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ORIGINAL PAPER

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Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine by novel fungi isolated from unexploded ordnance contaminated marine sediment

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Abstract Undersea deposition of unexploded ordnance (UXO) constitutes a potential source of contamination of marine environments by hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Using sediment from a coastal UXO field, Oahu Island, Hawaii, we isolated four novel aerobic RDX-degrading fungi HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5, tentatively identified as members of *Rhodotorula*, *Bullera*, *Acremonium* and *Penicillium*, respectively. The four isolates mineralized 15–34% of RDX in 58 days as determined by liberated $^{14}CO_2$. Subsequently we selected *Acremonium* to determine biotransformation pathway(s) of RDX in more details. When RDX (100 μM) was incubated with resting cells of *Acremonium* we detected methylenedinitramine (MEDINA), N_2O and HCHO. Also we detected hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) together with trace amounts of hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX). Under the same conditions MNX produced N_2O and HCHO together with trace amounts of DNX and TNX, but we were unable to detect MEDINA. TNX did not degrade with *Acremonium*. These experimental findings suggested that RDX degraded via at least two major initial routes; one route involved direct ring cleavage to MEDINA and another involved reduction to MNX prior to ring cleavage. Nitrite was only detected in trace amounts suggesting that degradation via initial denitration did take place but not significantly. Aerobic incubation of *Acremonium* in sediment contaminated with RDX led to enhanced removal of the nitramine.

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Introduction

Various naval military activities including testing, training, and demilitarization have resulted in the deposition of munitions and unexploded ordnance (UXO) in seas and waterways. Munitions compounds leached from ruptured UXO are considered as a major source of contamination because they can accumulate in sediment and aquatic organisms [13, 35]. Cyclic nitramines RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) are typical munitions compounds that are widely used by many naval defense departments worldwide. Besides being physically harmful both RDX and HMX are reported to be toxic [25, 31, 41] thus necessitating their removal from affected marine environments.

Biodegradation of RDX and HMX in water and soil [2, 3, 5, 9, 10, 12, 15, 16, 19, 32, 33] has been extensively reported, but less information is available on the biodegradation of the two nitramines in marine environments. Despite recent advances made towards the understanding of biodegradation of cyclic nitramines under anaerobic conditions [3, 5, 6, 8, 42–44] little progress is made on the aerobic side particularly in shallow marine sediments. For instance, so far no aerobic marine fungus has been reported for the degradation of RDX and HMX.

Since both RDX and HMX are considered to be oligomers, ($(CH_2NNO_2)_n$; $n=3$ or 4, respectively) of the same repeating structural moiety, CH_2NNO_2, we selected RDX as a typical munitions compounds to determine its aerobic biodegradability in a marine medium. Previous studies showed that RDX [33, 34] can be degraded using *Phanerochaete chrysosporium*, but little information was provided on the mechanism of degradation. The objective of the present study is to first

isolate fungi that can degrade RDX from an UXO-contaminated marine sediment, sampled from Hawaii [27], and second to determine the biodegradation pathway(s) of the chemical. We hope that understanding the role of sediment fungi in biodegradation of RDX under aerobic conditions will provide additional insight into the ultimate fate of these chemicals in real world marine environments.

Materials and methods

Chemicals and media

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (99% pure), uniformly labeled [UL-^{14}C]-RDX (chemical purity, > 98%; radiochemical purity, 96%; specific radioactivity, 28.7 μCi mmol^{-1}), uniformly ring-labeled [^{15}N]-RDX (98% pure) and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (99% pure) were provided by Defense Research and Development Canada (DRDC), Quebec, Canada. [^{14}C]-HCHO (specific activity, 53 μCi mmol^{-1}) was from Aldrich, Canada. Hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) (98% pure) was provided by Dr. R. J. Spanggord from SRI International (Menlo Park, CA, USA), and methylenedinitramine (MEDINA) was purchased from the rare chemical department, Aldrich, Canada. No reference standard was available for hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX). All other chemicals were reagent grade.

The medium for isolation and maintenance of fungi was marine fungal medium (pH 5.0) that contained (in 1 L deionized water) 3 g yeast extract (Difco), 3 g malt extract (Difco), 5 g Bacto peptone (Difco), 10 g glucose and 40 g sea salts (Sigma). Solid marine fungal medium was prepared by adding 2% agar. All media used were sterilized either by autoclaving at 121°C for 20 min or by passing through a Stericup filter (0.22 μm, Millipore).

