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Fate of CL-20 in sandy soils: Degradation products as potential markers of natural attenuation

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Two key intermediates of CL-20 degradation are potential markers of its natural attenuation in soil.

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ABSTRACT

Hexanitrohexaazaisowurtzitane (CL-20) is an emerging explosive that may replace the currently used explosives such as RDX and HMX, but little is known about its fate in soil. The present study was conducted to determine degradation products of CL-20 in two sandy soils under abiotic and biotic anaerobic conditions. Biotic degradation was prevalent in the slightly acidic VT soil, which contained a greater organic C content, while the slightly alkaline SAC soil favored hydrolysis. CL-20 degradation was accompanied by the formation of formate, glyoxal, nitrite, ammonium, and nitrous oxide. Biotic degradation of CL-20 occurred through the formation of its denitrohydrogenated derivative (*m*/*z* 393 Da) while hydrolysis occurred through the formation of a ring cleavage product (*m*/*z* 156 Da) that was tentatively identified as $CH_2{=}N{-}C({=}N{-}NO_2){-}CH{=}N{-}CHO$ or its isomer $N(NO_2){=}CH{-}CH{=}N{-}CO{-}CH{=}NH$. Due to their chemical specificity, these two intermediates may be considered as markers of *in situ* attenuation of CL-20 in soil.

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1. Introduction

The polycyclic nitramine 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (China Lake 20; CL-20), is one of the most powerful high energy materials and is being considered as a possible replacement for the currently used cyclic nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Geetha et al., 2003; Simpson et al., 1997). The manufacturing and usage of munitions has resulted in severe contamination of both soils and groundwater (Pennington and Brannon, 2002; Best et al., 2006). Toxicological studies showed that CL-20 did not adversely affect terrestrial plants and indigenous soil microorganisms (Gong et al., 2004) but was highly toxic to the earthworm *Eisenia andrei* (Robidoux et al., 2004) and potworms *Enchytraeus crypticus* and *Enchytraeus albidus* (Dodard et al., 2005; Kuperman et al., 2006). These findings suggested that the fate of CL-20 in soil required investigation prior to its large-scale production in order to determine the potential adverse impacts of accidental release of CL-20 in the environment.

Several studies have investigated the abiotic and biotic degradation of CL-20 in aqueous media (Table 1, Fig. 1). These studies showed consistently the formation of nitrite and formate ions, usually at respective stoichiometries of ~2 and ≤2. Although less reported in the literature other products of CL-20 include ammonia (NH_3), nitrous oxide (N_2O), glyoxal (CHOCHO), and glycolate ($CH_2(OH)COO^-$) (Table 1). In addition to these end products, early intermediates have been tentatively identified using a combination of LC/MS and amino- or nitro-labeled [^{15}N]-CL-20 (Fig. 1). The detection of these intermediates allowed proposing three initial CL-20 transformation routes prior to ring cleavage (Fig. 1): (1) the loss of one nitro group (denitration), (2) the reduction of one or two nitro group(s) to nitroso group(s) (nitroreduction), and (3) the transformation of a nitramine group into an amino group (denitrohydrogenation).

Although degradation of CL-20 in aqueous media, including formation of degradation products has been studied extensively, only little information is available on the degradation products of CL-20 in soil. CL-20 was reported to biodegrade in soil under aerobic (Trott et al., 2003; Crocker et al., 2005; Panikov et al., 2007) and anaerobic (Strigul et al., 2006; Panikov et al., 2007) conditions. Several strains capable of degrading CL-20, including *Agrobacterium* sp. strain JS71 (Trott et al., 2003), *Pseudomonas* sp. strain FA1 (Bhushan et al., 2003), and *Clostridium* sp. EDB2 (Bhushan et al.,

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Table 1
CL-20 products and their normalized molar yields obtained after chemical or enzymatic degradation[a]

Reaction	Products						Reference
	NO_2^-	N_2O	NH_3	$HCOO^-$	CH_2OHCOO^-	CHOCHO	
In water							
Hydrolysis (pH 10)	1.9	0.9	0.8	0.5	ND[b]	ND	Balakrishnan et al. (2003)
Hydrolysis (pH 9.5)	2.2–3.5	ND	ND	0.75	ND	ND	Szecsody et al. (2004)
Alkaline hydrolysis	2	ND	ND	ND	ND	ND	Karakaya et al. (2005)
Photolysis (300 nm)	3.9[c]	ND	0.8	2.0	ND	ND	Hawari et al. (2004)
Reduction by Fe^0	0.04[d]	2.3	1.1	0.4	0.4	0.4	Balakrishnan et al. (2004b)
Nitroreductase	1.8	3.3	1.3	1.6	ND	1.0	Bhushan et al. (2004a)
Monooxygenase	1.7	3.2	0.6	1.5	ND	ND	Bhushan et al. (2004b)
In soil/water							
Alkaline sterile soil	2	ND	ND	+[e]	ND	ND	Balakrishnan et al. (2004a)
Subsurface sediments	1.4–5.0	ND	ND	0.4–1.9	ND	ND	Szecsody et al. (2004)

[a] Values were calculated from the product concentrations obtained at the end of each experiment, and the stoichiometries are calculated based on the number of moles of product observed for each mole of reactant consumed.
[b] ND, not determined.
[c] $NO_2^- + NO_3^-$.
[d] Two equivalents of NO_2^- were initially formed but subsequently reduced by Fe^0.
[e] Detected but not quantified.

2004c), have been isolated from soils and sediments. Beside its susceptibility to microbial degradation, CL-20 can also degrade abiotically in soils that are alkaline (Balakrishnan et al., 2004a) or that contain ferrous iron or clays (Szecsody et al., 2004). However, only formate ($HCOO^-$) and nitrite (NO_2^-) have been identified as CL-20 products in two of these studies with soil (Balakrishnan et al., 2004a; Szecsody et al., 2004).

Our objective was to determine the fate of CL-20 under abiotic and biotic conditions in two sandy soils that differ in organic carbon content and pH. We aimed at elucidating the degradation pathway(s) of CL-20 by identifying the intermediate and end products formed during its degradation in soil, and selecting those products, which could be used as markers for monitoring the natural attenuation of CL-20 in soil in case of its accidental release in the environment.

