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Development of a biodegradable ethylene glycol dinitrate-based explosive

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ABSTRACT

Bacteria capable of degrading ethylene glycol dinitrate (EGDN) were isolated under aerobic and, carbon and nitrogen-limiting conditions from EGDN contaminated soil and rivers. EGDN degradation was monitored using HPLC and UV–Vis spectrometer. Among the isolated strains *Bacillus subtilis* GN was the best, completely degrading 6.6 mM EGDN with the concomitant release of nitrite and EGMN within 72 h. Furthermore, the level of toxicity of EGDN as measured by the bioluminescent bacteria, *Vibrio fischeri* was reduced by 80% when 100% of the 6.6 mM EGDN was degraded. An environmentally friendly "biodegradable explosives", was achieved by adsorbing *B. subtilis* GN spores onto the wood flour, an ingredient of the explosive. The incorporation of *B. subtilis* GN spores into the explosive formulation did not affect the quality of the explosive as confirmed by the almost unchanged detonation velocity (3410 ms^{-1} compared to 3500 ms^{-1} of the control), autoignition temperature, Abel test, shock and friction sensitivity test. It was also possible to achieve rapid degradation of the residues after detonation upon exposure to air and moisture.

1. Introduction

Nitro group containing explosives (nitrate esters and nitro aromatics) form the most important group of explosives extensively produced and used worldwide in the mining, construction and military industries. The use of these compounds as explosives dates back to as early as the 1830s [1]. These explosives are used alone or as mixtures with other explosives. Among these explosives, ethylene glycol dinitrate (EGDN) also known as nitro glycol has been manufactured over the last 100 years [2]. EGDN-based explosive formulations are major causes of ecotoxicity in the environment while human exposure results in vasodilation with the subsequent effect of the development of throbbing headache or blood pressure decreases [1]. Despite the danger posed by EGDN-based explosives, especially the residues scattered over a wide area after detonation, or explosives which fail to detonate and persist long after intended periods in the soil, no remediation strategies have been developed except for the treatment of production effluents which has also be investigated in our lab [3]. Although a number of studies have shown the possibility of degrading other sister nitrate ester and nitroaromatic-based explosives [4–7], very few published reports have shown EGDN degradation by microorganisms [8,9]. Further, the available reports on EGDN degradation have just been limited to monitoring EGDN disappearance without attempting to investigate if the resulting products were none toxic. Whether environmental safety is improved by a particular waste treatment method or not, can only be determined by assessing the toxicological effects of treated material on living things [10]. In this work the efficiency of the degradation process is assessed for the first time using the bioluminescence toxicity testing method which uses *V. fischeri*. This is a well established, reliable, sensitive, standardized (EN ISO 11348) and fast method to assess the acute toxicity of chemicals [11]. Suitability of *V. fischeri* as a test organism to evaluate explosive degradation process has been reported for nitroaromatic explosives [6,12,13].

This work is therefore aimed at isolating effective EGDN degrading microorganisms as well as investigating the EGDN degradation process by the isolated microorganisms. This study also attempts for the first time, to monitor toxicity during the biodegradation of EGDN as well as exploring the possibility of incorporating EGDN degrading microorganisms into EGDN-based explosive formulations, to achieve rapid degradation of the explosive residues after detonation. The challenge is to make the microorganism effective

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soon after detonation in order to remove any residues resulting from explosion which pose both environmental and health problems.

2. Materials and methods

2.1. Chemicals and microorganisms

All chemicals were of analytical grade obtained either from Merck or Sigma–Aldrich. The explosive ethylene glycol dinitrate and ethylene glycol mononitrate were supplied by Austin Powder GmbH in St. Lambrecht, Austria. Bacteria capable of growing in the presence of EGDN were isolated from EGDN contaminated soils from St. Lambrecht, Austria and identified by German Collection of Microorganisms and Cell Cultures (DSMZ), Brauschweig, Germany.

