Compound-Specific Isotope Analysis of RDX and Stable Isotope Fractionation during Aerobic and Anaerobic Biodegradation

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Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a common contaminant at explosives production sites. Here, we report on the use of compound-specific isotope analysis of RDX to obtain δ\(^{15}\)N and δ\(^{18}\)O enrichment factors during biodegradation in batch cultures. A new preparation method has been developed based on RDX purification using thin-layer chromatography. RDX is then subjected to an elemental analyzer coupled with an isotope-ratio mass spectrometer (EA-IRMS). The precision of the method shows standard deviations of 0.13% and 1.18% for δ\(^{15}\)N and δ\(^{18}\)O, respectively, whereas the accuracy of the method has been checked routinely, adhering to external standards. The method was applied to RDX samples subjected to biodegradation under aerobic or anaerobic conditions. Enrichment factors under aerobic conditions were −2.1% and −1.7% for δ\(^{15}\)N and δ\(^{18}\)O, respectively, and under anaerobic conditions, −5.0% and −5.3% for δ\(^{15}\)N and δ\(^{18}\)O, respectively. The results of this study provide a tool for monitoring natural attenuation of RDX in a contaminated environment.

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is an explosive that is frequently found as a pollutant of soil and groundwater adjacent to its production plants. Subsurface soil and groundwater contamination by RDX is a global problem. It has been reported in the U.S., Germany, the UK, Canada, Australia, and in Israel. Throughout the rest of the world, the extent of explosives contamination is either unknown or not available to the public (1).

Biodegradation is a desirable, natural attenuation process for RDX in soil and groundwater. Different microorganisms have been reported to degrade RDX and various degradation pathways have been suggested (2–7 Figure 1).

Under anaerobic conditions, two distinct pathways for the biodegradation of RDX have been proposed: (i) A transformation pathway, accompanied by accumulation of the subsequent nitroso derivates hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). This pathway was initially presented by McCormick et al. (5). (ii) A pathway that involves initial denitrification followed by ring cleavage, formation of methylenedinitramine (MEDINA) and the eventual formation of nitrous oxide as a major end product. This pathway was postulated by Hawari et al. (6) following biodegradation experiments with municipal anaerobic sludge. The mechanism of denitration under anaerobic conditions was further investigated by Jackson et al. (7), who reported the role of the napA gene in encoding cytochrome P450 under these conditions and the formation of MEDINA.

Under aerobic conditions, in contrast to the anaerobic biodegradation of RDX, nitro derivates are not normally detected. Thus, under aerobic conditions, ring cleavage is identified as an important initial step (8, 9). Early studies by Hawari et al. (2) stated that ring cleavage of the RDX molecule under aerobic conditions is the result of either N–N or C–H bond cleavage, which leads to the formation of unstable intermediates that spontaneously decompose. Later reports provided evidence that N–N bond cleavage and denitrification are the initial steps in the RDX-biodegradation pathway by the Rhodococcus strain DN22 (3, 4). This pathway was later documented for Rhodococcus strain YH1 as well (10). Investigation of the mechanism governing the degradation pathway revealed that it is linked to the activation of cytochrome P450 by xplA (7, 11, 12), similar to denitration under anaerobic conditions. Aerobically, however, denitrification leads to the formation of 4-nitro-2,4-diazabutanal (NDAB) instead of MEDINA (7). Exceptions for the occurrence of this pathway under aerobic conditions were reported for fungal strains by Sheremata and Hawari (13) and by Bhattacharjee et al. (14) in which MNX was formed followed by ring cleavage and the formation of MEDINA. Van Aken et al. (15) also detected MNX when RDX was biodegraded under aerobic conditions. It was proposed that the polar metabolite produced from the degradation of RDX has the molecular formula O\(_2\)NNH\(_2\)NHNO\(_2\), (i.e., MEDINA). Theoretically, MNX can potentially be degraded to NDAB as well, if ring cleavage occurs following removal of the NO group, rather than the nitro group. This, however, has never been reported.
and gaining information on the degradation pathway involved (e.g., ref 19). The rationale underlying this concept is that as biodegradation proceeds, the remaining pool of pollutants becomes enriched in the heavier isotopes due to chemical bonds of lighter isotopes reacting at a minutely faster rate. The remaining substrate fraction therefore changes its isotopic composition. The extent of isotope enrichment of the remaining contaminant pool enables further quantification of the extent of biodegradation along a contamination plume (20). If stable isotope compositions of several elements of a given compound are analyzed simultaneously, one can obtain information on the initial biochemical steps in the biodegradation pathway or even distinguish between the different possible pathways involved.