2. Materials and methods

2.1. Chemicals

Crystalline CL-20 (CAS 135285-90-4; ε-isomer, purity 99.3%, as determined by HPLC), amino-labeled and nitro-labeled [^{15}N]-CL-20, and uniformly labeled [^{14}C]-CL-20 (radiochemical purity, 98.8%; chemical purity 96.7%, specific activity of 0.75 mCi/

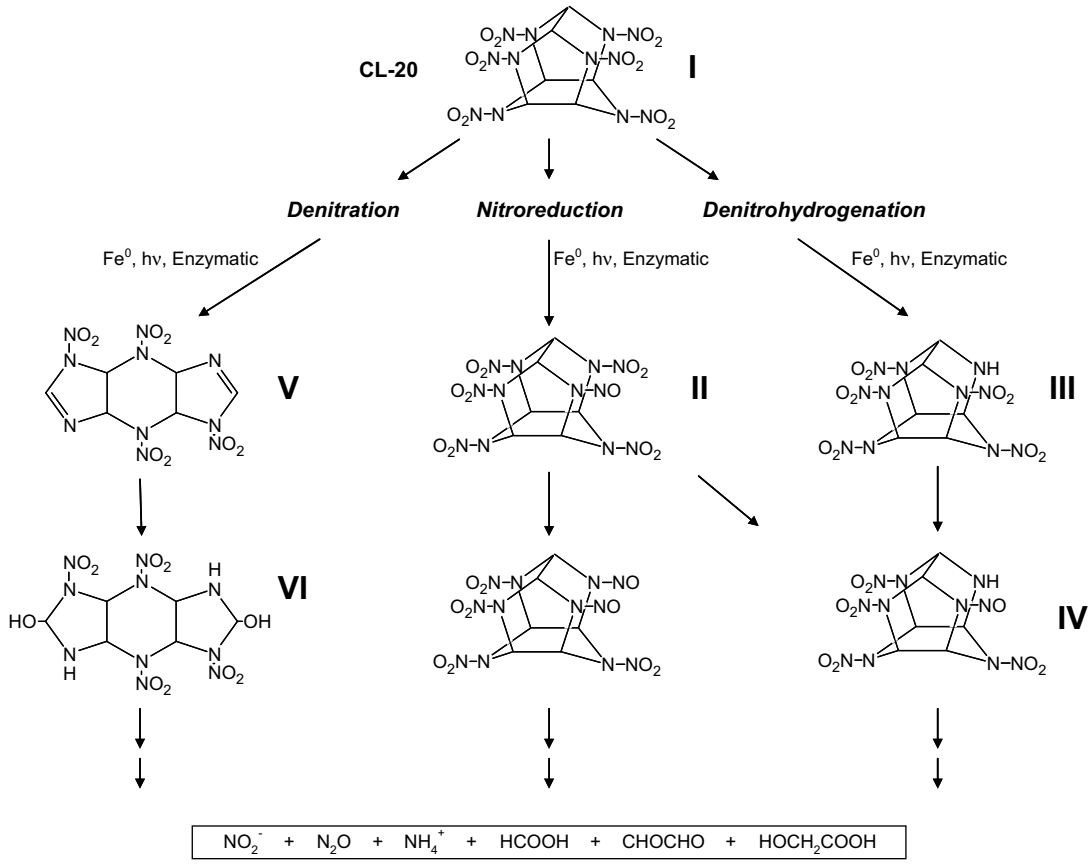

Fig. 1. Potential transformation routes for CL-20 degradation (data from Balakrishnan et al. (2004b), Bhushan et al. (2004a–c) and Hawari et al. (2004)).

Table 2
Physical and chemical properties of soils[a] used in this study

Soil	Granulometry			TOC[b] (%)	pH	K_d (L kg^{-1})[c]
	% Sand	% Silt	% Clay			
SAC	98.6	1.3	0.1	0.08	8.1	2.43 ± 0.04
VT	83	12	4	2.3	5.6	15.06 ± 0.42

[a] VT, varennes topsoil; SAC, soil from agriculture Canada.
[b] Total organic carbon on a dry basis.
[c] Soil–water distribution coefficient measured for CL-20 (data from Balakrishnan et al. (2004a)).

g) were manufactured and provided by ATK Thiokol Propulsion (Brigham City, UT, USA). All other chemicals were of reagent grade and used without purification. Formate, nitrite, and ammonium were obtained from Alltech (Deerfield, IL, USA); glyoxal and glycolic acid were obtained from Sigma (Oakville, ON, Canada); and potassium hydroxide (KOH) was obtained from EM Science (Gibbstown, NJ, USA). Acetonitrile (CH_3CN, HPLC grade) was from Fisher (Nepean, ON, Canada). Deionized water was obtained with a Milli-Q^{UV} plus (Millipore, Mississauga, ON, Canada) system.

2.2. Soils

Two soils were used in this study: a sandy agricultural soil (VT) originating from Varennes, Quebec, Canada, and a sandy soil provided by Agriculture Canada (SAC). Selected physical and chemical properties of the two soils are shown in Table 2. Each soil was passed through a 2 mm sieve and air dried in a fume-hood before use. A portion of each soil was sterilized by gamma irradiation from a ^{60}Co source (50 kGy) over 2 h at the Canadian Irradiation Center (Laval, Quebec).

2.3. Abiotic and biotic degradation of CL-20 in soil

Degradation of CL-20 under anaerobic conditions was investigated separately in VT and SAC soil over six-month periods. VT or SAC soil (2.0 g) and CL-20 (0.32 or 1.20 μmol introduced from an acetone stock solution (10,367 mg L^{-1}) and corresponding to 70 or 263 mg CL-20 kg soil^{-1}) were mixed in autoclaved 20-mL headspace glass vials. Solvent was evaporated before adding sterile deionized water (1 mL). Each soil was saturated with water. Bottles were sealed with butyl rubber stoppers, degassed under vacuum for 5 min then charged with oxygen-free argon, which was passed through a sterile Millex PTFE 0.20-μm filter. This was repeated four more times. The bottles were kept static, in the dark, at 22 ± 1 °C. At various time intervals, four replicates were sacrificed. Before opening the bottles, gaseous products were sampled with a gas-tight syringe and analyzed as described below. Two samples were treated with acetonitrile (9 mL), sonicated for 18 h, and filtered through a Millex-HV 0.45-μm syringe filter (Millipore Corp., Bedford, MA) for CL-20 and early intermediates analysis. Deionized water (9 mL) was added in the other two samples, which were then filtered and analyzed for various products (HCHO, CHOCHO, CH_2OHCOO^-, NH_4^+, NO_2^-, $HCOO^-$). Abiotic degradation experiments were conducted using the same procedure but with gamma irradiated soil. Controls consisting of VT or SAC soil incubated in the presence of deionized water without CL-20 were included in analytical determinations at each time interval.