2.2. Isolation of microorganisms

Soil samples (1 g) were added to 250 ml flasks containing basal salt medium (100 ml). The basal salt medium contained ($g l^{-1}$) K_2HPO_4 0.1, $(NH_4)_2 SO_4$ 0.4, $MgSO_4{\cdot}7H_2O$ 0.05, KCl 0.2, $CaCl_2{\cdot}2H_2O$ 0.005, $FeCl_2{\cdot}H_2O$ 0.005, 0.1 glucose dissolved in tap water was prepared. The flasks were incubated for a few days at 30 °C while shaking at 120 rpm. The samples were then subcultured on nutrient agar plates and incubated at 30 °C to isolate single colonies or obtain pure cultures.

2.3. Liquid culture conditions

For liquid cultures, bacteria were grown in a medium containing (per litre) K_2HPO_4, 3.5 g; KH_2PO_4, 2.0 g; $MgSO_4$, 0.22 g; glucose, 4.0 g; $CaCl_2{\cdot}2H_2O$, 2 g NH_4 clammonium chloride, supplemented with 5 ml salt medium and 6.6 mM EGDN final concentration. Experiments were performed in 250 ml conical flasks containing 100 ml medium. The inoculation of liquid cultures was done using an over night pre-grown culture. The cultures were then incubated at 30 °C while shaking at 120 rpm.

2.4. Biodegradation of EGDN by B. subtilis GN under different conditions

Unless otherwise specified, the medium and culture conditions describe above were used except for the parameter under investigation. To investigate if the organism was able to use EGDN as carbon or nitrogen source the nutrient under investigation (glucose or nitrogen) was substituted for EGDN in the medium described above. In addition the effect of glucose as a carbon source and ammonium chloride as a nitrogen source was investigated at different concentrations. Samples were regularly withdrawn and used to monitor EGDN degradation, nitrite release, biomass and another part extracted for HPLC analysis.

2.5. Monitoring EGDN degradation

2.5.1. HPLC analysis

Samples for HPLC analysis were removed at regular intervals by withdrawing 2 ml from the liquid cultures. The withdrawn samples were centrifuged at 13,000 rpm for 10 min. Part of the withdrawn samples (1 ml) was mixed with 20 μl sulphuric acid and incubated at room temperature for 20 min to precipitate the proteins, then centrifuged at 13,000 rpm for 10 min followed by filtering through a 0.2 μm pore-size solvent resistant paper. The filtrates were analyzed by HPLC System from Dionex© equipped with P580 pump, an ASI-100 auto sampler and a PDA-100 Photodiode Array Detector. The stationary phase was a reverse phase column, Hypersil HS C18 column (5 μm; 4.6 mm × 250 mm), and the mobile phase a 40:60 (v/v) methanol/H_2O deionised at flow rate of 1 ml min^{-1}.

2.5.2. Nitrite and nitrate analysis

The release of nitrite was monitored using the sulphanilamide method with slight modifications: 5 g sulphanilamide was dissolved in a 500 ml volumetric flask with 20 ml concentrated HCl and nitrite-free deionised water [14]. Further 0.5 g N-(1-naphtyl)ethylenediamine hydrochloride was dissolved in 500 ml nitrite-free deionised water. Both reagents were stored in a dark bottle at 4 °C. Shortly before the experiment sulphanilamide and N-(1-naphtyl)ethylenediamine hydrochloride were mixed in the ratio of 2:1, respectively. The culture samples were prepared by centrifugation at 13,000 rpm for 15 min to remove cells. The supernatant (200 μl) was mixed with 800 μl sulphanilamide: N-(1-naphtyl)ethylenediamine hydrochloride solution and incubated for 20 min at room temperature. Nitrite release was determined by monitoring the development of a pink colour at 540 nm using a Kontron UV–Vis spectrophotometer (Vienna, Austria). Nitrate production was analyzed by Spectroquant® Norvo400 at 340 nm, using a nitrate-cuvette test Kit from Merck.

