Biodegradation of the nitramine explosives hexahydro-1,3-5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine in cold marine sediment under anaerobic and oligotrophic conditions

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Abstract: The in situ degradation of the two 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), was evaluated using a mixture of RDX and HMX, incubated anaerobically at 10 °C with marine sediment from a previous military dumping site of unexploded ordnance (UXO) in Halifax Harbor, Nova Scotia, Canada. The RDX concentration (14.7 mg·L^{-1}) in the aqueous phase was reduced by half in 4 days, while reduction of HMX concentration (1.2 mg·L^{-1}) by half required 50 days. Supplementation with the carbon sources glucose, acetate, or citrate did not affect the removal rate of RDX but improved removal of HMX. Optimal mineralization of RDX and HMX was obtained in the presence of glucose. Using universally labeled (UL)-[^{14}C]RDX, we obtained a carbon mass balance distributed as follows: CO_2, 48%–58%; water soluble products, 27%–31%; acetonitrile extractable products, 2.0%–3.4%; and products covalently bound to the sediments and biomass, 8.9% (in the presence of glucose). The disappearance of RDX was accompanied by the formation of the mononitroso derivative hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and formaldehyde (HCHO) that subsequently disappeared. In the case of HMX, mineralization reached only 13%–27% after 115 days of incubation in the presence or absence of the carbon sources. The disappearance of HMX was also accompanied by the formation of the mononitroso derivative. The total population of psychrotrophic anaerobes that grew at 10 °C was 2.6 × 10^3 colony-forming units·(g sediment dry mass)$^{-1}$, and some psychrotrophic sediment isolates were capable of degrading RDX under conditions similar to those used for sediments. Based on the distribution of products, we suggest that the sediment microorganisms degrade RDX and HMX via an initial reduction to the corresponding mononitroso derivative, followed by denitration and ring cleavage.

Key words: biodegradation, nitramine explosives, marine sediment, psychrotrophic bacteria.

Résumé : La dégradation in situ de deux explosifs nitraminés: le hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) et le octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), a été évaluée en faisant usage d'une mélange de RDX et de HMX incubé dans des conditions anaérobies à 10 °C en présence de sédiments marins provenant d'un ancien site d'enfouissement militaire de munitions explosives non explosées (UXO) au port maritime d'Halifax. La concentration de RDX (14,7 mg·L^{-1}) dans la phase aqueuse a été diminuée de moitié en 4 jours, alors que la réduction des concentrations de HMX (1,2 mg·L^{-1}) de moitié a demandé 50 jours. L'enrichissement avec les sources de carbone tel que le glucose, l'acétate ou le citrate n'a pas eu d'impact sur le taux d'élimination du RDX mais a amélioré l'élimination du HMX. La minéralisation optimale sur RDX et du HMX a été obtenue en présence de glucose. Grâce au (UL)-[^{14}C]RDX, nous avons obtenu les proportions de la distribution de la masse du carbone suivantes: CO_2, 48 % – 58 %; produits solubles dans l'eau, 27 % – 31 %; produits solubilizables à l'acétonitrile, 2,0 % – 3,4 %; produits liés de façon covalente aux sédiments et à la biomasse, 8,9 % (en présence de glucose). La disparition du RDX a été accompagné par la formation du dérivé mononitroso, le hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), et de formaldéhyde (HCHO) qui ont disparu par la suite. Dans le cas du HMX, la minéralisation n'a atteint que 13 % – 27 % après 115 jours d'incubation en présence ou en absence de sources de carbone. La disparition de HMX a également été accompagné de la formation du dérivé mononitroso. La population totale d'anaérobes psychrotrophes croissant à 10 °C était de 2,6 × 10^3 unité formant des colonies·(g de sédiment poids sec)$^{-1}$. Certains isolats psychrotrophes des sédiments furent

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capables de dégrader le RDX dans des conditions semblables à celle utilizées pour les sédiments. En s'appuyant sur la distribution des produits, nous suggérons que les microorganismes des sédiments dégradent le RDX et le HMX par un réduction initiale vers le dérivé mononitroso correspondant, suivi par la dénitration et le clivage des cycles.

