2]. Naphthalene is the simplest polynuclear aromatic hydrocarbon (PAH) consisting of two benzene rings [1]. It is produced mainly by the refining of petroleum and is found mostly in creosote [3]. Naphthalene is a groundwater contaminant commonly found in wood preservation facilities [4,5]. It has been demonstrated that the naphthalene degrades aerobically [6,7], under anoxic conditions [8-10], under reducing conditions iron [11], under denitrifying conditions [12,13] and sulfates under reducing conditions [14,15].

Carbon has two stable isotopes $^{13}C$ and $^{12}C$, and a radioactive isotope, $^{14}C$ ($T_{1/2}$ = 5,730 years). The natural abundance of $^{13}C$ is 1.11% [16]. For kinetic reasons, there is a preference of microorganisms to break $^{12}C$-$^{12}C$ bonds rather than $^{12}C$-$^{13}C$ bonds during the biodegradation of organic compounds [17]. Therefore, the relationship $^{13}C/^{12}C$ of the compound being degraded could increase during a biodegradation process. Several studies have shown a change in this stable carbon isotope ratio during biodegradation of chlorinated carbon solvents [18-20]. However, the BTEX compounds produce very small stable carbon isotopic fractionation during biodegradation [20-22]. The gasoline additive, MTBE, also produces a very small stable carbon isotopic fractionation during biodegradation [23]. Richnow *et al.* [24] found a very low carbon isotopic fractionation on naphthalene in a field study.

To study this phenomenon under controlled laboratory conditions, naphthalene biodegradation can be reproduced in microcosms experiments. It would be expected that the $^{13}C/^{12}C$ ratio on naphthalene would increase over time as biodegradation takes place. The objective of this study is, through laboratory experiments, to determine

the fractionation of stable carbon isotopes during aerobic and anaerobic biodegradation of naphthalene.

## 2. Theory

Variations of $^{13}C/^{12}C$ in nature are very small. It is very difficult to measure absolute isotope abundances with a precision that is sufficient to detect small variations in isotope ratios. Measuring $^{13}C/^{12}C$ ratios relative to laboratory standards with a known isotope ratio, related to international standards, can be done with sufficient precision using stable isotope mass spectrometers. The unit in which the abundance of an isotope is reported is delta ($\delta$): the ratio of the isotopic composition of the analysed sample with respect to the standard. In the case of carbon, the international standard is known as VPDB (Vienna Pee Dee Belemnitella). It is expressed as parts per mil (‰) [16].

$$\delta^{13}C = \frac{\left(^{13}C/^{12}C\right)_{Sample} - \left(^{13}C/^{12}C\right)_{VPDB}}{\left(^{13}C/^{12}C\right)_{VPDB}} \times 1000\ ‰ \quad (1)$$

Craig [25] suggested that the small isotope fractionation observed during vaporisation or precipitation of water was governed by a Rayleigh distillation. The same Rayleigh behavior is observed in other isotope reactions. The Rayleigh distillation can be approximated using the delta per mil notation as [16]:

$$\delta - \delta_o \cong \varepsilon \ln(f) \quad (2)$$

where:

$\delta$ = is the isotope ratio on a reservoir with respect to a standard in parts per mil

$\delta_o$ = is the initial isotope ratio at the reservoir with respect to a standard in parts per mil

$\varepsilon$ = is the fractionation factor

$f$ = is the fraction of reactant remaining in the reservoir

Therefore, for a given isotope reaction (fractionation) that exhibits a Rayleigh distillation behavior, a corresponding enrichment factor ($\varepsilon$) can be calculated. This can be done by plotting several observations of the isotope ratio ($\delta$) against the natural logarithm of the corresponding fraction of reactant remaining [ln($f$)]. This will form a straight line of the following form:

$$\delta = \varepsilon \ln(f) + \delta_o \quad (3)$$

where the enrichment factor ($\varepsilon$) is the slope of the line. Using the Rayleigh distillation approach the enrichment factor ($\delta$) for naphthalene fractionation during biodegradation was calculated.