Sediment sampling

Marine sediment was sampled from a coastal region (Station ORD2, UXO field) of Oahu Island, Hawaii (15–21 m deep) using 4 L plastic cores (30 cm long). The containers were first washed with acetone and sea water. The sediment was scooped into the plastic core by a diver and subsequently sealed at both ends. Characterization of the sediment was previously described [27]. Briefly, the sediment was sandy mixed with coral reef and heavily degraded UXO. The temperature and pH at the sampling site were 26°C and 8, respectively. The sediment contained trace amounts of 2,4,6-trinitrotoluene (TNT) ($<$ 0.01 mg kg^{-1}), 2,4-dinitrotoluene (0–2.5 mg kg^{-1}) and 2,6-dinitrotoluene (0–0.1 mg kg^{-1}). Neither RDX nor HMX was detected. The sediment core was kept at 4°C prior to use. Only sediment in the middle of the core was used for isolating fungi.

Isolation and identification of fungi

Fungi were enriched by incubating sediment (10 g) in marine fungal medium (100 ml) in 500 ml Erlenmeyer flask on a rotary shaker (80 rpm) at room temperature for 7 days. Chloramphenicol (0.2 g l^{-1}) was added to the medium as ethanol stock solution after passing through a 0.2 μm filter to inhibit bacterial growth. The undiluted fungal enrichment culture (10 μl) was then spread on marine fungal agar plates followed by incubation at room temperature for 1 week. Several morphologically different fungal colonies were picked and further purified by streaking on fresh solid marine fungal medium. Four strains named HAW-OCF1, HAW-OCF2, HAW-OCF3, and HAW-OCF5 that were tested positive for their potential to degrade RDX under aerobic conditions were selected for further identification (see below).

Unicellular fungal strains (yeast) were characterized by their colonial (color, texture) and cellular (size, arrangement, shape and budding pattern) morphology, and physiological properties (assimilation of carbon and nitrogen sources and formation of starch) as described previously [4, 38]. Filamentous fungi were characterized by their colony morphological properties, mycelia features and spore patterns [22, 24].

The potential production of extracellular lignin peroxidase (LiP) and manganese peroxidase (MnP) by filamentous fungi was determined in nitrogen limited basic mineral medium supplemented with glucose (10 g l^{-1}) and NaCl (3.5% w/v) using previously described methods [36, 37, 39]. The basic mineral medium (g l^{-1}) was composed of KH_2PO_4 (2.0), $CaCl_2{\cdot}2H_2O$ (0.14), $MgSO_4{\cdot}7H_2O$ (0.70), $FeSO_4{\cdot}7H_2O$ (0.07), $ZnSO_4{\cdot}7H_2O$ (0.05), and $MnSO_4{\cdot}H_2O$ (0.04). To determine whether RDX can serve as N or C source we prepared two growth assays; one assay contained RDX (225 μM) and glucose (1 g l^{-1}) in basic mineral salt medium containing NaCl (3.5%) and another assay contained RDX (225 μM) and ammonium chloride (0.1 g l^{-1}). Initial OD_{600nm} was 0.05–0.07.

Fungal genomic DNA was prepared using Q1Aamp DNA mini Kit (Qiagen GmbH, Hilden, Germany). The 18S rRNA genes were amplified using HotStart PCR reagent kit (Qiagen) and subsequently sequenced on ABI PRISM®. The forward and reverse 18S rRNA primers for strain HAW-OCF1 were AAGGGTTCGATTCCGGAGAGG and CGACGGGCGGTGTGTACAAAG, for strains HAW-OCF2 and HAW-OCF3 were AAGGGTTCGATTCCGGAGAGG and CGACGGGCGGTGTGTACAAAG, and for the fourth strain HAW-OCF5 were CGGCGACGCTTCWTTCAAAT and TAGCGACGGGCGGTGTGTAC, respectively. PCR programs were as follows: 95°C, 12 min; 94°C, 30 s; 50°C, 30 s; 72°C, 1.5 min; 72°C, 7 min; 35 cycles. The obtained 18S rRNA sequences of HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5 were 1207b, 1212b, 1204b and 1234b, respectively. The sequences were then compared to known sequences in GeneBank by BLAST. The gene sequences of

the isolates and those of closely related fungi in the GeneBank were aligned using ClustalX (1.81). The Neighbor-joining method in the MEGA2 package based on the pair wise nucleotide distance of Kimura 2-parameter was used to build the Phylogenetic tree (the number of bootstrap repetitions was 4,000) [23]. GeneBank accession numbers of 18S rRNA gene sequences for strains HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5 were AY887944, AY887945, AY887946 and AY887947, respectively.

Biotransformation of RDX by growing fungal isolates

RDX biotransformation by growing fungi was conducted on a rotary shaker (80 rpm) at room temperature. Freshly grown (7 days) marine fungal agar plugs (5 × 5 mm) were inoculated in 50 ml marine fungal medium in 125 ml foam-plugged serum bottles. RDX (100 μM) was then added to above medium using an acetone stock solution. RDX remained and metabolites formed were measured as described below. To monitor production of metabolites in the headspace, two microcosms were sealed by rubber septa with air in headspace. To maintain aerobic conditions, air (10 ml) was introduced into the serum bottles every 3–4 days using a gas tight syringe through a 0.22-μm membrane filter (Millipore). The abiotic control was composed of autoclaved fungal agar plugs medium and RDX which was added following the autoclaving step.