2.6. Monitoring toxicity during EGDN biodegradation by B. subtilis GN

2.6.1. Growth conditions for V. fischeri

Toxicity testing was performed using *V. fischeri* 7151 (toxicity test of water and sewage samples DIN38412, Teil 34) obtained from DSMZ, Germany. The growth medium was as recommended by DSMZ, prepared as follows: 10 g beef extract and 10 g peptone were dissolved by heating in 250 ml tap water and pH adjusted to 7.8, further boiled for 10 min and again pH re-adjusted to 7.3 before adding 20 g agar. Artificial sterile sea water was prepared by dissolving 28.13 g NaCl, 0.77 g KCl, 1.60 g $CaCl_2{\cdot}2H_2O$, 4.80 g $MgCl_2{\cdot}6H_2O$, 0.11 g $NaHCO_3$, 3.50 g $MgSO_4{\cdot}7H_2O$ in 1000 ml distilled water. These were then autoclaved separately at 121 °C for 20 min. In case of the agar plates, 750 ml sterile sea water was then mixed with the 250 ml peptone–beef extract–agar solution after cooling down to 50 °C. Liquid medium was prepared by combining beef extract–peptone solution without agar with the artificial sea water after cooling to room temperature. The agar plates were incubated at 23 °C while the liquid culture was incubated at room temperature and 90 rpm for approximately 16–18 h before use.

2.6.2. In vitro toxicity test

Samples withdrawn at different intervals during EGDN biodegradation were centrifuged 13,000 rpm for 15 min to remove the cells and then mixed with a 16–18 h old fluorescing *V. fischeri* culture. Samples were prepared in triplicate with test population in special glass cuvettes at room temperature. At the end of 30 min exposure time, bacterial luminescence was measured using a photomultiplier (Berthold Biolumat LB 9500 Luminescence analyzer, Wildbad, Germany). The percentage bioluminescence in samples was compared with the control.

2.7. Preparation of B. subtilis GN for incorporation into explosive and long-term viability studies

A sporulation medium containing ($g l^{-1}$) 8 g bacterial nutrient broth, 10 ml KCl (10%, w/v) 10 ml $MgSO_4$ (1, 2% w/v) and 0.5 NaOH (1 M) was prepared and autoclaved at 121 °C for 15 min. The medium was left to cool down to 50 °C before supplementing it with 1 ml $Ca(NO_3)_2{\cdot}4H_2O$; 1 ml MnCl (0.01 M) and 1 ml $FeSO_4$ (1 mM) [15]. The medium was then inoculated using *B. subtilis* GN culture in its exponential phase and further incubated at 30 °C until the late exponential phase. After which the cultures were then

supplemented with 50 g sterile wood flour to enable the organism to adsorb on to the flour and further incubated for 4 days while monitoring spore formation. Spore formation was monitored microscopically using malachite green dye technique. The cultures were harvested by carefully decanting excess medium and transferred into 1-l plastic sterile bottles and plugging with sterile non-adsorbent cotton wool before freezing them at −20 °C. The Labconco Freeze Dry System/FreeZone© 4.5 Litre Benchtop Model 77500 (Vienna, Austria) was used to freeze dry the samples. The freeze drier was used at a temperature of −48 °C and at a vacuum pressure of 3×10^{-4} mbar. The freeze-dried samples of *B. subtilis* GN immobilized on wood flour were transported to Austin Powder GmbH for incorporation into the explosive. Long-term survival of the microbes was assessed after lyophilisation and storage for up to 2 years by plating on agar plates and counting the colonies.