## 3. Methods

### 3.1. Microcosms Experiments

Sampling of soil and groundwater for microcosms were collected and preserved as described by Lesser (2013) [26]. Microcosm experiments were done under aerobic and anaerobic conditions with sterile controls. The anaerobic microcosm experiments were performed without amendment, and with sulphate and nitrate amendments. The purpose of adding sulphate and nitrate was to promote anaerobic degradation of naphthalene by adding possible electron acceptors which may have become depleted in site groundwater. All the experiments were done in triplicate. These microcosms received 20 g of aquifer material and approximately 55 ml of groundwater in 60 ml hypovials with no remaining headspace. Initial site groundwater did not contain enough naphthalene to perform the experiments, so the addition of naphthalene was necessary (approximately 12 mg/l). The appropriate additions of sulphate and nitrate were made via syringe adding 1 ml from stock solutions (73.96 mg/l of sodium sulphate and 180.43 mg/l of potassium nitrate respectively). Anaerobic microcosms were assembled in an anaerobic chamber. A total of 144 microcosms were performed as shown in **Table 1**, which includes the characteristics and time of sampling for each microcosm.

The microcosms were periodically sacrificed and the aqueous phase was analyzed for the concentration of organic constituents, dissolved oxygen, and nitrate or sulphate where appropriate. The samples from the microcosm experiments to be analyzed for stable carbon isotopes were collected in 4.8 ml glass vials with a Teflon coated septum and leaving no headspace. They were preserved with sodium azide at a rate of 1 μL per 1 ml of sample, and stored at 4˚C until isotopic analyses were performed.

### 3.2. Laboratory Analysis

The concentrations of organic and inorganic compounds in water samples were analyzed by the Organic Geochemistry Laboratory at the University of Waterloo. Organic compounds were analyzed by gas chromatography and inorganic compounds by ion chromatography (anions) and by a plasma spectrometer (cations).

For the stable carbon isotope ratio analysis, an isotope ratio mass spectrometers (IRMS) was employed. These apparatuses are designed to measure mass differences in compounds such as $CO_2$. For isotope analyses of organic compounds contained in aqueous samples, the organic compounds must first be extracted from the sample using headspace and/or SPME techniques. Each compound must then be isolated using a gas chromatograph (GC). Then the carbon contained in the compound is converted into $CO_2$ in a combustion interface. Water from the combustion is trapped and the $CO_2$ is swept into an IRMS.

For this study a solid-phase microextraction technique (SPME) was used for extraction of naphthalene from the

**Table 1. Summary of microcosms prepared, characteristics and sampling times.**

| Microcosm Condition | Total Microcosms | Sampling time for each triplicate | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Aerobic-Control | 24 | 0 hr | 17.5 hr | 24 hr | 41.5 hr | 48 hr | 64 hr | 74.5 hr | 92.3 hr |
| Aerobic | 24 | 0 hr | 17.5 hr | 24 hr | 41.5 hr | 48 hr | 64 hr | 74.5 hr | 92.3 hr |
| Anaerobic-Control | 24 | 0 d | 7 d | 28 d | 65 d | 98 d | 231 d | 288 d | NA |
| Anaerobic-Unamended | 24 | 0 d | 7 d | 28 d | 65 d | 98 d | 231 d | 288 d | NA |
| Anaerobic-sulphate-amended | 24 | 0 d | 7 d | 28 d | 65 d | 98 d | 231 d | 288 d | 288 d |
| Anaerobic-nitrate-amended | 24 | 0 d | 7 d | 28 d | 65 d | 98 d | 231 d | 288 d | NA |

d = days; NA = Not analyzed.

aqueous samples. Chai *et al.* [27] and Potter *et al.* [28] outline the SPME technique to extract PAHs from aqueous samples. Hunkeler and Aravena [29] compare the SPME extraction technique with other methods and point out its accuracy for $\delta^{13}C$ determination.