Mineralization was conducted as described previously [19]. Briefly, marine agar plugs (5 × 5 mm) of fungal isolates were inoculated in 120 ml serum bottles containing 10 ml of marine medium and RDX (100 μM) spiked with [UL-^{14}C]-RDX and fitted with test tubes containing 1 ml KOH (0.5 N) to capture liberated $^{14}CO_2$. The KOH solution was sampled with a gas tight syringe once every 3–4 days to measure $^{14}CO_2$ released using a Tri-Carb 4530 liquid scintillation counter (LSC, model 2100 TR, Packard Instrument Company, Meriden, CT, US). Air (10 ml) was introduced after each sampling cycle as described before. At the end of the mineralization experiment which lasted 58 days, the aqueous phase of the cultures obtained by filtration was analyzed for remaining radioactivity (^{14}C) [33]. Formaldehyde, a degradation product of RDX, was tested for mineralization under similar conditions using [^{14}C]-HCHO. Biotransformation of nitrite (5 mM) by growing fungi was conducted under similar conditions as used for RDX. The abiotic control was composed of [UL−^{14}C]-RDX and medium only. All tests were performed in duplicates.

Biotransformation of RDX, MNX and TNX by resting cells of HAW-OCF3

Strain HAW-OCF3 was first grown in eight 500 ml Erlenmeyer flasks, each containing marine fungal medium (125 ml) and RDX (100 μM). After 1 week of incubation, the fungal biomass was collected under aseptic conditions by vacuum filtration (Millipore vacuum filter, Billerica, USA; 0.22 μm) followed by three washings with 100 ml of NaCl (3.5% w/v). Biomass (wet weight 20.4 g; dry weight 1.6 g) was re-suspended in 100 ml aqueous NaCl solution (3.5% w/v) at pH 5. Aliquots of cell suspensions (5 ml) were subsequently added into serum bottles (20-ml) followed by the addition of RDX (100 μM). The serum bottles were then sealed with rubber caps and incubated on a rotary shaker (80 rpm) at room temperature. After certain time intervals (0, 1, 2, 3, 4, and 5 h) biomass was filtered off and the filtrate was analyzed for remaining RDX and its produced metabolites. Before opening the bottles the headspace was analyzed for gaseous products such as N_2O. To confirm the source of N_2O we prepared microcosms using the ring-labeled [^{15}N]-RDX under the same conditions described for RDX. Transformations of MNX (100 μM) and TNX (100 μM) were conducted, separately following similar incubation procedures as those used for RDX. Abiotic controls contained either RDX or MNX in autoclaved cell suspensions. All tests were carried out in duplicates.

Biotransformation of RDX in sediment slurry bioaugmented with strain HAW-OCF3

Sediment slurry was first prepared by mixing unsterilized wet sediment (10 g) with marine medium (25 ml) in 125 ml serum bottle followed by the addition of RDX (100 μM). The pH of sediment slurry was adjusted to 5 to be compatible with the fungal optimal growth condition or to 7 to mimic the actual pH found in seawater. In one case the sediment slurry was bioaugmented with the isolate HAW-OCF3 (205 mg wet biomass representing 16 mg dry weight biomass) and incubated on a rotary shaker (80 rpm) at room temperature. In another case the sediment slurry was not inoculated with HAW-OCF3. Abiotic controls contained autoclaved sediment and RDX. The growth of fungi in the non-sterilized microcosms was determined by visual observation of the formation of mycelia. RDX removal and formation of metabolites in the aqueous phase was monitored using the same procedures used for RDX transformation with strain HAW-OCF3 in marine liquid cultures (see below). All tests were carried out in duplicates.

Analyses of RDX and its products

Aliquots of the aqueous phase of RDX biotransformation medium was filtered through a 0.45-μm-pore-size Millex-HV filter. RDX and its reduced products MNX, DNX and TNX were analyzed by HPLC (Waters) fitted with a Supelcosil LC-CN column (4.6 mm ID × 25 cm) (Supelco) and a Model 996 photodiode array detector [19]. The mobile phase was a methanol/water gradient at