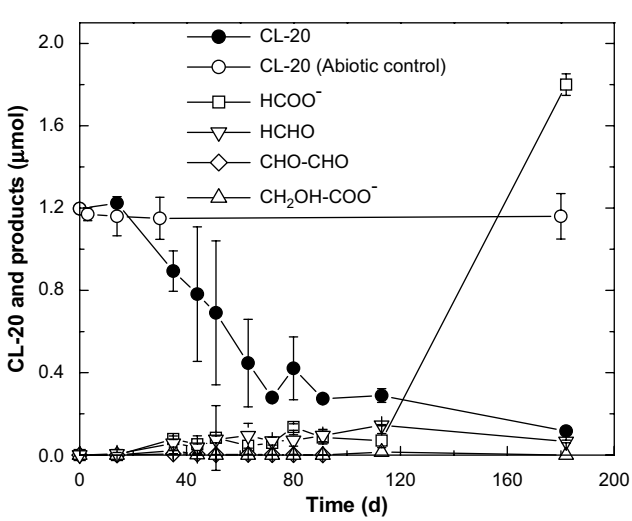

Fig. 2. Time course of biotic degradation of CL-20 with VT soil under anaerobic conditions. Abiotic control was conducted with sterile soil. Data are mean and standard error ($n = 2$).

2.4. ^{14}C partitioning experiments

Uniformly labeled [^{14}C]-CL-20 was used to determine the rate and extent of CL-20 mineralization in VT or SAC soils. Sterile or non-sterile dry soil (2 g), and deionized sterile water (1 mL) were added to 20-mL headspace glass vials

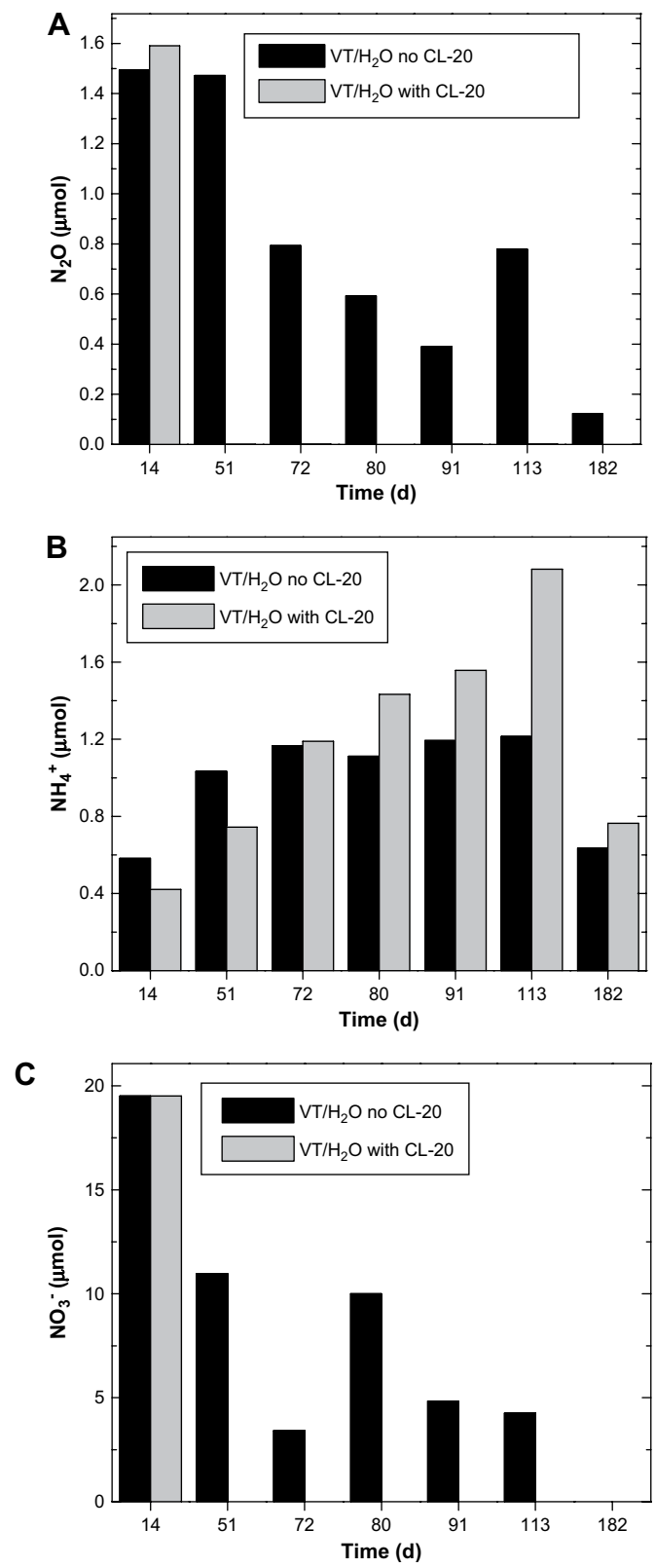

Fig. 3. Concentrations of nitrous oxide (A), ammonium (B), and nitrate (C) in non-sterile VT:H_2O (2 g:1 mL) systems with or without CL-20 amendment.

containing a CO_2 trap (1 mL KOH 0.5 M). Bottles were sealed with butyl rubber stoppers, degassed under vacuum for 5 min then charged with oxygen-free argon, which was passed through a sterile Millex PTFE 0.20-μm filter. The degassing–refilling step was repeated four times before the radiotracer, [^{14}C]-CL-20 (20 μL of an acetone stock solution (4750 mg L^{-1}), 0.071 μCi, 0.217 μmol), was introduced. All bottles were incubated in the dark at 22 ± 1 °C under static conditions. Formation of $^{14}CO_2$ was monitored in the KOH trap using a Tri-Carb 4530 liquid scintillation counter (model 2100 TR; Packard Instrument Company, Meriden, CT). Mineralization experiments were carried out in triplicate.