The SPME fiber used in this study is coated with Polydimethylsiloxane (Supelco, Bellefonte, PA, USA). Different film coating thicknesses were employed: 7 µm, 30 µm and 100 µm. The amount of analyte that can be sorbed is related to coating thickness. The thicker the coating, the greater the sensitivity and the lower concentrations can be analyzed. The IRMS system has a limited range of concentrations that can be analyzed, therefore when samples are highly concentrated a thinner coating film must be used or the samples must be diluted.

The fiber is contained in a fiber-holder which resembles a syringe. The needle is used to penetrate the septa of the sample vials and then the fiber is lowered into the water. Prior to lowering the fiber, a magnetic stir bar had been deposited inside the vial, and 0.5 ml of water had been removed in order to leave a headspace and avoid the needle touching the sample. All magnetic stir bars were washed with pentane, dried at 100˚C for 30 min and deposited into the vials using a sterilized Teflon coated magnet. Then, the sample is stirred for 20 minutes, until naphthalene reaches equilibrium between the water sample and the silica fiber.

Then the fiber is withdrawn into the needle and the needle is removed from the vial. The needle is then used to penetrate the GC septum, and the fiber is lowered into the GC inlet. The Gas Chromatograph (GC) model HP 6890 has an injector (split/splitless) equipped with a SPME liner from Supelco (Bellefonte, PA, USA). The column used (Rtx5) was 60 m long × 0.25 mm I.D. and 1.0 µm coating film thickness (5% diphenyl-95% dimethyl polysiloxane) from Restek Corporation (Bellefonte, PA, USA).

The analytes are thermally desorbed from the fiber and swept onto the GC column. The inlet temperature was 270˚C with a purge time of 1 minute (*i.e.* it runs in splitless mode for 1 minute before turning into split mode). The desorption time was 20 minutes (*i.e.* the total time the fibre is in the injector). The column is temperature programmed as follows: 80˚C for 2 min, then the temperature is increased 20˚C per min, up to 200˚C. Then the temperature remains at 200˚C for 12 min. Each sample was run twice to measure the reproducibility of the results. The GC separation of naphthalene from the rest of the organics found in the sample was confirmed by using neat compounds.

The GC column is connected to a quartz tube filled with copper oxide [combustion interface (Micromass UK Limited, Manchester, UK)] with an operating temperature of 850˚C. The combustion interface converts the organic compounds to $CO_2$ and $H_2O$. A liquid nitrogen cold trap set at –100˚C is used to retain the water while the $CO_2$ gas is swept with helium by capillary tubing into the isotope ratio mass spectrometer (IRMS) model Isochrom (Micromass UK Limited, Manchester, UK).

To measure the reproducibility and accuracy of $\delta^{13}C$ measurements, a naphthalene standard was prepared. Seven subsamples from the standard were run. Each subsample was run twice and the average was calculated as it was done for the real samples. The standard deviation obtained was 0.083‰, then the 95% confidence interval was calculated to be twice the standard deviation (0.166‰). This is the reported precision for each sample [30].

## 4. Results and Discussion

### 4.1. Aerobic Microcosms

Because the microcosm experiments were performed by analyzing bottles at different times each bottle is, on its own, a single experiment. Each experiment has then a different initial concentration ($C_o$) depending on the sorptive characteristics of the soil particles on each bottle. It is not practical to attempt to measure this variability due to the nature of the experiment, but this is assumed to have minimum effects on the final results of the ex-

periment (biodegradation of naphthalene with time). Hence, the first set of triplicates was assumed to represent the initial concentration of all samples ($C_o$) in each of the conditions of the microcosm experiments for each condition shown in **Table 1**. Control microcosms showed no decrease in the concentration of naphthalene.