a flow rate of 1.5 ml min^{-1}. Analytes were identified by comparison with reference standards materials. Methylenedinitramine (MEDINA) was also analyzed by HPLC (Waters) but the column was an ICSep Ice-Ion-310 fast organic acid column (6.5 × 150 mm) kept at 35°C (Transgenomic, San Jose, CA, USA) equipped with a photodiode array detector [15]. The mobile phase was 2 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. Formaldehyde was analyzed as described previously as its 2,4-pentanedione derivative using an HPLC system fitted with a 5 μm Supelcosil LC-8 column (4.6 mm ID × 25 cm) (Supelco) maintained at 40°C and a FL3000 fluorescence detector (ThermoFinnigan, excitation at 430 nm and emission at 520 nm) [20]. The mobile phase was an acetonitrile–water gradient of 15–27%, at a flow rate of 1.5 ml min^{-1}. NO_2^- analysis was performed using a colorimetric method described by the Environmental Protection Agency (EPA) method 345.1 [14]. N_2O was analyzed by an Agilent 6890 GC system coupled to an electron capture detector (300°C) [20]. Analyses of $^{15}N^{14}NO$ (45 Da) and $^{14}N^{14}NO$ (44 Da) were carried out with a Hewlett Packard 5890 GC coupled to a 5970 quadrupole mass spectrometer. A GS-GasPro (30 m × 0.32 mm) capillary column (J & W Scientific) was used under splitless condition [33].

Results and discussion

Identification of RDX-degrading marine fungi

Four morphologically different marine aerobic fungi (HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5) were isolated from Hawaii sediment for their potential to degrade RDX under aerobic conditions. The fungi grew on peptone and yeast extract, and did not utilize RDX as either a carbon or a nitrogen source in basic salts medium.

The morphological and physiological features of HAW-OCF3 were consistent with those of filamentous fungi [22, 24, 28] (Table 1). Strain HAW-OCF3 had typical features of *Acremonium*: very thin (1–3 μm) septate mycelia, unbranched and separated spore-bearing structures (phialides) with rice-granule-shaped conidia (1–3 × 5–6 μm). Phylogenetic analyses of 18S rRNA gene sequences also showed HAW-OCF3 to be most similar to *Acremonium* sp. strain CSSF-1 (98.8% similar) [40] and *Acremonium*-like hyphomycete strain KR21-2 (99.8% similar) (Fig. 1) [26], thus the strain was identified as *Acremonium* sp. Strain CSSF-1 is a fungus found in symbiotic association with photoautotrophic *Chlolorella* [40] whereas strain KR21-2 is a Mn-oxidizing aquatic fungus found on river pebbles [26].

Strain HAW-OCF5 produced white colonies at an early stage (2 days) of growth but later turned blue–green. The fungus showed septate hyphae (2.5–4 μm) and formed round, unicellular and unbranched chains of conidia (2.5–5 μm in diameter) at the tips of the branched conidiophores, thus was considered to be similar to

Table 1 Morphological and biochemical properties of RDX-degrading marine fungi

	HAW-OCF1	HAW-OCF2	HAW-OCF3	HAW-OCF5
Colonial morphology (4 days old)				
Size (mm)	2–4	2–4	4–6	10–16
Pigmentation	Red	Cream	Off-white	Blue–green
Texture	Smooth	Smooth	Powdery	Rough
Appearance	Mucoid	Mucoid	Raised, filamentous	Cottony, filamentous
Cellular morphology (4 days old)				
Size (μm)	3–4 × 3–6	2–3 × 3–5	1–1.5 × 3–8	2–3 × 3–14
Shape	Ovoid	Ovoid	Filamentous	Filamentous
Cellular arrangement	Single, pair, chain	Single, pair	Mycelia with conidia	Mycelia with conidia
Budding/spore pattern	Multilateral	Unilateral	Conidia (2–3 × 4–6 μm)	Conidia (1.5–2 × 5 μm)
Mycelium appearance	Absent	Absent	Septate and branched	Septate and branched
Assimilation of carbon sources				
Galactose	–	+	+	+
Erythritol	–	–	+	+
D-Ribose	–	+	+	+
Maltose	–	+	+	+
L-Rhamnose	+	+	–	+
Cellobiose	–	+	–	–
Trehalose	+	+	–	+
D-Mannitol	–	+	+	+
Lactose	–	+	+	+
D-Xylose	–	–	+	+
Inositol	–	+	+	+

Absence and presence of growth are indicated by (–) and (+), respectively

Penicillium spp. Phylogenetic analyses of 18S rRNA gene sequence showed that strain HAW-OCF5 was most closely related to *Penicillium* spp. (*Penicillium chrysogenum*, 100%; *Penicillium italicum*, 99.9%) (Fig. 1).