The increasing use of CSIA in recent years is closely related to the development of analytical capabilities, enabling the isotopic analysis of a specific organic compound. The analytical procedure for CSIA normally comprises gas chromatographic separation of individual compounds, which are then transformed on line to analyte gases for the different elements, such as CO₂ for carbon, N₂ for nitrogen, H₂ for hydrogen, or CO for oxygen. The analyte gases are subsequently transferred on line to an isotope-ratio mass spectrometer (GC-C-IRMS; ref 21). To date, the CSIA concept has focused mainly on aromatic hydrocarbons and chlorinated solvents (18).

Although RDX is found worldwide to be an environmental pollutant, its isotopic composition and isotope fractionation have received only scant attention. In the forensic literature, some publications relate to the isotope composition of RDX (22, 23). However, the environmental aspects of RDX isotope composition have rarely been considered. DiGnazio et al. (24) related δ²⁵N values of nitrate in the groundwater to RDX degradation, but noted that they had no other isotopic data to confirm this correlation. Lately, Bordeleau et al. (25) related δ¹⁵N values of nitrate in the groundwater to RDX degradation as well. Beller et al. (26) reported isotope signatures of NO₃⁻ and NO₂⁻ formed by RDX photolysis. Hoffsommer et al. (27) reported δ²H isotope effects following RDX alkaline hydrolysis. The use of CSIA of RDX in environmental case studies is not documented in the literature. Among other reasons, this is probably due to the difficult analysis of explosives such as RDX with CSIA because of the requisite GC separation step: explosives usually decay at elevated temperatures such as those applied in GC separations. Furthermore, even fragments cannot be used for stable isotope analysis (a concept which is usually applied in GC/MS analysis of explosives concentrations), because the decay reaction into fragments is connected with stable isotope effects, which make isotopic analysis difficult. Nevertheless, the application of stable isotope fractionation analysis to quantify the extent of degradation, and to gain information on the degradation pathways involved in RDX-contaminated field sites, may be very promising.

Therefore, the objectives of this study were to develop a suitable method for δ¹⁵N and δ¹⁸O stable isotope analysis in aerobic and anaerobic biodegradation of RDX by microorganisms originating from an RDX contaminated site.

Materials and Methods

Biodegradation Experiments. Batch biodegradation experiments were conducted to analyze isotope fractionation effects in aerobic and anaerobic degradation pathways of RDX. A sterile 2 L volume of aqueous medium was prepared in a 3 L Erlenmeyer flask for aerobic experiments and in 2.5 L glass bottles for anaerobic experiments. In both cases, the mineral medium consisted of a phosphate buffer (1.0 g/L K₂HPO₄ and 0.5 g/L KH₂PO₄, pH 7.1), 0.2 g/L MgSO₄, and 2.5 mL/L of trace elements solution (2.5 mg/L FeSO₄, 5 mg/L CaCl₂, 1.25 mg/L MnSO₄, 0.25 mg/L CuSO₄, and 0.25 mg/L Na₂MoO₄). Under aerobic conditions, cyclohexanone served as the carbon source (1.0 mL/L). Under anaerobic conditions, sodium acetate served as the carbon source (1.0 g/L). RDX was added to the aqueous media at an initial concentration of 16–22 mg/L, and served as the sole nitrogen source. The aerobic batch experiments were conducted in eight replicates for oxygen, and eight replicates for nitrogen isotope analysis.
The anaerobic batch experiments were conducted in six replicates for oxygen and six replicates for nitrogen isotope analysis.