The aerobic microcosm experiments show that naphthalene was consumed aerobically in less than 60 hours (**Figure 1**). These results are in agreement with previous studies that have shown aerobic biodegradation of naphthalene [6,7]. These data show that after 48 hours there were significant differences in the amount of naphthalene consumed (from 50% to 95%). The best estimate of naphthalene aerobic biodegradation was taken as the average of biodegradation in the triplicate microcosms.

Apparent biodegradation rates were calculated using zero-and first-order kinetics as follows:

$$\lambda_o = \frac{[C/C_o]-1}{t} \quad (4)$$

$$\lambda_1 = \frac{\ln[C/C_o]}{t} \quad (5)$$

where:

$t$ = time

$C$ = concentration at time $t$

$C_o$ = initial concentration

$\lambda_o$ = zero order kinetics apparent biodegradation rate [$t^{-1}$]

$\lambda_1$ = first order kinetics biodegradation rate [$t^{-1}$].

The apparent biodegradation rates were calculated by fitting the naphthalene data. The first-order decay rate ($\lambda_1$) was 2.4 days$^{-1}$, and the zero-order apparent decay rate ($\lambda_o$) was 0.72 days$^{-1}$.

Taking into account the results of the biodegradation of naphthalene, 13 microcosms samples were analyzed for $\delta^{13}$C. The results show a slight $\delta^{13}$C enrichment during aerobic biodegradation of naphthalene. The $\delta^{13}$C increased from approximately −25.5‰ to −25.1‰ (enrichment of 0.4‰ ± 0.08‰), only in one sample where 95% of the naphthalene had been consumed. The data clearly shows that this enrichment is only apparent after most of naphthalene has been degraded (**Figure 2**).

The statistical significance of the $\delta^{13}$C enrichments observed during biodegradation of naphthalene was calculated using the statistic test $T$ [31]. This test determines if there is any difference between two random variables from approximate normal distributions. The statistic test T is calculated as follows [31]:

$$T = \frac{\left(\bar{X}_a - \bar{X}_b\right)}{\sqrt{S_p^2\left(1/n_a + 1/n_b\right)}} \quad (6)$$

where:

$T$ = $T$ statistic value

$X_a$ = Mean value $a$

$X_b$ = Mean value $b$

$n_a$ = number of repetitions for value $a$

$n_b$ = number of repetitions for value $b$

and $S_p^2$ is a pooled variance calculated as:

$$S_p^2 = \frac{(n_a-1)s_a^2 + (n_b-1)s_b^2}{n_a + n_b - 2} \quad (7)$$

where:

$S_a^2$ = variance of population $a$

$S_b^2$ = variance of population $b$

with $(n_a + n_b - 2)$ degrees of freedom.

The $\delta^{13}$C for each microcosm were compared using this statistical test. For the sterile controls, except in one case, the $\delta^{13}$C values were statistically equal. The sample from the aerobic microcosm where most of naphthalene had been biodegraded was statistically different from the other 3 samples with a lower degree of biodegradation. Therefore, the isotopic enrichment observed during the aerobic biodegradation, though small, was statistically significant.

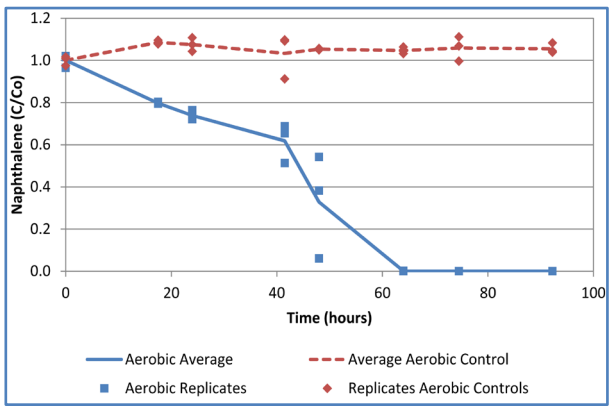


**Figure 1. Normalized naphthalene concentrations for the aerobic microcosms experiments. Triplicates results are shown, and the lines show the averages.**