Macroscopic, microscopic, and physiological features of isolates HAW-OCF1 and HAW-OCF2 (Table 1) were consistent with those described for unicellular fungi, i.e., yeast [4, 38]. Strain HAW-OCF1 was similar to *Rhodotorula* or *Rhodosporidium* by the characteristic red color of its colony, absence of ballistospores and by its inability to assimilate inositol [4]. Phylogenetic analyses of 18S rRNA gene sequence of strain HAW-OCF1, showed strain HAW-OCF1 to be most similar to *Rhodotorula* spp. (similarities: *Rhodotorula mucilaginoa*, 99.4%, *Rhodotorula glutinis*, 98.51%; *Rhodotorula graminis*, 97.8%) (Fig. 1). Therefore we identified HAW-OCF1 as a member of *Rhodotorula*. Finally strain HAW-OCF2 had non-aromatic and cream-colored colony, formed symmetrical ballistospores and produced starch. These features were consistent with those of the genus *Bullera* [4]. This strain also displayed the highest similarity by 18S rRNA gene sequence to *Bullera* spp. (*Bullera unica*, 99.3%; *Bullera alba*, 98.6%) (Fig. 1), suggesting its affiliation to *Bullera*.

Strains of *Rhodotorula* [11], *Bullera* [17], *Acremonium* [1, 29] and *Penicillium* [30] were previously isolated from

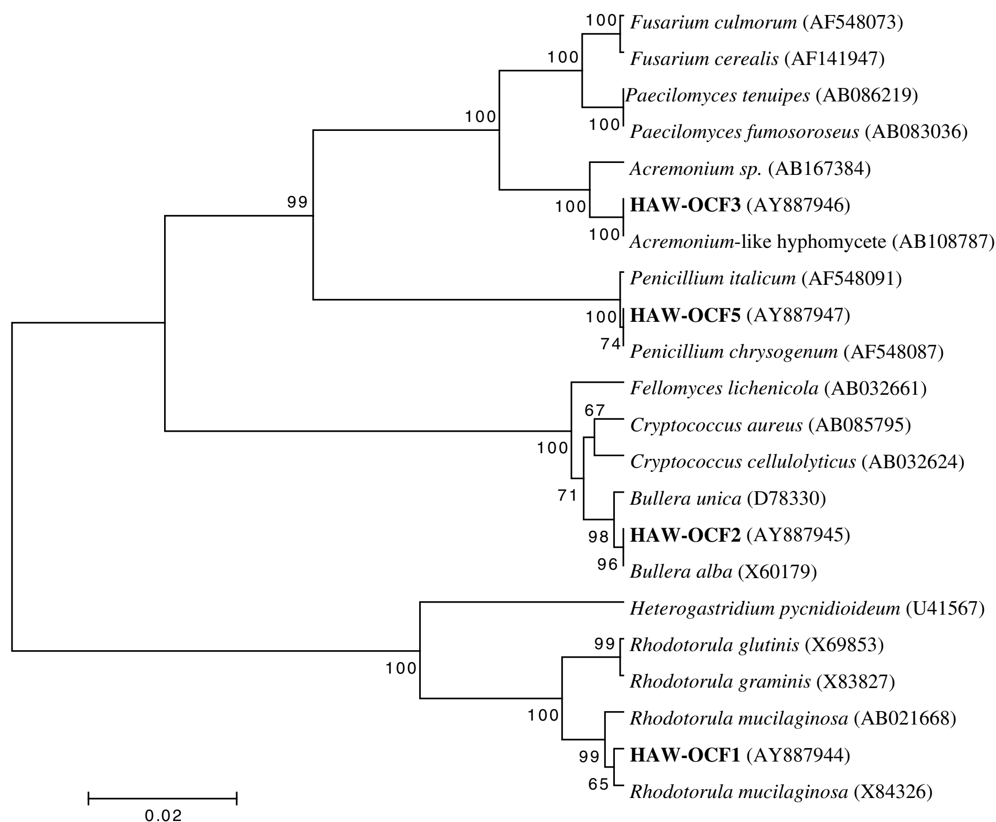

Fig. 1 Phylogenetic tree of 18S rRNA genes of fungal isolates from Hawaii sediment. The phylogenetic tree was generated based on pair wise nucleotide distance of Kimura 2-parameter using the Neighbor-joining method included in the MEGA2 software package. The *bar* indicates a difference of two nucleotides per 100 bases. In brackets are the GenBank accession numbers of the18S rRNA gene. The number of bootstrap repetitions was 4,000. The number beside the node is the statistical bootstrap value (values lower than 50 are not shown)