Mots clés : biodégradation, explosifs nitraminés, sediments marins, bactérie psychrotrophes.

[Traduit par la Rédaction]

Introduction

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) are powerful and highly energetic chemicals that are widely used in the production of explosives and nuclear warheads (Haas et al. 1990; Myler and Sisk 1991). Various military activities, including manufacturing, land and sea testing and training, and demilitarization activities, have resulted in severe contamination of terrestrial and marine environments. Leaching from unexploded ordnance (UXO) is considered a major source of sediment contamination in seas and waterways. These toxic contaminants can subsequently migrate from sediments and accumulate in other aquatic organisms, causing adverse ecological effects (Yinon 1990; Robidoux et al. 2000).

Bacteria in soil (Young et al. 1997*a*, 1997*b*), anaerobic sludge (McCormick et al. 1981; Hawari et al. 2000, 2001, 2002; Zhao et al. 2002, 2003*a*, 2003*b*), fresh water sediments (Beller 2002; Boopathy et al. 1998), and horse manure (Kitts et al. 1994) were reported to degrade RDX and HMX under oxygen-limited conditions. We found that bacteria from anaerobic sludge could degrade RDX and HMX to nitrous oxide (N_2O) and formaldehyde via initial denitration (Hawari et al. 2000, 2001; Halasz et al. 2002; Zhao et al. 2002, 2003*a*, 2003*b*). Little data are currently available on the fate and degradation of explosives from UXO in marine sediments.

The objective of the present study was to evaluate the biodegradation of RDX and HMX in marine sediment from a dumping site, located at the Emerald Basin (215 m deep, 50 nautical miles from Halifax Harbor, Nova Scotia, Canada), where warships unloaded live ordnance during wartime. Traces of explosives were recently found in the sediment and water column from this harbor (Darrach et al. 1998). Marine sediment is mostly anaerobic with low amounts of nutrients (oligotrophic) and high salt content (Karl and Dore 2001; Bowman 2001). Also marine sediments are subjected to high pressure under cold temperatures. Psychrotrophic bacteria that can grow at low temperatures (7–18 °C) have been found in marine sediments (Knoblauch et al. 1999). Such bacteria are either psychrophilic with low temperature growth optima (<15 °C) or psychrotrophic (grow at low temperature but have a normal temperature optima). The biodegradability of the two explosives in the sediment was therefore evaluated under oligotrophic and anaerobic experimental conditions at 10 °C.

A Remotely Operated Vehicle carried by the Canadian Navy Deep Seabed Intervention System used an aluminum container to collect the sediment. The sediment was sealed with seawater and kept at 4 °C during shipment and storage. The sediment (pH 6.5) was composed of silt (90%, particle size 2–50 μm), clay (8.3%, particle size <2 μm), and sand (1.3%, particle size >50 μm). The water content of wet sediment was 62%. Other sediment characteristics are described in Table 1.

Table 1. Components (g·(kg sediment dry mass)$^{-1}$) of Halifax Harbor marine sediment.

Component	Mass
Total organic C	12.0
Total S	2.1
NH_3	0.0077
Al	15.0
Ca	24.0
Fe	40.0
Na	10.0
Mg	8.8
K	5.3
Mn	0.5
Sr	0.1
Ti	0.5
Zn	0.1

RDX (99% pure), HMX (99% pure), universally labeled (UL)-[^{14}C]RDX (chemical purity, >99%; radiochemical purity, 96%; specific radioactivity, 28.7 μCi·mmol^{-1}), and universally labeled (UL)-[^{14}C]HMX (chemical purity, >94%; radiochemical purity, 91%; specific radioactivity, 93.4 μCi·mmol^{-1}) were provided by Defense Research and Development Canada (DRDC), Valcartier, Quebec, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (98% pure) was provided by R.J. Spanggord from SRI International (Menlo Park, Calif., U.S.A.). All other chemicals used were of reagent grade.