Two microcosms were sacrificed after 97 d for VT and 185 d for SAC to determine the ^{14}C distribution between gaseous, aqueous, and solid phases. Water (9 mL) was added to each microcosm, which was shaken periodically and manually over a 4-h period at room temperature. After settling down, 1 mL of the aqueous phase was decanted. The soil suspension was then acidified by the addition of 1 mL 0.1 M HCl and periodically agitated over a 24-h period before sampling. The aqueous phase was then removed and the soil was extracted with 10 mL of CH_3CN (65 h at room temperature). Extracts were analyzed by liquid scintillation counting. When the sum of radioactivity of all extracts represented less than 95% of the radioactivity introduced, the soil was burned to evaluate the amount of radioactive carbon irreversibly bound to soil.

2.5. Analytical methods

CL-20 was analyzed by HPLC connected to a photodiode array (PDA) detector as described previously (Monteil-Rivera et al., 2004). Nitrous oxide (N_2O) was measured as previously described (Sheremata and Hawari, 2000). Glyoxal was determined as its derivatized product with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) (Balakrishnan et al., 2004b). Ammonium, nitrite, nitrate, formate, and glycolate were analyzed by ion chromatography (IC) (Balakrishnan et al., 2004b) in water extracts after removing the glyoxal by precipitation with PFBHA. Methanol (CH_3OH) was analyzed on a Hewlett Packard 6890 gas chromatograph coupled to an FID using a Hayesep Q micropacked column (2 m × 0.03 mm, Supelco, Oakville, ON, Canada) (detection limit, 0.1 mg L^{-1}).

Intermediate products of CL-20 were analyzed by LC/MS using a Bruker benchtop ion trap mass detector attached to a Hewlett Packard 1100 Series HPLC system equipped with a DAD detector. The samples were injected into a 5 μm-pore size Zorbax SB-C18 capillary column (0.5 mm ID by 150 mm; Agilent, Mississauga, ON, Canada) at 25 °C. The solvent system was composed of a CH_3CN/H_2O gradient (30–70% v/v) at a flow rate of 15 μL min^{-1}. For mass analysis, negative electrospray ionization mode was used to produce deprotonated molecular ions $[M–H]^-$; or nitrate adduct ions $[M + NO_3]^-$. The mass range was scanned from 40 to 550 Da. Detected intermediates were tentatively identified using amino and nitro-labeled [^{15}N]-CL-20. LC/MS analyses were also conducted in the presence of trifluoroacetic acid (0.1 μL L^{-1} in water) because as it was observed in our laboratory with RDX, HMX, CL-20, and their nitroso derivatives, trifluoroacetate anion (TFA) forms adduct ions $[M + TFA]^-$ with cyclic nitramines, which facilitates their identification.

[^{14}C]-CL-20 and its products were separated by HPLC using a Discovery C18 column (Supelco) and a solvent system composed of a CH_3CN/H_2O gradient (30–70% v/v). Radioactivity was determined in each collected fraction by liquid scintillation counting.

3. Results and discussion

3.1. Transformation of CL-20 in VT soil

3.1.1. Time courses

Incubation of CL-20 under non-sterile anaerobic conditions led to the disappearance of the parent chemical with only 10% of the

Table 3
Distribution of ^{14}C[a] in microcosms containing [^{14}C]-CL-20 (0.217 μmol; 0.071 μCi) and sterile or non-sterile VT or SAC soils after anaerobic incubation

Conditions	Gaseous phase	Aqueous phase	Solid phase			Total
			10 mM HCl extract	CH_3CN extract	Soil combustion	
VT-Non-sterile[b]	2.9 ± 0.9	14.6 ± 2.7	~0	80.6 ± 0.6	ND[c]	98.1 ± 4.0
SAC-Non-sterile[d]	67.5 ± 3.6	19.9 ± 3.5	~0	1.3 ± 0.5	4.6 ± 0.8	93.3 ± 0.3
SAC-Sterile[d]	50.1 ± 2.9	34.5 ± 3.8	~0	1.6 ± 0.5	5.2 ± 1.4	91.4 ± 0.5

[a] Values are reported in % of total ^{14}C added. Values are presented as mean ± standard error of duplicate samples.
[b] Incubation time: 97 d.
[c] ND, not determined.
[d] Incubation time: 185 d.