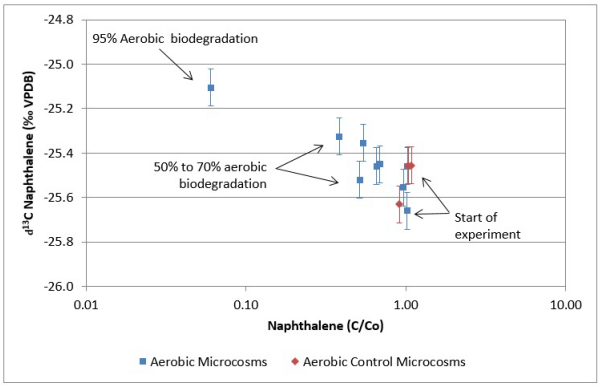


**Figure 2. Naphthalene $\delta^{13}$C for aerobic biodegradation microcosms. Naphthalene concentration is normalized and the bars represent the analytical error.**

Other researchers have also observed small isotopic enrichments on naphthalene in the final stages of their aerobic biodegradation [32]. Comparing with the results obtained for other compounds biodegraded aerobically, benzene was found to have an isotopic enrichment of 1.1‰ [21], and aerobic biodegradation of toluene did not exhibit any significant fractionation [20].

The enrichment factor for aerobic biodegradation of naphthalene was calculated. The concentration for each bottle was normalized with respect to the initial concentration ($C_o$) and this normalized value is in fact the fraction of remaining reactant ($f$). These normalized concentrations were plotted against the $\delta^{13}C$ and a least squares regression was obtained, which is equivalent to the Rayleigh distillation curve. Using Equation (3), an enrichment factor of ($\varepsilon$) of −0.16 was calculated, with a correlation factor ($R^2$) of 0.8. This correlation factor, although reasonable, is poor for a controlled microcosm experiment, and the enrichment factor ($\varepsilon$) is very low. Therefore, this isotopic enrichment may not be sufficient to demonstrate biodegradation of naphthalene in field situations, where many other processes such as advection, dispersion and adsorption, also help to reduce the concentrations of contaminants.

## 4.2. Anaerobic Microcosms

The anaerobic microcosms were also performed in triplicate. The results show that after 288 days the microcosms with no amendments, amended with nitrate and amended with sulfate, had consumed respectively 30%, 50% and 60% of naphthalene on average, compared to control microcosms (Figure 3). Essentially all the naphthalene was consumed in 4 in microcosms. The concentration of naphthalene in the sterile controls changed only slightly during the experiments; therefore much of the change in naphthalene concentration in the active microcosms is attributed to biodegradation.

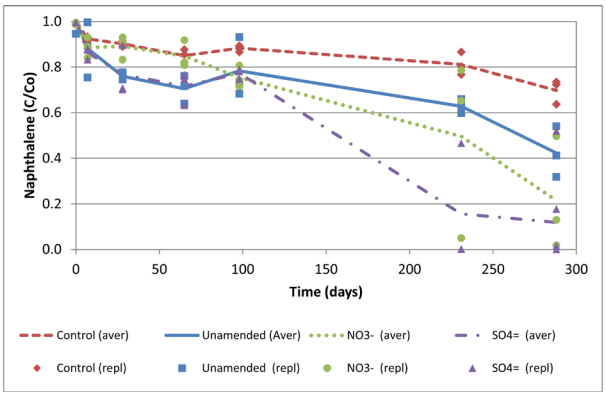


**Figure 3. Normalized concentrations of naphthalene for anaerobic microcosms experimenents (sterile control, nitrate-amended, sulfate amended and unamended). Replicates results are shown (repl), and the lines show the averages (aver).**