2.8. Incorporation of B. subtilis GN into the explosive formulation

The immobilized *B. subtilis* GN was incorporated into the explosive at Austin Powder GmbH, St. Lambrecht, Austria. The detonation of the explosive was evaluated using the standard detonation procedure which is normally done by the company. Aluminium sample collecting plates (5× 10 cm) were planted around the explosive shortly before detonation in order to trap detonation residues. In parallel, the detonation velocities, autoignition temperature, shock sensitivity test, friction sensitivity test and the Abel test were investigated. After detonation the residues trapped on the aluminium plates were recovered by washing with 10 ml of water and used to detect if *B. subtilis GN* survived the detonation process by plating 100 μl on nutrient broth agar plates. Similarly, 1 g of long-term stored (2 years) explosive was mixed with 10 ml of water followed by plating 100 μl onto agar plates. In both cases, recovered *B. subtilis* GN was used to inoculate liquid cultures as described above.

3. Results and discussion

3.1. Isolation of microorganisms, EGDN biodegradation and toxicity monitoring

A number of bacterial isolates growing on EGDN contaminated soil and river water were isolated. However, only four of the isolated strains identified as *B. subtilis* (and named *B. subtilis* GN), *Bacillus* sp., *Agrobacterium radiobacter* and *Serratia fonticola* were capable of degrading EGDN. Growth and EGDN degradation efficiency by these bacterial strains is shown in Fig. 1. Although the biomass of all the four microorganisms was not affected by the concentration of EGDN used, the degradation efficiency varied significantly. *B. subtilis* GN was the most efficient degrading 80% of the 6.6 mM EGDN after 72 h.

Although *A. radiobacter* was capable of effectively degrading glycerol trinitrate [16], it was not effective in transforming EGDN in this study. Nevertheless, Ramos et al. [8] reported mineralization of EGDN by a mixture of *Arthrobacter ilicis* and *Arthrobacter radiobacter* resulted in mineralization. It was found out that *A. ilicis* was responsible for initiating the degradation while *A. radiobacter* facilitated mineralization. The biodegradation of EGDN was shown to progress by elimination of the nitro groups from the organic molecule to generate ethylene glycol, which was then mineralized [8]. The observed accumulation of EGMN confirms the ability to remove nitrite from EGDN. Nonetheless, the accumulation of EGMN