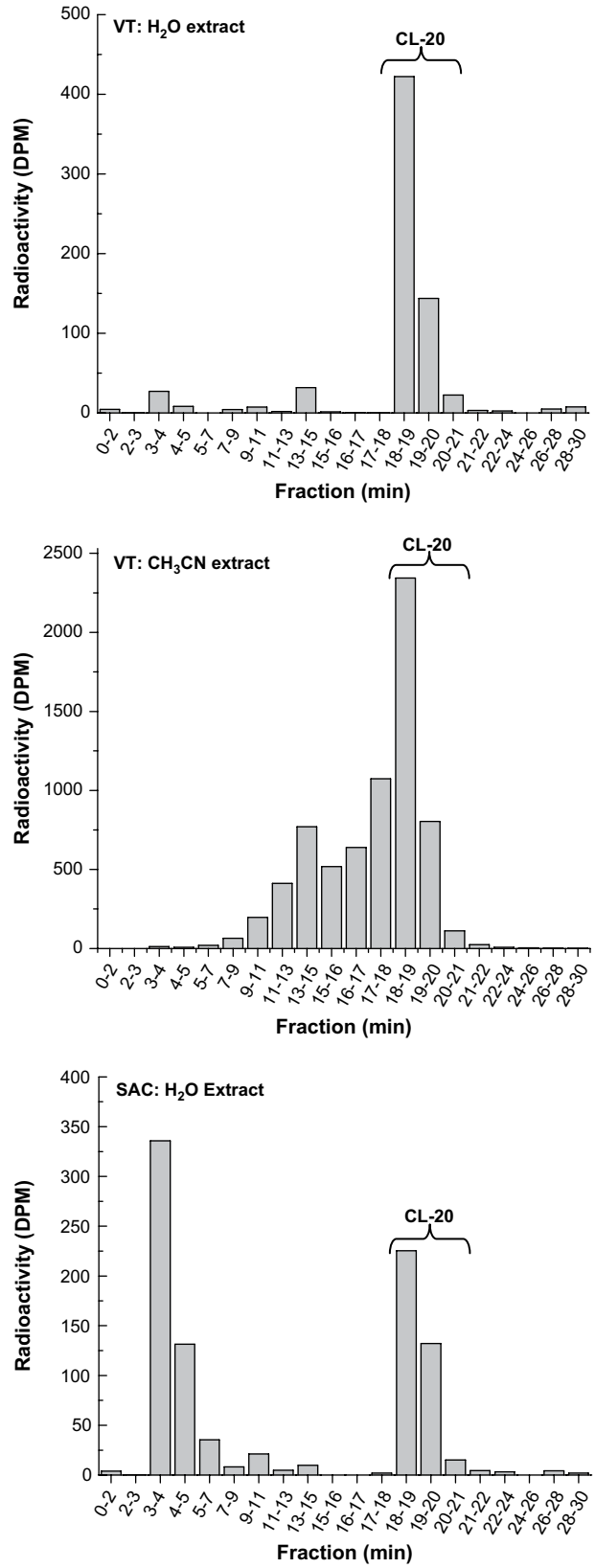

Fig. 4. Distribution of ^{14}C measured by radiochemical HPLC in aqueous and acetonitrile extracts after anaerobic incubation of [^{14}C]-CL-20 in non-sterile VT soil (97 d) or SAC soil (185 d). CH_3CN extract was not analyzed for SAC soil due to its low content of ^{14}C (~1%).

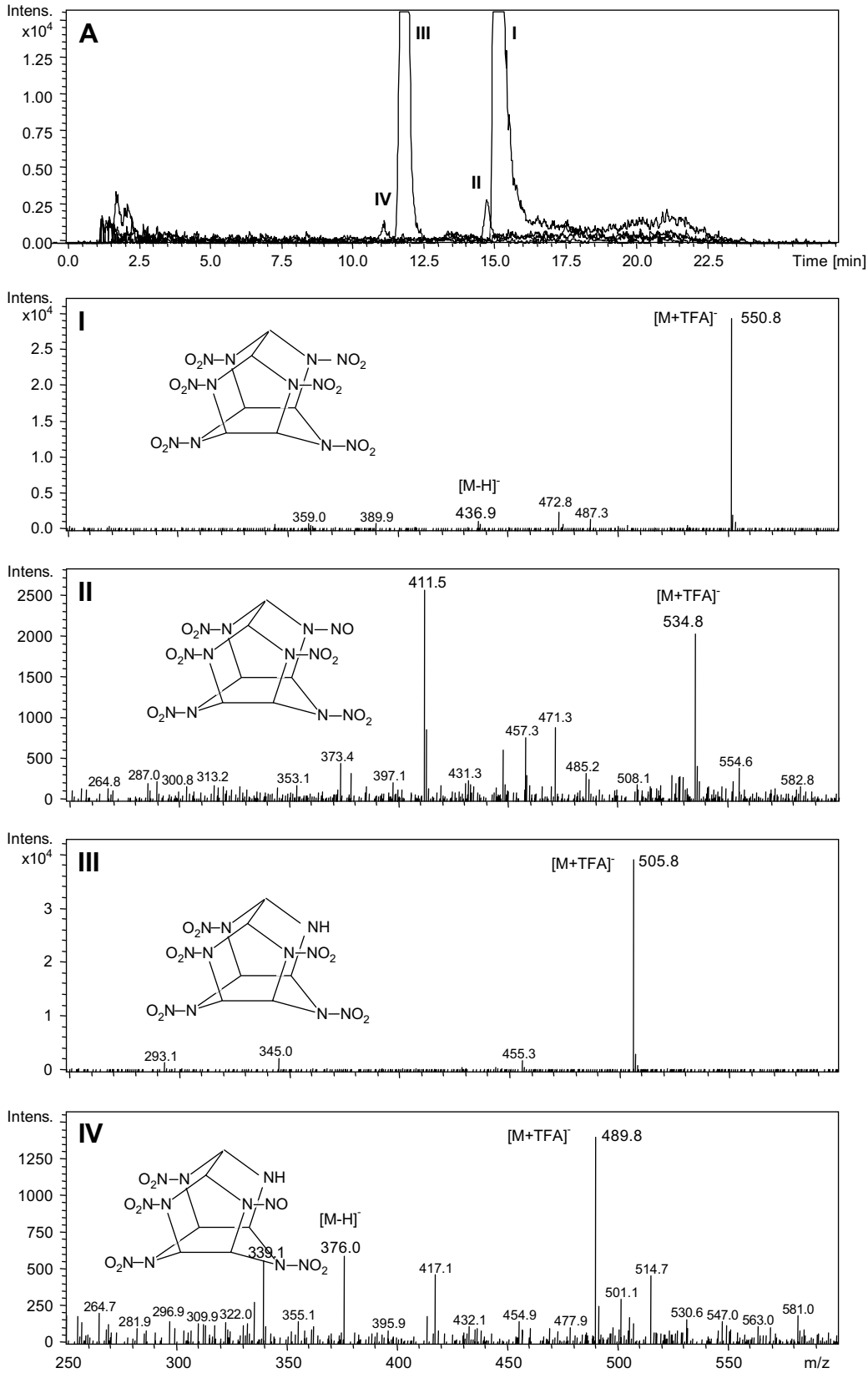

Fig. 5. LC-MS extracted ion chromatogram (A) and mass spectra of CL-20 (**I**) and its initial intermediates (**II–IV**) observed in the acetonitrile extract after 143 d of anaerobic incubation with VT soil.

nitramine remaining in the soil after six months (Fig. 2). The first-order degradation rate constant calculated after the lag phase was 0.018 d^{-1} ($t_{1/2} = 38.5$ d). Under sterile conditions, very little degradation of CL-20 occurred in VT soil with 96.4% of the nitramine recovered after 6 months (Fig. 2). The persistence of CL-20 in sterile controls indicated that the degradation in non-sterile soil was biological. With a greater organic C content (2.3%), VT soil was expected to support microbial activity without nutriment amendment. Moreover, its acid pH prevented hydrolysis of CL-20 from occurring.