For the RDX-biodegradation experiments under aerobic conditions, *Rhodococcus* strain YH1 isolated from RDX-contaminated soils at an industrial site (28) was used. Inoculation was carried out by adding to each 2 L sterile mineral medium 1 mL of a stationery starter culture of *Rhodococcus* strain YH1. For RDX degradation under anaerobic conditions, sediments excavated from the saturated zone of the same industrial site were used as a source for RDX-degrading bacteria. Initially, 3–5 g of sediment was used for inoculating a starter mineral medium (2 L), which was kept under anaerobic conditions for 1 month. The bacterial community was then harvested by centrifugation (Laboufe 400, Heraeus Inst., Germany) at 3500 RCF, and the pellet was suspended in 100 mL of sterile water, which was then equally divided between the 12 replicates. Aerobic cultures were constantly stirred on a rotary shaker at 175 rpm, 30 °C. Anaerobic cultures were incubated in an anaerobic chamber (Coy Laboratory, Grass Lake, MI) with a gas mixture of 94% \( \text{N}_2 \) and 6% \( \text{H}_2 \) at room temperature. To produce anaerobic conditions, four cycles of purging with the gas mixture, alternated with the application of vacuum, were applied to the bottles before they were introduced into the glovebox. Bottles were then sealed with a cup and kept static in the glovebox for the entire incubation period. This particular way of handling the anaerobic cultures enabled easy collection of high-volume samples, without disturbing the anaerobic conditions.

Samples for isotope analysis were collected into Erlenmeyer flasks during the incubation in increasing volumes; this assured a minimal mass of 600 mg RDX in each sample. RDX concentration in the samples was monitored by high-performance liquid chromatography (HPLC; Agilent 1100 series, Palo Alto CA) according to EPA method 8330 (29). The RDX in the samples was liquid—liquid extracted into dichloromethane (DCM). The DCM extracts were reduced to a final volume of 0.5–1.0 mL under nitrogen flow at 30 °C (TurboVap Zymark, Hopkinton, MA).

**RDX Purification by Thin Layer Chromatography (TLC).** RDX in the DCM extracts was purified from its degradation products by TLC method, using silica gel on glass TLC plates (10 × 20 cm) with an internal fluorescent indicator (Fluka, Buch, Switzerland). The mobile phase in the TLC method consisted of double-distilled water, methanol, and acetone (HPLC grade) in a volumetric ratio of 1:1:0.1. Plates were placed vertically in covered glass tanks, and the mobile phase was allowed to rise up the plate as a prewash. When the wetting front reached the top, the plate was taken out of the tank and air-dried for 30 min. After drying, RDX samples, dissolved in DCM, were applied in a line 1.5 cm above the bottom of the plate, using a disposable glass Pasteur pipet. When the solvent evaporated, plates were put back into the tanks until the mobile phase again reached the top of the plate. Plates were then removed and air-dried for 30 min. For greater separation between RDX and its degradation products, a second cycle of wetting and drying was subsequently carried out. In this second cycle, plates were put back into the tanks until the mobile phase again reached the top of the plate. They were then removed from the tank, and air-dried for 30 min. RDX spots were detected by UV light and scraped off the plate into a glass vial.

**Sample Preparation for Isotope Analysis.** Purified RDX was eluted from the scraped silica gel by adding 3–4 mL of DCM and shaking on a rotary shaker at 1 h. Up to 2 mL DCM solution from each sample was collected in a 2 mL glass vial using a glass syringe. The collected DCM solution was allowed to evaporate at room temperature to a final volume of approximately 1 mL. To ensure the absence of reduced degradation products in the samples, a fraction from the DCM solution was analyzed by HPLC following EPA method 8330 (29). RDX standard for HPLC was prepared from solid powder obtained from the Israel Military Industry (Ramat Hasharon). MNX, DNX, and TNX were purchased from SRI International (Menlo Park, CA). In samples in which the presence of the degradation products MNX, DNX, and TNX was found to be greater than 5% of the RDX mass, the solution was subjected to an additional separation procedure on TLC plates, as described above.

For δ\(^{18}\)N analysis, the DCM solution was injected into two duplicate tin capsules for liquids, and for δ\(^{18}\)O analysis the DCM solution was injected into two duplicate silver capsules for liquids (Hekatech, Wegberg, Germany). Before closing the capsules, DCM was allowed to evaporate, leaving an expected minimal mass of 120 μg purified RDX.

**Isotope Analysis Technique.** Isotope analysis was carried out by an elemental analyzer (EA) (Euro Vector SPA, Milan, Italy) coupled with a combustion unit (Hekatech, Wegberg, Germany) followed by IRMS (Thermo Fisher Scientific, Bremen, Germany). The temperature of the oxidation tube in the EA was 1000 °C and the temperature of the reduction tube was 600 °C. The temperature of the combustion tube was 1480 °C.