Anaerobic biodegradation of a mixture RDX and HMX at 10 °C in sediment

Biodegradation of a mixture of RDX (0.59 mg) and HMX (0.048 mg) in 10 g of wet sediment (dry mass, 3.8 g) and 30 mL of nitrogen-free marine medium A in 100-mL serum bottles was conducted at 10 °C under an atmosphere of nitrogen. Anaerobic conditions were monitored with a BBL indicator (VWR Canlab, Mississauga, Ont., Canada). The marine medium A was composed of 25 g·L^{-1} of NaCl, 5 g·L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.2 g·L^{-1} of $CaCl_2 \cdot 2H_2O$, 0.1 g·L^{-1} of KCl, 30 mg·L^{-1} of $FeSO_4 \cdot 7H_2O$, and 12 mmol·L^{-1} Na_2HPO_4 (pH 7.2). In some cases, microcosms were supplemented with 0.1 g·L^{-1} of the following carbon sources: glucose, sodium acetate, or sodium citrate. Autoclaved sediment (autoclaved for two consecutive days at 120 °C for 30 min) was used as control to determine degradation of RDX and HMX under abiotic conditions. The RDX and HMX in the aqueous phase were monitored over a period of 4 months. RDX, HMX, and their products were analyzed by previously described methods (Hawari et al. 2000, 2001).

Fig. 1. Anaerobic removal of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (0.59 mg) (A) in the presence of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (0.048 mg) in marine sediment at 10 °C, and production of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (B) and formaldehyde (HCHO) (C). Medium: 30 mL of marine medium and 10 g of wet sediment.

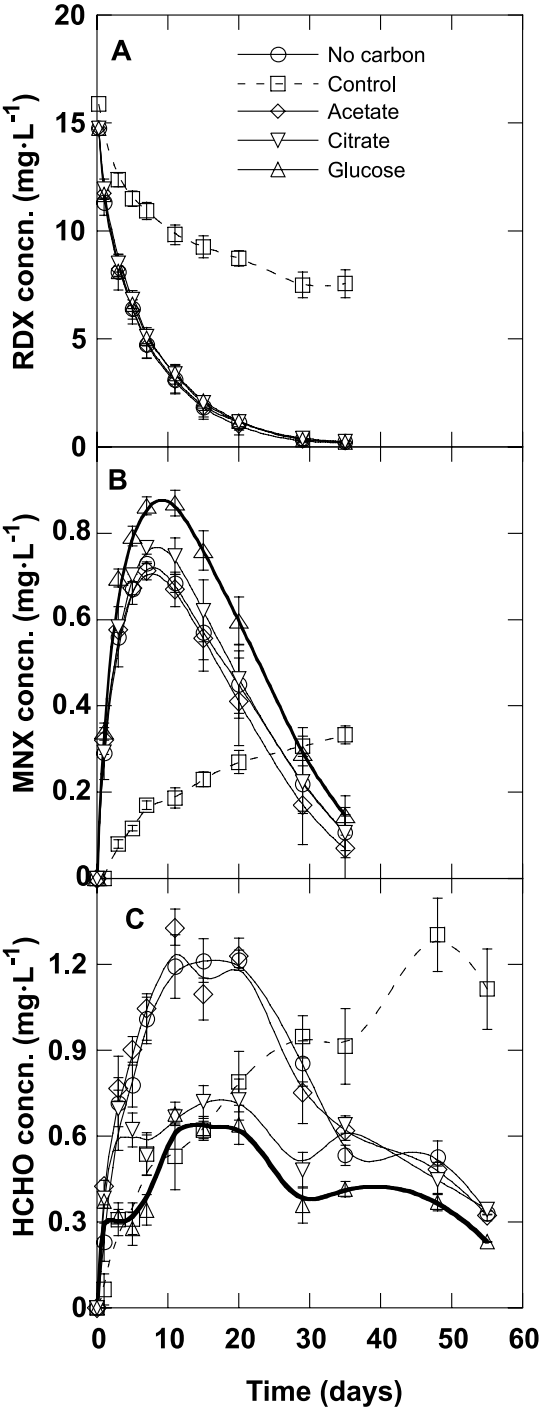
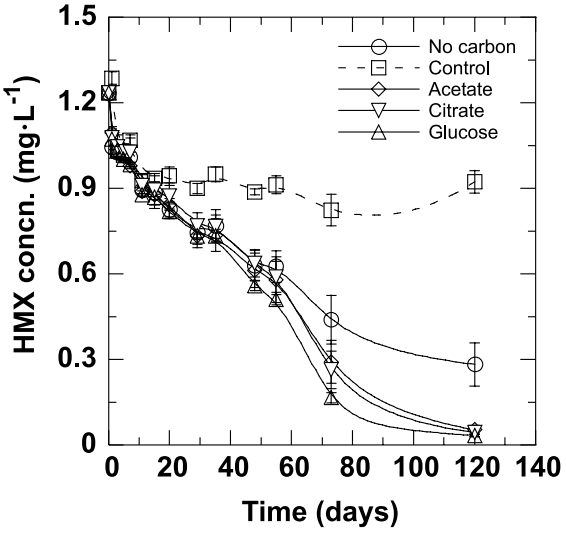