Apparent zero-order decay rate constants ($\lambda_o$) were calculated by subtracting the apparent mass loss in sterile controls, obtaining 0.26, 0.77 and 0.58 $yr^{-1}$ for the unamended, sulphate-amended and nitrate-amended respectively. The first-order decay rate constants ($\lambda_1$) obtained were 0.51, 1.50 and 2.23 $yr^{-1}$ for the unamended, sulphate-amended and nitrate-amended respectively. These decay rates are considerably lower than those obtained for aerobic degradation. Blum *et al.* [11] obtained a $\lambda_o$ of 1.39 $yr^{-1}$, similar to what was found in this study; however other studies report $\lambda_o$ of 5 to 31 $yr^{-1}$ [8].

In the nitrate- and sulfate amended microcosms, concentrations of nitrate and sulphate were also analyzed to monitor their activity as electron acceptors. These data show that the decrease in naphthalene concentration in the sulphate-amended microcosms correlates with a decrease in sulphate concentrations (data not shown). This suggests that biodegradation of naphthalene occurred under a sulphate-reducing conditions. Similarly a decrease in naphthalene concentration on the nitrate-amended microcosms also correlated with a decrease on nitrate concentrations (data not shown), suggesting that biodegradation occurred under denitrifying conditions.

Based on these concentration results, 20 microcosms were selected to be analyzed for the $\delta^{13}C$. The results show that under denitrifying conditions the $\delta^{13}C$ of naphthalene increased from −25.2‰ to −23.9‰ (enrichment of 1.3‰ ± 0.3‰) after naphthalene biodegraded in 95% (Figure 4). The enrichment factor ($\varepsilon$) according to the Rayleigh distillation was −0.3, with a correlation factor ($R^2$) of 0.7. Similarly to aerobic microcosms, the correlation factor is acceptable and the enrichment. is low. This means that under field conditions, this enrichment under denitrifying conditions would not be appreciated easily. The $\delta^{13}C$ of each microcosm were compared with statistic test $T$ [31]. For sterile controls $\delta^{13}C$ values were showed to be statistically equal. For the

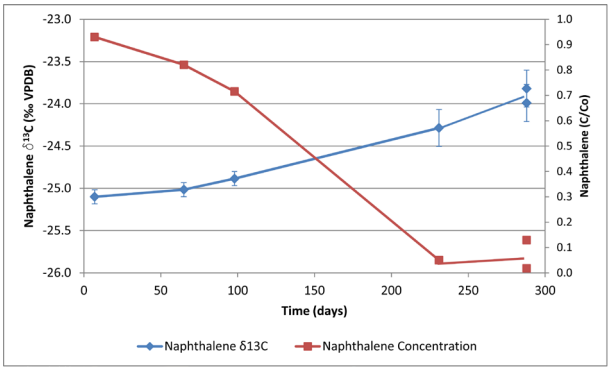


**Figure 4. Naphthalene $\delta^{13}C$ during anaerobic biodegradation microcosmos under denitrifiying conditions (nitrate amended). Naphthalene concentration is normalized and the bars represent the analytical error.**

active nitrate-amended microcosm, most $\delta^{13}C$ values were statistically different.

For the unamended microcosm a slight enrichment was detected in naphthalene $\delta^{13}C$ from −25.2‰ to −24.5‰ (0.7‰ ± 0.3‰ enrichment) after a biodegradation of approximately 65% (after 288 days, Figure 5). The enrichment factor ($\varepsilon$) according to the Rayleigh distillation, was −0.62 with a correlation factor ($R^2$) of 0.8. As in the previous cases, the correlation factor is acceptable but the enrichment factor is low, which would prove difficult to be verified in field situations. For these unamended microcosms, just one $\delta^{13}C$ results proved to be statistically different from the rest using the statistic test *T*.

For reductive sulphate-amended microcosms, no significant changes were seen in the $\delta^{13}C$ results.