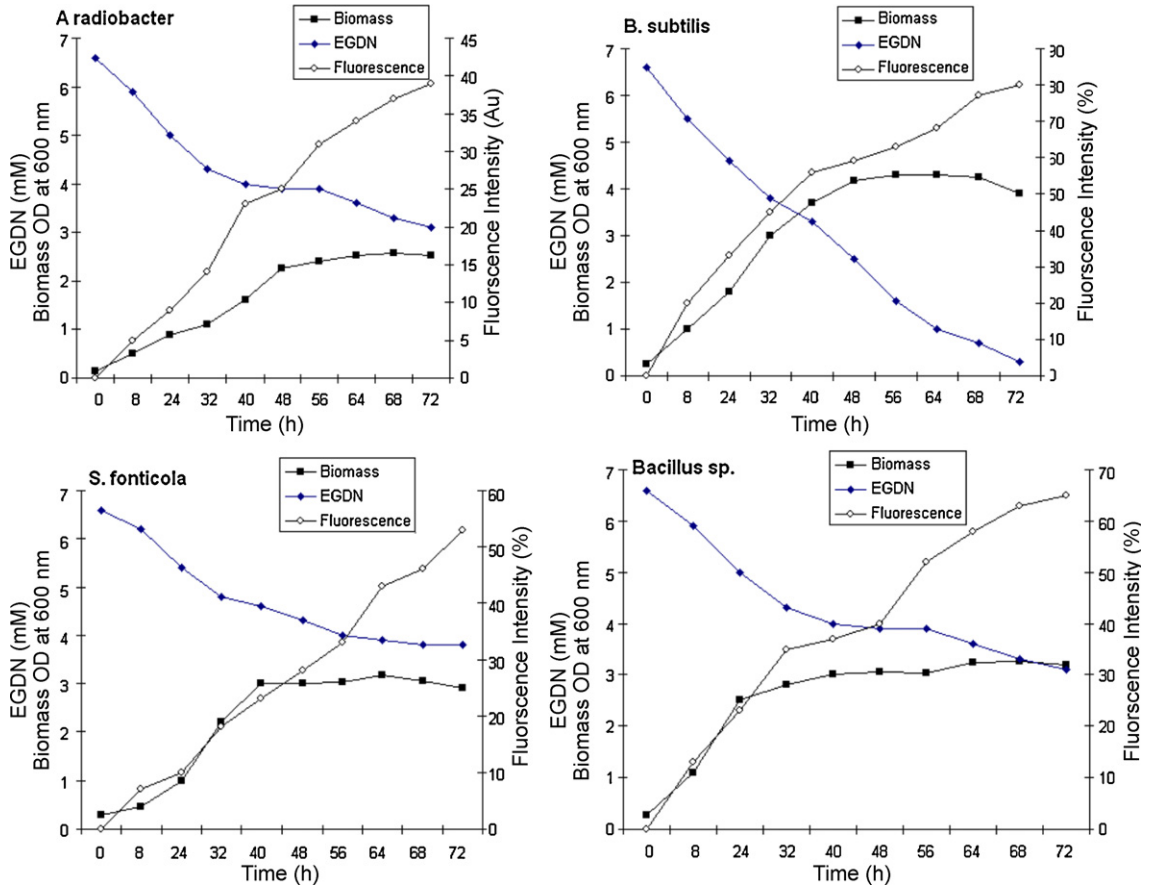

Fig. 1. Shows the growth and EGDN biodegradation activities of the respective bacterial strains isolated from EGDN contaminated sites and the acute toxicity measurements. Results are an average of three independent experiments with a standard deviation of ±3.