Figure 1A shows the anaerobic removal of RDX (0.59 mg) in the presence of HMX (0.048 mg) in the marine sediment suspension at 10 °C. The concentration of RDX in the aqueous phase decreased to half after 4 days of incubation and disappeared from the aqueous phase with or without supplementation of carbon sources after 35 days of incubation. No RDX was found remaining in the sediments following extraction with acetonitrile, indicating that essentially all the RDX was degraded. In the control with autoclaved sediment, approximately 50% of the RDX in the aqueous phase was lost after 115 days, presumably because of abiotic reactions.

Fig. 2. Anaerobic removal of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (0.048 mg) in the presence of hexahydro-1,3,5-trinitro-1,3,5-triazine (0.59 mg) in marine sediment at 10 °C. Medium was the same as in Fig. 1.



Removal of HMX (0.048 mg) in the same RDX–HMX mixture is shown in Fig. 2. When RDX removal was almost complete after 35 days of incubation, only 40% of the HMX was degraded. HMX decreased to half its initial concentration (1.2 $mg \cdot L^{-1}$) after 50 days, ten times longer than that observed for RDX (Fig. 1), indicating that microorganisms in the sediment removed RDX preferentially to HMX. In the absence of additional carbon sources, the sediment decreased the HMX concentration to 24% of its initial concentration after 115 days. In the presence of additional carbon sources (glucose, acetate, and citrate) the sediment decreased HMX concentration to 4% of its initial concentration after 115 days, indicating that supplementation of additional carbon sources improved HMX removal. In the control with autoclaved sediment, HMX only decreased to 76% of its initial concentration after 115 days incubation.

Metabolite production

Disappearance of RDX in the RDX–HMX mixture was accompanied by the formation of MNX, which accumulated only transiently (Fig. 1B) with concurrent formation of formaldehyde (HCHO) (Fig. 1C). Since the initial HMX concentration was only about 10% of RDX and the amount of HMX removed was very small, most of the detected HCHO was presumed to originate from RDX. HCHO concentration in the medium with carbon supplementation was lower than that observed in the media without supplementation, indicating an active metabolism of HCHO in the former cases. HCHO was presumed to be mineralized to carbon dioxide (CO_2). No di- or tri-nitroso derivatives of RDX were

Fig. 3. Anaerobic mineralization of universally labeled [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (A) and universally labeled [^{14}C]octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (B) in the RDX–HMX mixture in marine sediment at 10 °C. Medium: 15 mL of the marine medium and 5 g of wet sediment.