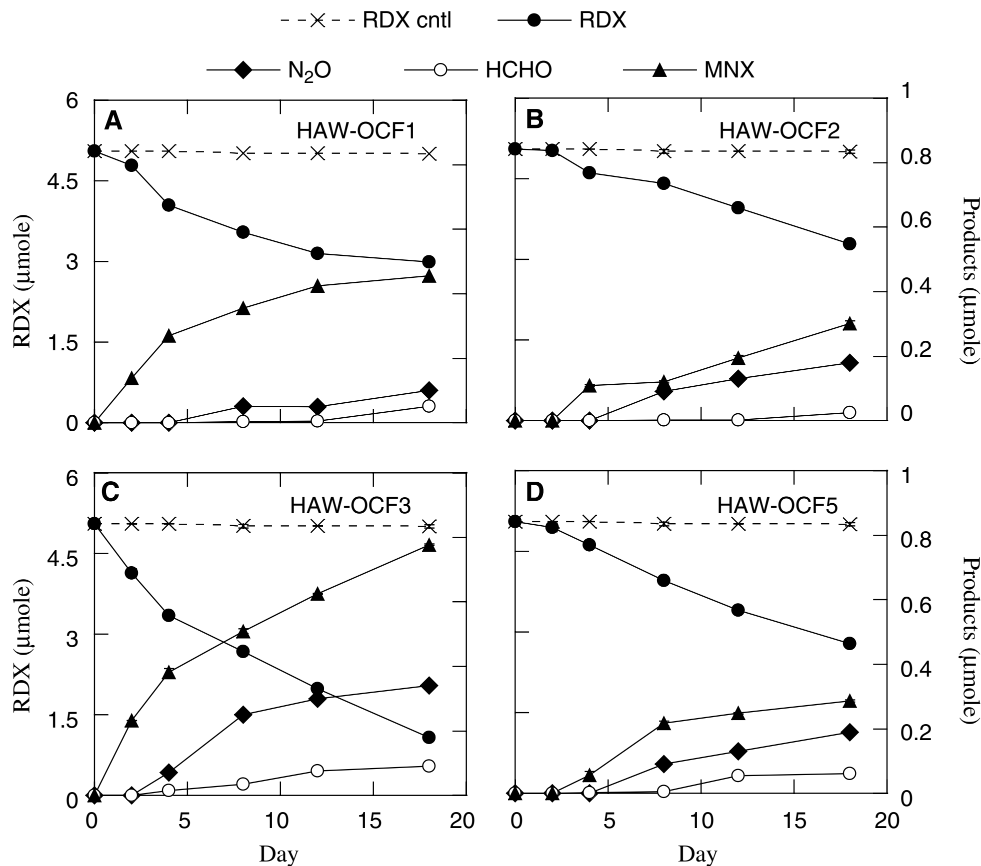

Fig. 2 Biodegradation of RDX by fungal isolates growing in marine fungal medium. The abiotic control was prepared using biomass killed by autoclaving

marine sources, but none was reported for its potential to degrade RDX. Other marine fungi such as *Candida* spp. and *Yarrowia lipolytica* were reported to biotransform 2,4,6-trinitrotoluene (TNT) [21] and hydrocarbons [45], respectively. Terrestrial *P. chrysosporium* was reported to degrade RDX and HMX under ligninolytic conditions [16, 33, 34], but the present RDX-degraders including *Acremonium* sp. HAW-OCF3 did not show any LiP or MnP activity.

Biodegradation of RDX and its metabolites by fungal isolates: insight into mechanisms of degradation

As shown in Fig. 2 the four marine fungal isolates HAW-OCF1, HAW-OCF2, HAW-OC3 and HAW-OCF5 degraded 40, 35, 75 and 45% of RDX in 18 days, respectively. The removal of RDX with growing cells of the fungi was accompanied by the formation of MNX, HCHO and N_2O (Fig. 2). Whereas Fig. 3 shows that the four isolates HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5 mineralized 15, 15, 34 and 24% of RDX, respectively after 58 days of incubation as determined by liberated $^{14}CO_2$. At the end of experiment (58 days) the carbon mass balance measured as the sum of $^{14}CO_2$ and water soluble ^{14}C containing compounds were relatively high and ranged from 89, 79, 83 to 70%, respectively, for HAW-OCF1, HAW-OCF2, HAW-OCF3 and HAW-OCF5. When [^{14}C]-HCHO was incubated with one of these isolates, namely, HAW-OCF3 more than 40% mineralization was obtained as determined by liberated $^{14}CO_2$. This indicated that RDX mineralization proceeded via the intermediary formation of HCHO. The abiotic control composed of [UL-^{14}C]-RDX and medium, did not show any degradation.

During RDX biotransformation with resting cells (wet weight 20.4 g l^{-1}; dry weight 1.6 g l^{-1}), we detected methylenedinitramine (MEDINA), $O_2NNHCH_2NHNO_2$, in addition to MNX, N_2O, HCHO and traces of DNX and TNX (Fig. 4a). It is known that MEDINA is unstable in aqueous solution and eventually decomposes to produce HCHO and N_2O [18]. Nitrite was detected but in trace amounts (0.3% of RDX removed). When uniformly ring-labeled [^{15}N]-RDX was incubated with the fungus we detected both ^{14}N–^{14}NO (*m*/*z* at 44 Da) (19.1%) and ^{15}N–$^{14}NO_2$ (*m*/*z* at 45 Da) (80.9%), suggesting that nitrous oxide originated mainly from ^{15}N to $^{14}NO_2$ group and to a lesser extent from the peripheral $^{14}NO_2$ groups.