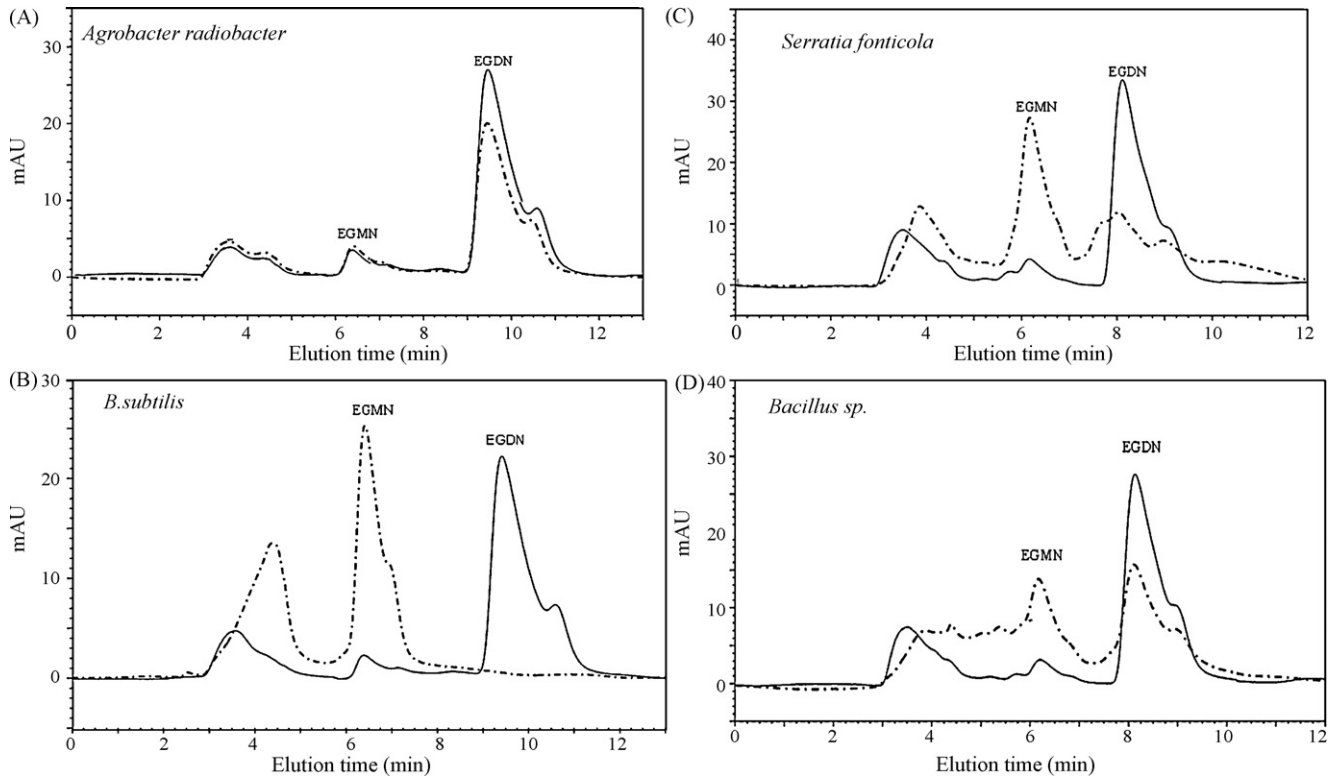

Fig. 2. Degradation of 6.6 mM EGDN and the release of EGMN by (A) *A. radiobacter*, (B) *B. subtilis* GN, (C) *S. fonticola* and (D) *Bacillus* sp.

as a dead end metabolite is certainly bad news and future efforts are directed at discovering new effective microorganisms. However, acute toxicity studies showed a progressive decrease in toxicity as evidenced by the increase in fluorescence intensity with increasing incubation time (Fig. 1). Fluorescence emission by *V. fischeri* was total inhibited during the first 1 h of incubation in all cultures. However, 80% of the bioluminescence was restored when all the 6.6 mM EGDN was transformed in *B. subtilis* GN cultures (Fig. 1). In cultures were biodegradation of EGDN was poor, inhibition of fluorescence was high. This study therefore demonstrates that indeed toxicity is reduced during biodegradation of EGDN. In a similar previous study, TNT degradation resulted in less toxic products as compared to the parent compound [14].

3.2. Degradation products

EGDN degradation activity resulted in the accumulation of ethylene glycol mononitrate (EGMN) in *Bacillus* sp., *B. subtilis* GN and *S. fonticola* cultures (Fig. 2). Since degradation of nitrite ester explosives has been reported to result predominantly in denitration activity releasing nitrite [16–18], the release of this compound was also monitored in this study (Fig. 3). High nitrite concentrations (2.56 mg l^{-1}) in *B. subtilis* GN cultures were detected during the first hours of incubation (Fig. 3). Interestingly the nitrite disappeared to insignificant levels after further 3 h of incubation in all cultures. The rapid increase in nitrite in the medium was also accompanied by a rapid decrease in EGDN.

3.3. Effect of glucose and nitrogen on EGDN degradation by B. subtilis GN

Since in previous reports it was found that *Arthrobacter* was able to use ethylene glycol as a C source [19], it was decided to investigate if *B. subtilis* GN (as the best EGDN degrading microorganism) was able to first denitrate EGDN, use the released nitrogen compound as a nitrogen source and then ethylene glycol as a C source. As shown in Fig. 4, both glucose and ammonium chloride were necessary for effective EGDN degradation. In cultures supplemented with 4 g glucose, 98% EGDN was degraded as compared to only less than 4.5% in the absence of glucose (Fig. 4A). Similarly, in the presence of 2 g ammonium chloride, 95% EGDN was degraded (Fig. 4B). EGDN biodegradation and growth of *B. subtilis* GN was very poor in the absence of either glucose or ammonium chloride (Fig. 4). In all the cultures accumulation of EGMN was the major characteristic. Both glucose and ammonium chloride were also necessary for growth of the *B. subtilis* GN as evidenced by poor growth in their absence. However, in similar previous studies Accashian et al. [20] and Meng et al. [18] reported denitration of glycerol trinitrate, provided as the sole C and N source while Pesari and Grasso [21] reported the use of ethyl acetate as a growth substrate during the degradation of glycerol trinitrate.