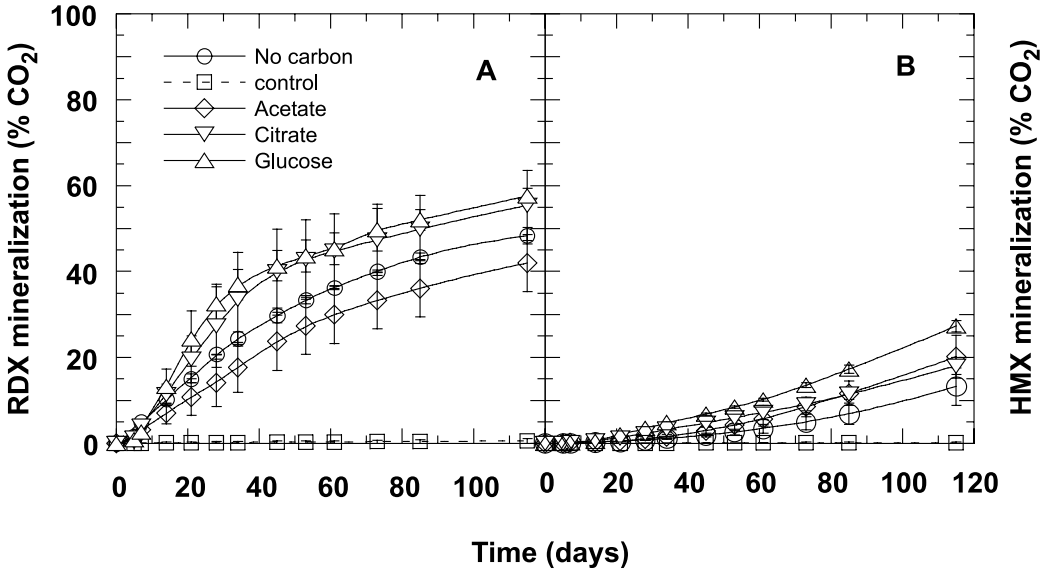


Table 2. Carbon (C) balance of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degradation in marine sediments.

		^{14}C distribution (%)					
C source	Initial ^{14}C in [^{14}C]RDX (%)	CO_2 (A)	C in aqueous phase (B)	C extracted with AcCN (C)	Subtotal (A+B+C)	C bound to sediment (D)	Recovery (A+B+C+D)
No C	100	48.3 (1.9)	27.4 (2.2)	3.2 (2.9)	78.9 (2.4)	ND	ND
Control	100	0.57 (0.48)	59.2 (3.9)	6.6 (1.1)	66.6 (2.4)	ND	ND
Acetate	100	42.1 (6.6)	31.1 (1.3)	3.0 (2.1)	76.0 (4.1)	ND	ND
Citrate	100	55.4 (8.2)	25.1 (0.6)	3.4 (3.3)	83.7 (5.1)	ND	ND
Glucose	100	57.6 (1.9)	27.1 (0.7)	1.9[a]	86.5	8.9[a]	95.4

Note: All results were obtained after 115 days of incubation. Values in parentheses are standard deviation of triplicate. ND, not determined; AcCN, acetonitrile.

[a]Average of a duplicate.

detected. MNX and HCHO observed in the control were presumably from abiotic reactions, and they did not degrade further.

The mononitroso derivative of HMX was also detected as a product during degradation of the mixture of RDX–HMX, indicating transformation of HMX. No di- or tri-nitroso derivatives of HMX were detected.

We only detected a trace of N_2O and nitrite in the present tests, likely due to their removal upon formation. Previously we reported that anaerobic degradation of RDX (Zhao et al. 2002, 2003*a*, 2003*b*) occurred via transformation of RDX to MNX followed by denitration. We also found that obligate anaerobic mixed cultures can remove nitrous oxide (N_2O) (Zhao et al. 2003*b*).