When MNX was incubated with resting cells of HAW-OCF3 (wet weight 20.4 g l^{-1}; dry weight 1.6 g l^{-1}), we observed N_2O and HCHO (Fig. 4b). Likewise only traces of DNX and TNX were detected. No significant MNX removal occurred in controls containing heat-killed fungal biomass (Fig. 4b). Under the same conditions, strain HAW-OCF3 removed little TNX within 5 h, indicating its resistance to biodegradation (data not shown).

Based on product distribution and time courses shown in Fig. 4 we proposed the occurrence of at least

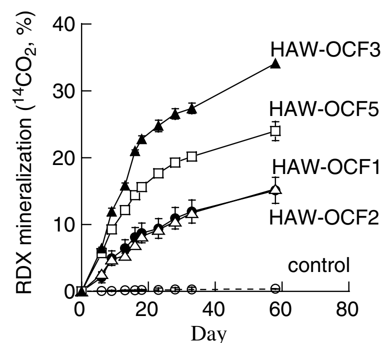

Fig. 3 [UL-^{14}C]-RDX mineralization by growing marine fungal isolates HAW-OCF1, HAW-OCF2, HAW-OCF3, and HAW-OCF5. Abiotic control was composed of [UL-^{14}C]-RDX and medium

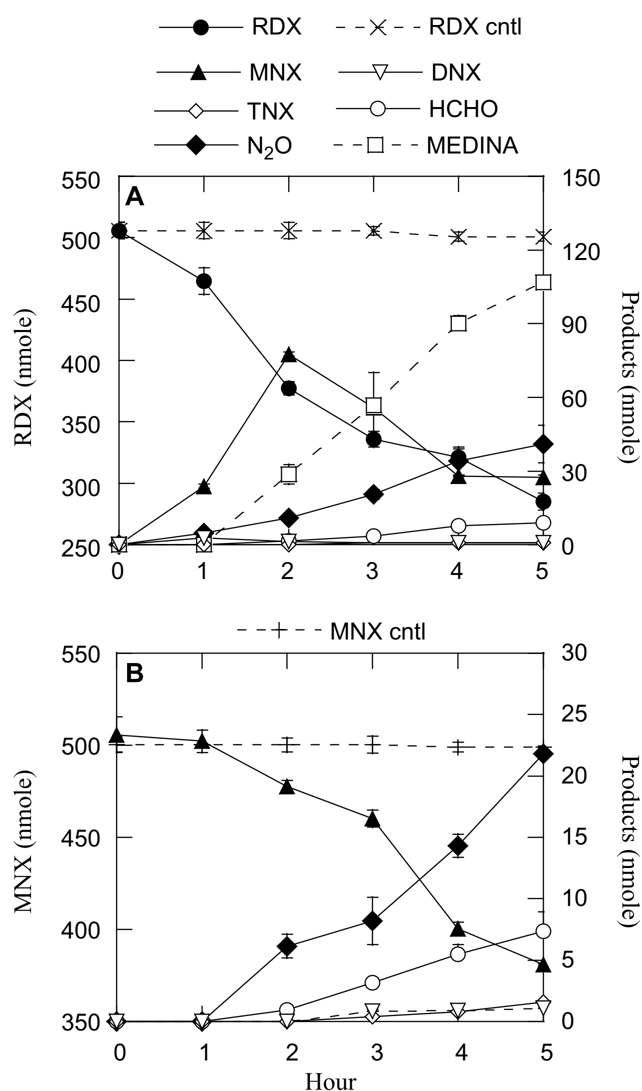

Fig. 4 Aerobic biotransformation of RDX (**a**) or MNX (**b**) by resting mycelia of isolate HAW-OCF3 suspended in 3.5% NaCl (5 ml, pH 5, biomass amount: 80 mg dry)