The disappearance of CL-20 was accompanied by the formation of formate, formaldehyde, glyoxal, and glycolate (Fig. 2). Formate, with 1.8 μmol formed (1.6 molar equivalents per CL-20 reacted), was the main C-containing product detected. Formaldehyde, glyoxal and glycolate were detected at low levels of 0.15, 0.005, and 0.02 molar equivalent at their maxima, respectively. Glyoxal and glycolate were transient products and their concentrations decreased after reaching their respective maxima between days 35 and 44. Although present in the system, N-containing products such as ammonium, nitrate, and nitrous oxide were not included in the time course because the three chemicals were also found in large amounts in controls containing only VT soil and water. However, the presence of CL-20 induced a rapid diminution of nitrate and nitrous oxide amounts compared to the controls without CL-20 (Fig. 3), suggesting that the energetic chemical significantly affected the microflora responsible for the nitrogen cycle. N_2O, for instance, was detected in all controls without CL-20, whereas it was only detected in the first set of CL-20-containing microcosms (day 14).

3.1.2. ^{14}C partitioning

Experiments were conducted with [^{14}C]-CL-20 to determine the partitioning of CL-20 carbon among the gaseous, aqueous, and solid phases. Abiotic controls were not carried out with [^{14}C]-CL-20 due to the low degradation rate measured under sterile conditions. Anaerobic mineralization of CL-20 in non-sterile VT soil was slow with only 2.4% of ^{14}C recovered as CO_2 after 97 d (Table 3). At the end of the mineralization experiment, sequential extraction, using water, 0.01 M HCl, and acetonitrile, was performed to determine the amount of ^{14}C reversibly or irreversibly bound to soil. A relatively small fraction of the ^{14}C was recovered from the aqueous phase (14.6%) (Table 3). The extraction in acid aqueous phase was done in order to release amino products that could have bound to soil humic matter through amide linkages. No ^{14}C was recovered from this acid washing. In contrast, most of the radioactivity (80.6%) was recovered in the acetonitrile extract suggesting that the insoluble fraction of CL-20 and its products was not covalently bound to soil. Given the high radiolabel recoveries measured (98.1%), combustion of the resulting soil was not performed. Radiochemical HPLC analysis of the aqueous extract showed that most of the radioactivity in this phase was attributable to CL-20 (18–21 min, 85%) (Fig. 4). A ^{14}C peak comprising 5% of the total radioactivity in the aqueous extract eluted at approximately 3.5 min, in the typical eluting zone for polar and/or ionized molecules. Formate and formaldehyde found in cold samples are probably contributing to this early ^{14}C peak. Radiochemical HPLC analysis of the acetonitrile extract showed a different pattern with CL-20 corresponding to about 45% of the radioactivity and the remaining 55% eluting over a broad zone between 10 and 18 min (Fig. 4). Tentative identification of these peaks was performed by LC/MS as described below.

3.1.3. Intermediates identification by LC/MS

LC/MS analyses were run in the presence of TFA. Fig. 5 represents the extracted ion chromatograms of CL-20 (**I**) and three suspected intermediates (**II–IV**) detected in the acetonitrile extract of VT soil after 143 d of incubation under non-sterile conditions. Peak **I** with a retention time (r.t.) of 15.2 min was that of CL-20 which appeared as a TFA adduct $[M+TFA]^-$ at 551 Da (Fig. 5, compound **I**). Peak **II**, appearing at 14.8 min, was only detected in trace amounts and showed a $[M+TFA]^-$ mass ion at 535 Da (Fig. 5, compound **II**), matching a molecular formula of $C_6H_6N_{12}O_{11}$. Using the ring or nitro-labeled [^{15}N]-CL-20, the previously detected mass ion for **II** was observed at 541 Da, an increase of six atomic mass units (amu) for both labeled compounds, thus confirming the involvement of the 12 CL-20 nitrogen atoms in compound **II.** The later was tentatively identified as the mononitroso derivative of CL-20. Beside this trace compound, a major intermediate, **III** (r.t. 11.9 min), with a $[M+TFA]^-$ mass ion at 506 Da (Fig. 5, compound **III**), matching a molecular formula of $C_6H_7N_{11}O_{10}$ was also observed. Using [^{15}N]-CL-20 labeled at either the amino or nitro groups, the previously detected mass ion gave $[M+TFA]^-$ mass ions at 512 and 511 Da, respectively, suggesting the involvement of the six amino ^{15}N atoms and only five of the six nitro ^{15}N atoms in the intermediate. Intermediate **III** was tentatively identified as the denitrohydrogenated product of CL-20. Finally, compound **IV**, appearing at 11.1 min, was also detected in trace amounts and showed a $[M+TFA]^-$ mass ion at 490 Da (Fig. 5, compound **IV**), matching a molecular formula of $C_6H_6N_{11}O_9$. It was tentatively identified as the nitroso derivative of compound **III**. Compounds **II**, **III** and **IV** have all been already observed when treating CL-20 with