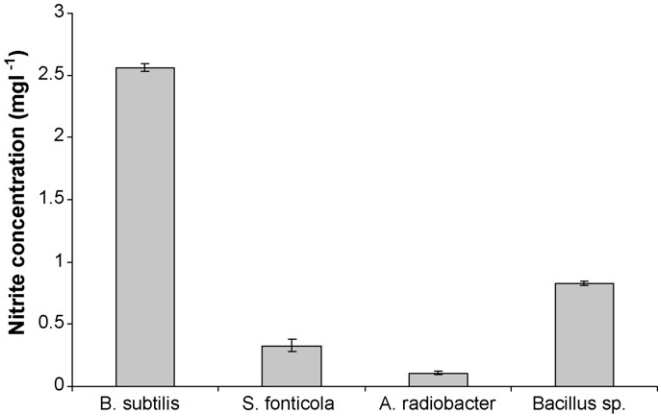

Fig. 3. Nitrite (mg l^{-1}) released during the incubation of 6.6 mM EGDN with the different microorganisms in their exponential phase.

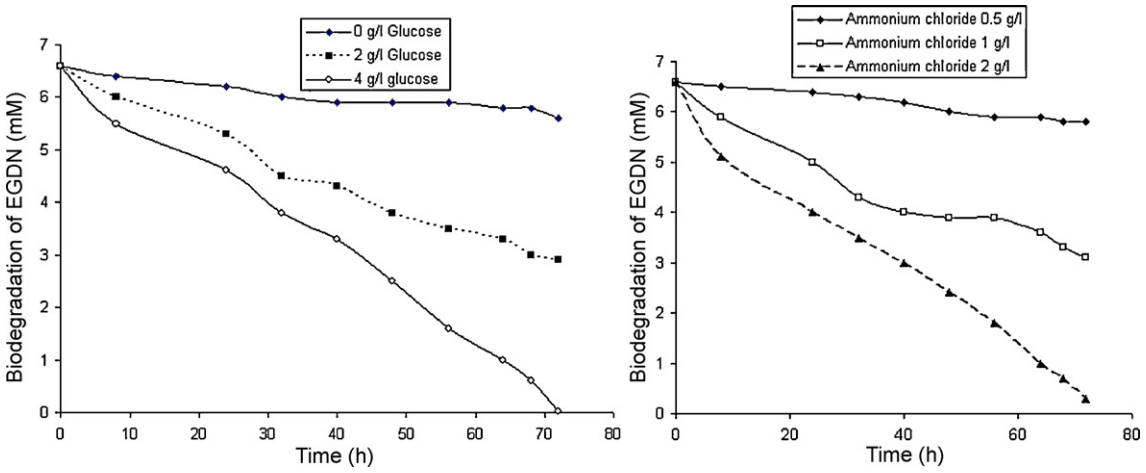

Fig. 4. Effect of different concentrations of glucose (0, 2 and 4 gl^{-1}) and ammonium chloride (0.5, 1 and 2 gl^{-1}) on EGDN degradation by *B. subtilis* GN. Results are an average of three independent experiments with a standard deviation of ±3.

3.4. Incorporation of B. subtilis GN into the explosive formulation and assessing its viability

B. subtilis GN spores immobilized on the woodflour were incorporated into an explosive at Austin Powder GmbH, St. Lambrecht, Austria. To investigate whether the incorporation of the microorganisms into the explosive formulation had an effect on the quality of the explosive, the detonation velocity which is one of the most important parameter of the explosive was investigated. This was compared to the reference, which in this case was the identical formulation without microorganism. The detonation velocity slightly decreased while in a previous study incorporation of *Pseudomonas putida* GG04 and *Bacillus* SF into TNT-based explosive led to a slight increase in the detonation velocity [22]. In this study, autoignition temperature, friction sensitivity and the Abel parameter remained unchanged after incorporation of *B. subtilis* GN spores (Table 1). The shock sensitivity (after 1 week of storage) decreased while after 4 years of storage this parameter was 5 J. In general, for this particular formulation this parameter varies between 2 and 5 J in commercial batches. This therefore indicates that the incorporation of microorganisms or their parts in to nitroaromatic or nitrate ester explosives does not negatively affect the explosive quality.