For calibration, before and after the analysis of each 46-sample batch, three different standards were analyzed in triplicate. Standards used for δ\(^{15}\)N calibration were caffeine (IAEA-600), and l-glutamic acid (USGS-40 and USGS-41). Standards used for δ\(^{18}\)O calibration were benzoic acid (IAEA-601 and IAEA-602) and sucrose (IAEA-CH6). In addition, a standard was analyzed after every seventh sample for calibrating shifts in the measurements. Caffeine standard was used for this purpose in the δ\(^{15}\)N measurements and benzoic acid was used for this purpose in the δ\(^{18}\)O measurements.

**Calculations.** The natural abundance of \(^{18}\)O and \(^{15}\)N is 0.205% and 0.368%, respectively. IRMS yields the isotopic composition of the investigated compound \( x \) relative to the isotopic composition of a standard. This isotope ratio, \( \delta \), is given in per mil units (‰) and is defined as

\[
\delta_x = \left( \frac{R_x - R_{std}}{R_{std}} \right) \times 1000
\]

where \( \delta \) is the isotope ratio of the investigated compound, and \( R_x \) and \( R_{std} \) are the ratios between the heavy and light isotopes in the investigated compound, and in the standard, respectively. The mathematical description of the relation between the extent of degradation and isotopic composition of the investigated compound can be performed by a Rayleigh distillation equation (30)

\[
\ln \left( \frac{R_x}{R_{true}} \right) = (\alpha - 1) \ln f
\]

with

\[
f = \frac{C_t}{C_0}
\]

where \( C_0 \) and \( C_t \) are the compound concentrations at times 0 and \( t \), respectively.

The stable isotope fractionation factor \( \alpha \) can be obtained by plotting the natural logarithm of the isotopic enrichment, \( (R_x/R_{true}) \), against the natural logarithm of the extent of degradation, \( f \). The linear slope of the obtained curve is thus \( (\alpha - 1) \). Enrichment factors (\( \varepsilon \)) were determined for the different degradation experiments, expressed as

\[
\varepsilon = (\alpha - 1) \times 1000
\]
when several atoms of the same element are present in a compound, those not taking part in the reaction will decrease the observable isotope fractionation (31). Thus, intrinsic isotope enrichment, $e_i$, can be defined as

$$e_i = \frac{\delta_i^s - \delta_i^{std.}}{\delta_i^{std.}} \cdot n$$  \hspace{1cm} (5)

where $n$ is the number of atoms in the element.

**Results and Discussion**

Previous studies suggested that aerobic biodegradation of RDX by *Rhodococcus* strain YH1, which was isolated from contaminated soils at the RDX production site (28), is initiated by denitration (10). Thus, under aerobic conditions, biodegradation was expected to be accompanied by accumulation of NDAB and by a primary isotopic effect of $\delta^{15}N$ enrichment, whereas no, or only very slight $\delta^{18}O$ enrichment would be observed. It was also hypothesized that under anaerobic conditions, with a mixed culture of contaminated soil originating from the same RDX production site, RDX biodegradation would follow the transformation pathway described by McCormick et al. (5), accompanied by accumulation of the nitroso derivatives MNX, DNX, and TNX and by a primary isotope effect for both $\delta^{15}N$ and $\delta^{18}O$. The present work was aimed at establishing a methodology for stable isotope analysis of RDX, and testing whether stable isotopic fractionation analysis of several elements ($N$ and $O$) can distinguish between anaerobic and aerobic degradation, or even support one or the other proposed reactions. As already noted, the use of GC-C-IRMS for CSIA analyses of RDX is difficult due to decomposition of the compound within the injector and the column, and the accompanying isotopic effect. Consequently, there was a clear need to establish a new technique for CSIA of RDX.

**TLC Separation.** The separation procedure using the silica-gel TLC plates and methanol–water–aceton mixture was sufficient to observe clear separation of RDX from its degradation products. The $R_t$ values using this method were 0.53, 0.63, 0.73, and 0.80 for RDX, MNX, DNX, and TNX, respectively. When the mass of RDX and its degradation products was high, the spots were smeared, and the separation procedure was therefore repeated. The additional separation cycle was found to be sufficient to decrease the concentration of the reduced degradation products in the RDX samples to less than 5%, as verified by HPLC.