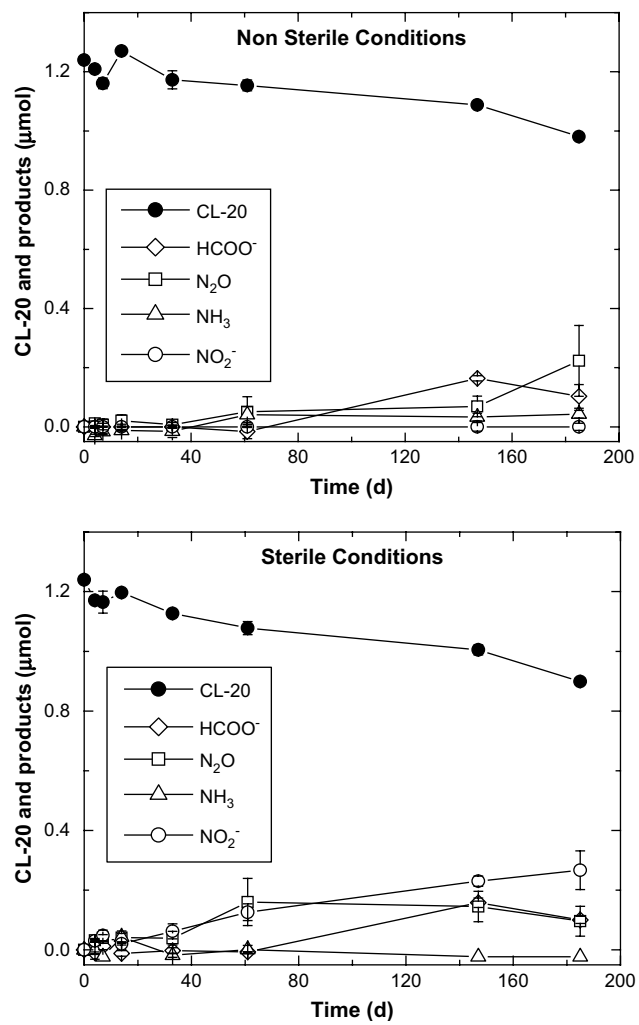

Fig. 6. Time courses of CL-20 degradation with SAC soil under abiotic or biotic conditions. Data are mean and standard error ($n = 2$).

Fe^0, UV light or enzymes (Fig. 1). Compounds **V** and **VI** specific of a denitration process (see Fig. 1) were, however, not detected in VT microcosms either because this degradation route was not active in that soil or because the two intermediates were too unstable to be detected.

These results showed that CL-20 was biodegradable in VT soil under anaerobic conditions and without nutriment amendments. Biotransformation of CL-20 has led to little mineralization and to formation of ring cleavage products. Most of the products were extractable with acetonitrile and consisted of compounds exhibiting the caged structure of CL-20. Among them, the denitrohydrogenated compound **III**, was detected as a major intermediate at all sampling times. Persistence of compound **III** in soil, and its distinct structure that differentiates it from naturally occurring chemicals, suggest that compound **III** has the potential for use as a marker of natural attenuation of CL-20 in anaerobic soil if the latter is released in the environment.

3.2. Transformation of CL-20 in SAC soil

3.2.1. Time courses

Biotic and abiotic transformation of CL-20 was also investigated in SAC soil which was previously shown to support hydrolysis of CL-20 under abiotic conditions (Balakrishnan et al., 2004a). Comparison of CL-20 loss under sterile and non-sterile conditions showed similar trends (Fig. 6), indicating a degradation dominated by abiotic processes, as it was expected from the low organic C content (0.08%) and slight alkalinity of this soil.

The transformation experiment was carried out starting from either 1.2 or 0.3 μmol of CL-20. In both cases, the amount of CL-20 degraded after 185 d was approximately equal to 0.3 μmol, corresponding to 25 or 100% of the initially introduced amount. The first-order degradation rate constants calculated from the biotic and abiotic experiments performed with 1.2 μmol of CL-20 were equal to 0.0012 d^{-1}. These rate constants were approximately 300 times lower than the rate constant of 0.29 d^{-1} determined by Balakrishnan et al. (2004a) when stirring 1.2 μmol of CL-20 with 1.5 g of SAC in 10 mL of water. These results demonstrated that using kinetics data determined in the laboratory to predict CL-20 degradation in the environment can lead to inaccurate estimates because the amount of water present in field soil will affect the rate of CL-20 degradation.

The disappearance of CL-20 was accompanied by the formation of formate, ammonia, nitrous oxide, and nitrite, with about one molar equivalent of the latter being accumulated under abiotic conditions for each mol of CL-20 reacted (Fig. 6). Under biologically active conditions nitrite ions were not detected indicating their consumption by indigenous microorganisms. Glyoxal was detected under sterile and non-sterile conditions but only during the first 2 weeks of monitoring and at low levels (<0.010 μmol).