B. subtilis GN was recovered from residues sticking onto the metal plates planted around the explosives before explosion and plated on agar plates leading to 29.8×10^7 colony forming unit (CFU) per ml recovered from washing the plates as compared to 33.5×10^7 CFU in the explosive before detonation. Similarly, a CFU of 27.7×10^7 per ml of *B. subtilis* GN was recorded from the explosives after 2 years of storage at room temperature. Both recovered and stored *B. subtilis* GN samples were able to degrade 61% and 54% of 6.6 mM EGDN, respectively after 72 h. Nyanhongo et al. [22] reported recovery of 28.5×10^5 CFU g^{-1} of *Bacillus* SF after 5 years of storage of the explosive at room temperature. Nevertheless, in a similar study, *P. putida* GG04 was inactive after 2 years of storage [22].

B. subtilis GN has therefore been shown to efficiently degrade up to 6.6 mM EGDN. Although a number of microorganisms were observed to grow in the presence of EGDN, a few organisms were able to degrade it. In similar previous studies *A. radiobacter* was shown to grow in the presence of EGDN but was not involved in its degradation [8]. EGDN biodegradation like in previous studies by Ramos et al. [8] resulted in the release of nitrite. The observed accumulation of EGDN may indicate that this is a dead end metabolite in the degradation process and therefore a need to work with those strains to effectively degrade EGDN or continue with the screening study. However, the acute toxicity tests with *V. fischeri* which showed a significant decrease in toxicity are quite encouraging. This is the first time that the degradation of EGDN has been proved to lead to non-toxic metabolites. *B. subtilis* GN was also shown to be able to use the released nitrite as a source of its nitrogen thereby incorporating back the nitrogen into its natural cycle. The fact that *B. subtilis* GN could neither use nor convert nitrate contradicts the mechanism proposed by White and Snape [23], who postulated that whole cells achieved denitration through hydrolytic cleavage of the nitrate ester followed by reduction of the resulting nitrate to nitrite by a nitrate reductase. In addition to its degradation capabilities, the natural attributes of *B. subtilis* GN (spore formation) makes this organism the best candidate for incorporation into the explosive formulation. This ensures long-term stability and survival of the organisms at the same time guarantee successful degradation of explosive residues resulting from detonation.

Summarizing our results, a number of microorganisms able to degrade EGDN were isolated. EGDN biodegradation varied from one bacterium to the other. The best EGDN degrading microorganisms

Table 1
The effect of incorporating *Bacillus subtilis* GN in the explosive mixture on different explosives parameters.

Properties tested	Formulation			
	Control 1 week storage	Containing *B. subtilis* GN 1 week storage	Containing *B. subtilis* GN 4 years storage	Minimum requirement[a]
Detonation velocity [m s^{-1}]	3500	3530	3410	–
Shock sensitivity [J]	5	3	5	2
Friction sensitivity [N]	360	360	360	288
Abel test [min]	>60	>60	>60	30
Autoignition temperature [°C]	>300	>300	>300	180

[a] According to internal company standards.

resulted in release of nitrite and accumulation of EGMN. Nevertheless, the degradation process was shown to result in a significant decrease in toxicity as measured with *V. fischeri*. The study also showed for the first time that *B. subtilis* GN spores could survive in an EGDN-based formulation allowing immediate EGDN transformation under appropriate conditions. This strategy of incorporating microorganisms or their parts into an explosive formulation is a very attractive option especially for formulations that are used in the mining and construction industry since incompletely detonated residues constitute a great occupational health hazard to workers. This study also shows that EGDN biodegradation products are less toxic, making it a very attractive alternative method for EGDN pollution control and prevention.

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