RDX and HMX mineralization and mass balances

Mineralization of UL-[^{14}C]RDX (83 700 dpm, 0.29 mg) in the presence of nonlabeled HMX (0.02 mg) or mineralization of UL-[^{14}C]HMX (51 350 dpm, 0.08 mg) in the presence of nonlabeled RDX (0.24 mg) was conducted in medium A (15 mL) containing sediment (wet mass, 5 g; dry mass, 1.9 g) under the same conditions as described above. Measurement of CO_2 collected in the KOH traps was conducted by following previously described protocols (Hawari et al. 2002). The sediment was centrifuged, and radioactivity in the supernatant (aqueous phase) and sediment (extraction with acetonitrile by sonification overnight) was measured. Carbon covalently bound to the sediments after acetonitrile extraction was recovered by the wet-combustion protocol, as described previously by Shen et al. (1998) and Thompson et al. (1998).

Optimal mineralization of RDX was found in the presence of glucose (Fig. 3A, Table 2). After 115 days, we obtained a carbon mass balance (95.4%) of RDX distributed as follows: CO_2 (57.6%), water-soluble products (27.1%), acetonitrile-extractable products (1.9%), and products covalently bound to biomass and sediment (8.9%) (Table 2).

In the case of HMX, only 13%–27% of the total C of initial HMX was mineralized after 115 days of incubation (Fig. 3B). Supplementation with glucose, citrate, or acetate improved HMX mineralization, with glucose resulting in the greatest extent of mineralization (Fig. 3B).

Our results clearly showed that sediment alone could anaerobically degrade the two explosives to CO_2 at 10 °C.

Supplementation of carbon sources did not significantly affect the removal rate of the two explosives, suggesting that the organic carbon (12 $mg \cdot kg^{-1}$) present in the sediment (Table 1) was sufficient to support growth of indigenous marine microorganisms to degrade RDX and HMX.

In RDX or HMX controls with autoclaved sediment, the two chemicals were not mineralized (Figs. 3A and 3B). However, earlier we showed that RDX could undergo slow abiotic hydrolysis in water without producing CO_2 (Zhao et al. 2002; Fig. 1A, this paper).

Preliminary results showed that some unidentified psychrotrophic anaerobic isolates from the marine sediment were capable of degrading RDX or HMX at 10 °C under conditions similar to those used to degrade RDX and HMX in the sediment.

Anaerobic mineralization of RDX at normal temperatures (room temperature and above) has been reported previously in mixed microbial populations, such as anaerobic sludge (Hawari et al. 2000), soil bacteria (Juck et al. 2003), and the sludge isolate *Klebsiella pneumoniae* SCZ-1 (Zhao et al. 2002). Anaerobic mineralization of HMX was found only in anaerobic sludge (Hawari et al. 2001). Although psychrophilic anaerobic bacteria belonging to the γ- and δ-*Proteobacteria* and to *Spirochaetales*, with optimal growth temperatures of 7–18 °C, have been previously isolated from marine sediment (Bowman 2001; Mountfort et al. 1998; Nogi et al. 1998), none have been described as degraders of RDX and HMX. The present results indicate that psychrotrophic microorganisms in marine sediment were capable of mineralizing RDX and HMX.

Detection of MNX in small amounts and the absence of di- or tri-nitroso derivatives as metabolites in RDX degradation in marine sediment indicate the similarity between product distributions of the marine sediment microorganisms and those found in anaerobic sludge (Hawari et al. 2002) and one of its facultative anaerobic isolates, strain SCZ-1 (Zhao et al. 2002). Thus, the sediment microorganisms might also degrade RDX and HMX via a similar reduction route to the corresponding mononitroso derivative followed by denitration and ring cleavage. Currently, we are characterizing selected marine isolates for metabolism of RDX and HMX to gain new insight into the biodegradation pathway of cyclic nitramines in marine sediment.

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