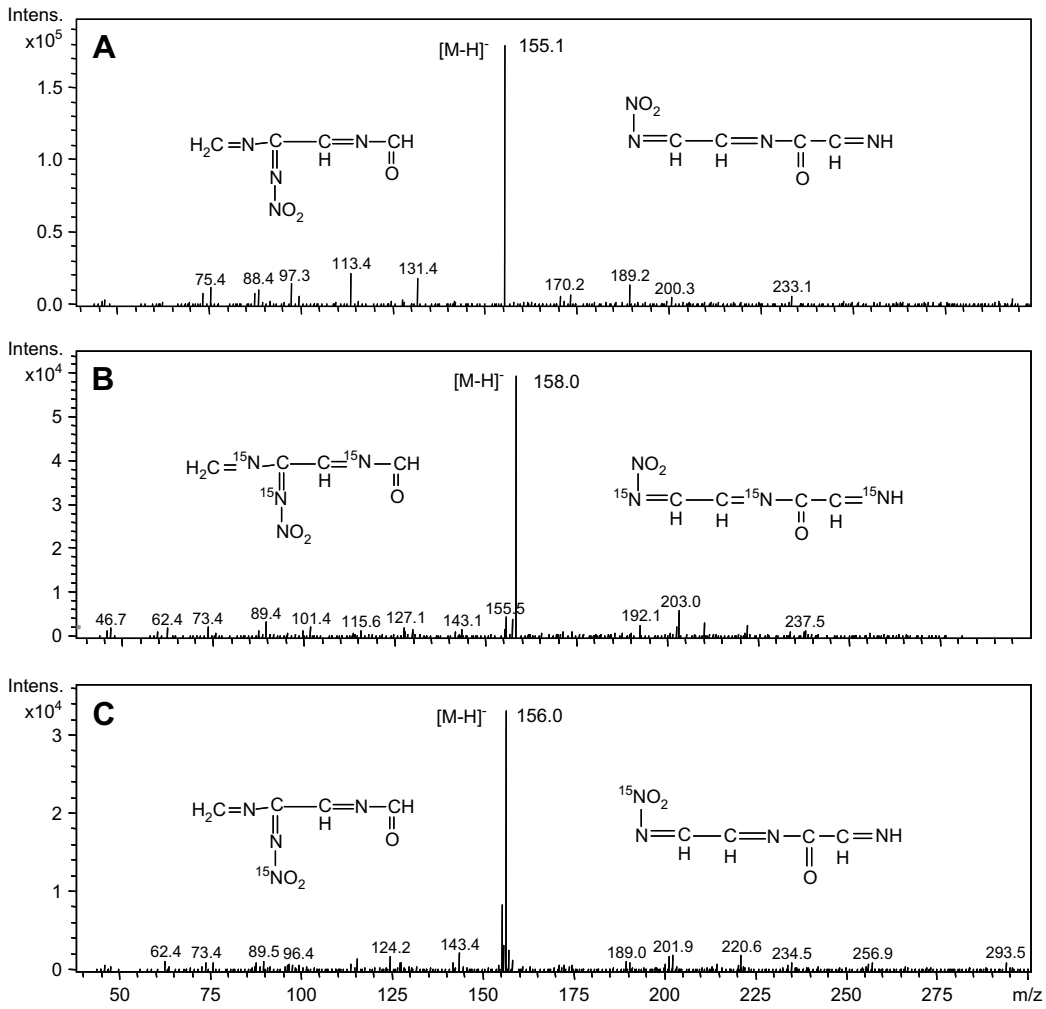

Fig. 7. LC/MS (ES-) mass spectra and proposed structures of degradation product (**VII**) observed after 7 d with SAC soil, using CL-20 (A), amino-labeled [^{15}N]-CL-20 (B), and nitro-labeled [^{15}N]-CL-20 (C).

3.2.2. ^{14}C partitioning

The fate of carbon was investigated using [^{14}C]-CL-20 under sterile and non-sterile conditions. Transformation of CL-20 led to high carbon recoveries of 50.1 and 67.5% in the KOH trap in sterile and non-sterile treatments, respectively, after 185 d (Table 3). In both cases, most of the remaining non-gaseous ^{14}C was present in the aqueous phase (Table 3). Small amounts of ^{14}C (~1%) were retrieved from the acetonitrile extract and close to 5% were recovered as irreversibly bound material. Radiochemical HPLC analysis of the aqueous phase of the non-sterile sample revealed two main zones of elution: one early peak (3–5 min) consisting of 50% of the total ^{14}C in the aqueous phase and corresponding to polar or ionized compounds, and a second peak (18–21 min, 38%) corresponding to unreacted CL-20. Formate and glyoxal determined in the cold samples likely contributed to the early peak.

The use of KOH to trap ^{14}C labeled CO_2 is a common method for measuring the mineralization of organic molecules. The large amount of ^{14}C in the KOH trap under abiotic conditions was unexpected and could result from sources other than complete mineralization of CL-20. Formate (p*K*a 3.75) is ionized at pH 8.1 and thus was not expected to volatilize in the present media. Glyoxal with a boiling point of 50.4 °C could volatilize partially into the headspace, and then be trapped by the KOH as it is known to react easily with bases (Yadav and Gupta, 2000). When we introduced an aqueous solution of [^{14}C]–CHOCHO in a closed flask containing a KOH trap for 21 d, we recovered 21% of the glyoxal radioactivity in the trap. However, attempts to identify glyoxal products in the trap were unsuccessful, and the contribution of glyoxal to the measured radioactivity remains hypothetical. Other volatile and unidentified chemicals might have contributed to the high radioactivity measured in the KOH trap, especially under abiotic conditions.

3.2.3. Intermediates identification by LC/MS

Intermediates resulting from abiotic degradation of CL-20 (1.2 μmol) in SAC soil (1.5 g in 10 mL H_2O) were monitored using LC/MS in the presence and absence of TFA. Analyses were conducted after 7 d incubation. A trace quantity of compound **III** was detected in the acetonitrile extract in the presence of TFA, suggesting that very few cyclic nitramine products were present in the system. Analyses of the aqueous phase without TFA showed a major signal (**VII**) eluting at 2.0 min with a $[M–H]^-$ mass ion at 155 Da (Fig. 7) matching a molecular formula of $C_4H_4N_4O_3$. When using the amino and nitro-labeled [^{15}N]-CL-20, the detected mass ions were observed at 158 and 156 Da (Fig. 7), respectively, confirming the involvement of three ring N atoms and one nitro N atom in intermediate **VII**. Compound **VII** could be derivatized with 2,3,4,5,6-pentafluorobenzylhydroxylamine, indicating that this compound contains one carbonyl group. We thus tentatively identified **VII** as $CH_2{=}N–C({=}N–NO_2)–CH{=}N–CHO$ or its isomer $N(NO_2){=}CH–CH{=}N–CO–CH{=}NH$. The concomitant formation of nitrite ions (see Fig. 6) suggests that compound **VII** can be a product of denitration or denitrohydrogenation. The absence of compound **III** in the medium suggests that denitration is the most probable abiotic pathway. Being also a unique CL-20 product, compound **VII** has the potential for use as marker of *in situ* natural attenuation of CL-20.

4. Conclusion

The present study demonstrated that CL-20 can be abiotically or biotically degraded in soil. Biotic degradation was prevalent in VT soil, which contained a greater organic C content and was slightly acidic, while the slightly alkaline SAC soil favored the hydrolysis of the cyclic nitramine. Biotic degradation of CL-20 occurred through the initial denitrohydrogenation route, with the formation of denitrohydrogenated derivative **III**, as the major product. Hydrolysis led to fast opening of the cage rings as revealed by the formation of a ring cleavage product, **VII**, that was tentatively identified as $CH_2{=}N–C({=}N–NO_2)–CH{=}N–CHO$ or its isomer $N(NO_2){=}CH–CH{=}N–CO–CH{=}NH$. Under both abiotic and biotic conditions, CL-20 degradation led to the formation of formate ($HCOO^-$) and glyoxal (CHOCHO). Nitrite (NO_2^-), ammonium (NH_4^+), and nitrous oxide (N_2O) were also formed under abiotic conditions but were not detected in biotic experiments. The detected key intermediates, including the denitrohydrogenated compound **III**, and the ring cleavage product **VII,** have the potential for use as markers of natural attenuation of CL-20 in soil in case of its accidental release in the environment.

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