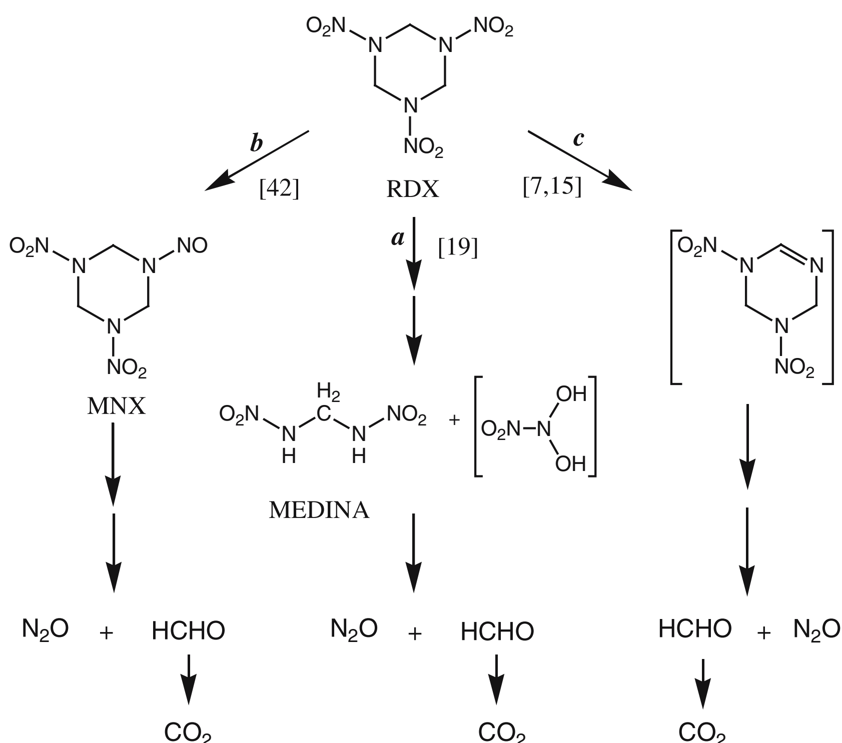

Fig. 5 Hypothetical pathways of aerobic RDX degradation in *Acremonium* sp. HAW-OCF3

two initial routes for the degradation of RDX; one route involved direct ring cleavage to form the key intermediate MEDINA (Fig. 5, *path a*) and another was suggested to involve reduction of RDX to MNX prior to ring cleavage (Fig. 5, *path b*). Direct ring cleavage of RDX was already reported during biodegradation of RDX with anaerobic sludge [19] whereas reduction to MNX prior to ring cleavage was reported with *Clostridium bifermentans* HAW-1 [42]. We were unable to detect nitrite by incubating RDX with growing cells of HAW-OCF3, but as we mentioned earlier traces of the anion (0.3% of RDX removed) were detected with resting cells. However, when $NaNO_2$ was treated with any of the above growing isolates, the NO_2^- anion disappeared, suggesting that degradation of RDX via initial denitration might have taken place but insignificantly (Fig. 5, *path c*). Initial denitration of the cyclic nitramine followed by ring cleavage has been previously reported to occur during aerobic biodegradation of RDX by *Rhodococcus* sp. strain DN22 [7, 15].

RDX degradation in sediment slurry bioaugmented with fungus HAW-OCF3

In live sediment slurry inoculated with fungal strain HAW-OCF3 at pH 5 or pH 7, RDX (100 µM) disappeared in both cases with similar rates. Figure 6 shows RDX removal in the slurry with pH 7 inoculated with 8.2 g l^{-1} of wet mycelium (representing 0.64 g l^{-1} dry weight) of strain HAW-OCF3. RDX removal in inoculated sediment was accompanied by the initial formation of mainly MNX (70%) and the two ring cleavage products N_2O and HCHO (Fig. 6). However, in the live sediment slurry without fungal inoculation, no RDX removal was found. We did not detect RDX removal in the non-inoculated control with sterile sediment. These results demonstrate that bioaugmentation of sediment

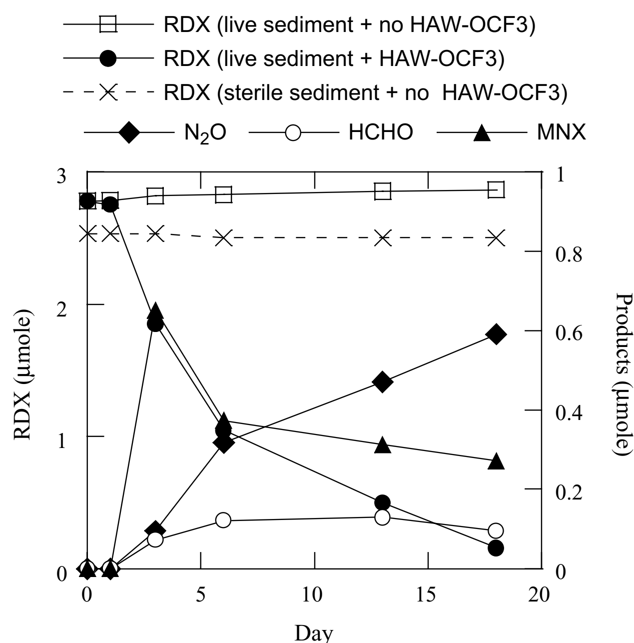

Fig. 6 Enhancement of RDX transformation in non-sterile sediment slurry by inoculation of fungal strain HAW-OCF3 at pH 7

with the marine fungus isolate HAW-OCF3 can be potentially used to remove RDX aerobically from contaminated marine environments.

Conclusion

The present study showed that Hawaii marine sediment contains fungi capable of aerobically biodegrading RDX to HCHO, CO_2 and N_2O through both direct ring cleavage and reduction to MNX prior to ring-cleavage.

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