To determine the method’s accuracy and precision, replicates of pure RDX from a stock solution (1.0 mg/mL RDX in HPLC-grade DCM) were subjected to the TLC preparation procedure and subsequently analyzed for $\delta^{18}O$ and for $\delta^{15}N$. The results were compared to the $\delta^{18}O$ and $\delta^{15}N$ composition of an RDX stock solution that was not separated by TLC (Table 1) to ensure that the TLC procedure itself does not cause stable isotope fractionation. The $\delta^{18}O$ and $\delta^{15}N$ isotope compositions of the TLC-treated RDX were found to be within the error range of the pure, non-TLC-treated RDX (Table 1). This suggests that the TLC procedure does not cause significant isotope fractionation, and that the procedure does not suffer from external contamination with respect to $\delta^{15}N$ or $\delta^{18}O$ composition. The TLC-treated samples gave less precise results in the $\delta^{18}O$ vs $\delta^{15}N$ analysis. Nevertheless, the average isotopic composition found for the TLC-treated RDX was similar to that of the pure RDX. Thus, it is suggested that the purification procedure is suitable for routine use of CSIA for RDX with respect to $\delta^{15}N$ and $\delta^{18}O$ compositions.

**Stable Isotope Fractionation in Aerobic and Anaerobic Cultures.** The analysis of $\delta^{15}N$ and $\delta^{18}O$ stable isotope fractionation during the biodegradation of RDX in batch experiments produced distinct fractionation trends and enrichment factors under aerobic and anaerobic conditions (Figure 2, Table 2).

Under aerobic conditions, clear $\delta^{15}N$ enrichment of RDX was detected. Although early reports indicated that both $N-N$ and C−H bond cleavage result in an unstable intermediate leading to rapid ring cleavage (2, 32), the clear $\delta^{15}N$ enrichment observed in this study was in accordance with later reports that $N-N$ bond cleavage, rather than C−H bond cleavage, is the first step in the aerobic biodegradation pathway of RDX by *Rhodococcus* strain YH1. Studies on the biochemical pathway of RDX biodegradation under aerobic conditions have revealed denitration (with the involvement of cytochrome P450) to be an initial step in various *Rhodococcus* strains. This was demonstrated by Fournier et al. (3) and Bhushan et al. (4) who studied the RDX-biodegradation pathway of *Rhodococcus* strain DN22, and by Seth-Smith et al. (12), who presented similar results with *Rhodococcus* strain 11Y. Later works identified the role of the gene *xplA* which encodes cytochrome P450 (7, 11, 12). The work of Nejidat et al. (10), who studied the aerobic biodegradation of RDX by *Rhodococcus* strain YH1, revealed the formation of a polypeptide which is identical to polypeptides encoded by xplA. Thus, it has been well established that denitration is the initial step in the aerobic biodegradation pathway by this strain. To further confirm the occurrence of this degradation pathway, we carried out biodegradation experiments under aerobic conditions using this strain. During these experiments, HPLC analyses for the detection of RDX, nitroso derivatives and NDAB were carried out (see the Supporting Information for the methods and complete results). In these experiments, accumulation of NDAB was observed following RDX degradation, whereas nitroso derivatives (among them MNX) were not detected. Mass-balance calculations revealed that the RDX was completely converted to NDAB within 100 h of incubation. From these results, it could be concluded that RDX biodegradation indeed followed the denitration pathway.

Nevertheless, in contrast to our expectation of a primary isotopic effect of $\delta^{15}N$ enrichment during denitration under aerobic conditions with no, or only very slight $\delta^{18}O$ enrichment, clear $\delta^{18}O$ enrichment was detected in RDX that was biodegraded under aerobic conditions. This raised the assumption that, in contrast to the knowledge gained on the aerobic degradation pathway used by strain YH1, $N-N$ bond cleavage is the initial step in this pathway rather than $N-N$ bond cleavage, as suggested above. However, if RDX biodegradation under aerobic conditions was initiated by an $N-N$ bond cleavage, inevitably, MNX would have formed.