Richnow *et al.* [24] found an isotopic fractionation of 1.2‰ naphthalene in an anaerobic aquifer. Kelley *et al.* [33] analyzed the $\delta^{13}C$ of several PAHs (including naphthalene) in an aquifer with anaerobic conditions. In their field data a very small enrichment in the $\delta^{13}C$ of naphthalene can be appreciated, however they do not seem to pay much attention to this result, perhaps because of the uncertainty associated with such low enrichments. The results of this study are in agreement with the results of Kelley *et al.* [33].

## 5. Conclusions

Aerobic microcosm showed that naphthalene degraded aerobically within 60 hours. Biodegradation rates obtained under aerobic conditions were: $\lambda_1 = 2.4\ d^{-1}$ and $\lambda_o = 0.72\ d^{-1}$. The $\delta^{13}C$ increased from −25.5‰ to −25.1‰ (enrichment of 0.4‰ ± 0.08‰) in a single sample in which 95% of the naphthalene was biodegraded. This result was statistically different from other 3 samples with a lower degree of biodegradation. The enrichment factor ($\varepsilon$) for aerobic biodegradation, according to Rayleigh distillation, was −0.16.

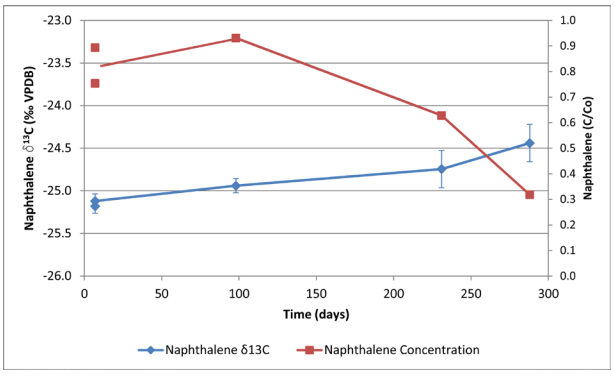


**Figure 5. Naphthalene $\delta^{13}C$ de during anaerobic biodegradation in unamended microcosmos. Naphthalene concentration is normalized and the bars represent the analytical error.**

Anaerobic microcosms show that after 288 days, the microcosms with no amendments, amended with nitrate and amended with sulfate had consumed respectively 30%, 50% and 60% of naphthalene on average, compared to control microcosms. Biodegradation rates obtained under anaerobic conditions were $\lambda_o$ of 0.26, 0.77 and 0.58 $yr^{-1}$ for unamended, nitrate-amended and sulphate-amended microcosms respectively. For the same cases, $\lambda_1$ was 0.51, 1.50 and 2.23 $yr^{-1}$.

Under the denitrifying conditions, the $\delta^{13}C$ of naphthalene increased from −25.2‰ to −23.9‰ (enrichment of 1.3‰ ± 0.3‰) after a 95% of naphthalene biodegradation, which an enrichment factor ($\varepsilon$) of −0.3 was obtained. For sterile controls, the $\delta^{13}C$ values were statistically equal. For the unamended microcosms, a slight enrichment on $\delta^{13}C$ napthhalene was detected, from −25.2‰ to −24.5‰ (enrichment of 0.7‰ ± 0.3‰) after a biodegradation of approximately 65% of naphthalene (after 288 days), with an enrichment factor ($\varepsilon$) of −0.62. For sulfate reducing microcosms, no significant changes were detected on the $\delta^{13}C$ of naphthalene.

The isotopic enrichment observed during aerobic and anaerobic biodegradation of naphthalene, although small, is statistically significant. It has been shown that some compounds that exhibit isotopic fractionation during biodegradation under laboratory conditions may only show a limited fractionation in field samples [23]. Therefore, the isotopic enrichment detected in this study for naphthalene may not be sufficient to demonstrate its biodegradation in field situations where many other processes such as advection, dispersion and adsorption, also help to reduce the concentrations of contaminants.

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