**TABLE 1. Isotopic Composition of RDX Standards with and without the TLC Purification**

<table>
<thead>
<tr>
<th>RDX following</th>
<th>Pure RDX without</th>
</tr>
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<tbody>
<tr>
<td>TLC procedure</td>
<td>TLC procedure</td>
</tr>
<tr>
<td>average $\delta^{15}N$</td>
<td>$-9.66%$ ($n = 7$)</td>
</tr>
<tr>
<td>average $\delta^{18}O$</td>
<td>$24.07%$ ($n = 13$)</td>
</tr>
</tbody>
</table>

This table shows the isotopic composition of RDX standards with and without the TLC purification procedure. The results indicate that the TLC procedure itself does not cause stable isotope fractionation. The $\delta^{15}N$ and $\delta^{18}O$ isotope compositions of the TLC-treated RDX were found to be within the error range of the pure, non-TLC-treated RDX (Table 1).
possible that a strong secondary isotopic effect occurs in this system as a consequence of the biochemical mechanism of the rate-determining step. Hengge and Cleland (34) and more recently, Hengge (35) studied secondary isotopic effects in phosphoryl-transfer reactions and later in sulfuryl-transfer reactions. Using labeled compounds, they observed, in certain reactions, secondary isotopic effects in the nonbridge oxygen atoms of the phosphate esters that were in the same range as the bridge oxygen atoms being cleaved. Certainly, RDX denitration has no similarity to phosphoryl or sulfuryl reactions; however, it was shown that strong secondary isotopic effects can indeed occur in certain reactions which are related to their mechanism.

Similar to isotopic enrichment during aerobic biodegradation, enrichment of both $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ was evident under anaerobic conditions (Figure 2). These results were expected, as N–O bond cleavage was identified as the first step in the reduction pathway under anaerobic conditions by the enriched culture. Ring cleavage as an initial step in RDX biodegradation under anaerobic conditions, as reported by Hawari et al. (6), was excluded as a possible pathway in this study following the observed sequential formation of MNX, DNX, and TNX during the degradation process (for mass-balance calculations, see the Supporting Information).

Reports in the literature on $\delta^{15}\text{N}$ or $\delta^{18}\text{O}$ enrichment following biodegradation are scarce. Three studies exhibiting some similarities with RDX biodegradation were performed by Hartenbach et al. (36), Tobler et al. (37), and Hofstetter et al. (38) who measured isotope enrichment during the abiotic reduction of different nitro-aromatic compounds. In this process, an N–O bond is cleaved in a nitro group that is external to the aromatic ring; this is somewhat similar to the anaerobic reduction pathway of RDX in which an N is external to the aromatic ring; this is somewhat similar to ring. In the three aforementioned studies, the intrinsic $\delta^{15}\text{N}$ enrichment factors, $\epsilon_i$, varied between $-28.0 \pm 0.8\%$ and $-41.9 \pm 1.1\%$. The intrinsic enrichment factors of $\delta^{15}\text{N}$ found in this work for anaerobic biodegradation of RDX ($-30.0 \pm 1.8$) are within the same range. However, neither of the aforementioned studies looked at $\delta^{18}\text{O}$ enrichment. Studies on fractionation involving N–N bond cleavage in similar organic compounds have not been reported in the literature.

Because the bacteria used in this study originated from an RDX-contaminated site, the results of the study point to two different potential RDX-biodegradation pathways at this site: an aerobic pathway which is initiated by a denitrification step, followed by ring cleavage (pathway A in Figure 1), and an anaerobic pathway, which is characterized by sequential transformation of RDX to its nitroso derivatives (pathway B in Figure 1). There is no evidence in this work for the existence of the other proposed pathways (6): initial ring cleavage under anaerobic conditions (pathway C in Figure 1) or initial C–H bond cleavage under aerobic conditions.

Anaerobic and aerobic degradation of RDX produce different isotope fractionation patterns (Figure 3), which optimally might constitute a valuable tool to distinguish between the two possible pathways in contaminated field sites (19). Theoretically, if RDX isotope values from the field were to lie on one of the curves in a two-dimensional isotope plot of $\delta^{15}\text{N}$ versus $\delta^{18}\text{O}$, the type of degradation pathway and the extent of biodegradation could be evaluated, providing that the parent values ($R_0$) of the original released compound were known. However, as the difference between the two patterns in the case of RDX biodegradation is not very pronounced, it might be difficult to perform such an evaluation. Thus, the $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ enrichment factors obtained in this study for RDX biodegradation under aerobic and anaerobic conditions, although valuable for quantifying the extent of biodegradation at RDX-contaminated field sites, may not be useful for distinguishing the relative importance of the biodegradation pathways involved at the site.

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**Supporting Information Available**

Experiments were carried out to determine the RDX degradation products under aerobic and under anaerobic