




Selecting the best electron donor and operational temperature for the rapid biotransformation of the insensitive munitions compound 2,4-dinitroanisole (DNAN) by anaerobic sludge

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ABSTRACT

2,4-Dinitroanisole (DNAN) is a toxic compound increasingly used by the military that can be released into the environment on the soil of training fields and in the wastewater of manufacturing plants. DNAN's nitro groups are anaerobically reduced to amino groups by microorganisms when electron donors are available. Using anaerobic sludge as the inoculum, we tested different electron donors for DNAN bioreduction at 20 and 30 °C: acetate, ethanol, pyruvate, hydrogen, and hydrogen + pyruvate. Biotic controls without external electron donors and abiotic controls with heat-killed sludge were also assayed. No DNAN conversion was observed in the abiotic controls. In all biotic treatments, DNAN was reduced to 2-methoxy-5-nitroaniline (MENA), which was further reduced to 2,4-diaminoanisole (DAAN). Ethanol or acetate did not increase DNAN reduction rate compared to the endogenous control. The electron donors that caused the fastest DNAN reductions were (rates at 30 °C): H₂ and pyruvate combined ($311.28 \pm 10.02 \mu\text{M}\cdot\text{d}^{-1}\cdot\text{gSSV}^{-1}$), followed by H₂ only ($207.19 \pm 5.95 \mu\text{M}\cdot\text{d}^{-1}\cdot\text{gSSV}^{-1}$), and pyruvate only ($36.35 \pm 2.95 \mu\text{M}\cdot\text{d}^{-1}\cdot\text{gSSV}^{-1}$). Raising the temperature to 30 °C improved DNAN reduction rates when pyruvate, H₂, or H₂ + pyruvate were used as electrons donors. Our results can be applied to optimize the anaerobic treatment of DNAN-containing wastewater.




Key words | aromatic amine, bioremediation, biotransformation, electron donor, emerging high explosive, nitroaromatic

HIGHLIGHTS

- DNAN reduction was utterly dependent on sludge's biological activity.
- Ethanol and acetate were ineffective electron donors compared to the endogenous condition.
- The best electron donors were H₂ combined with pyruvate, followed by H₂ only and pyruvate only.
- Increasing the temperature from 20 to 30 °C was a major game-changer when H₂, pyruvate, or H₂ + pyruvate were used as electron donors.

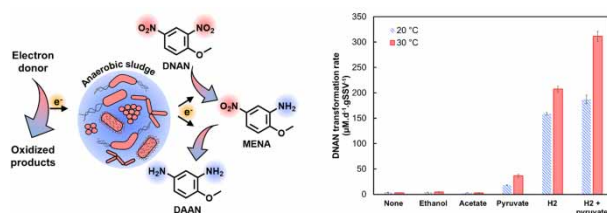
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GRAPHICAL ABSTRACT



INTRODUCTION

The munition industry has been replacing traditional explosives for insensitive high explosives compounds in the ordnance production for the military. This effort has resulted in the replacement of the traditional 2,4,6-trinitrotoluene (TNT) with 2,4-dinitroanisole (DNAN), which offers similar performance and more favorable properties, such as lower shock sensitivity and higher detonation temperatures, making the insensitive munitions compounds safer for manufacturing, transportation, and storage (Davies & Provatas 2006). As the use of insensitive munitions increases, industrial wastewater contaminated with these compounds will be produced during manufacture (Schroer 2018), which can be released into the soil, surface water and groundwater. Environmental contamination can also occur via incomplete or low-order detonations that scatter explosives on training ranges. Insensitive munitions are more likely to result in incomplete detonations than traditional explosives (Walsh *et al.* 2013), leaving centimeter-sized pieces of unexploded material on the ground of military training ranges (Taylor *et al.* 2015).

The water solubility of DNAN (276.2 mg·L⁻¹) is also higher than that of TNT (103.1 mg·L⁻¹) (Taylor *et al.* 2015) at 25 °C. Taylor *et al.* (2015) showed that DNAN is readily dissolved from insensitive munitions formulations by rainwater, being transported through the soil to groundwater. DNAN was found to be toxic to aquatic organisms, such as algae, cladocerans, and fish, and lethal to earthworms in soil (Dodard *et al.* 2013; Liang *et al.* 2013; Kennedy *et al.* 2015). Studies on rats have indicated that the acute toxicity of DNAN (50%-lethal dose of 199 mg·kg⁻¹) could be even higher than that of TNT (50%-lethal dose of 794–1,320 mg·kg⁻¹) (Davies & Provatas 2006). Therefore, there is a need to develop treatment methods for water and wastewater contaminated with DNAN.

To date, DNAN degradation has been studied using the Fenton oxidation process (Shen *et al.* 2013), alkaline

hydrolysis (Sviatenko *et al.* 2014), bimetallic reducing treatment (Koutsospyros *et al.* 2012), reduction by reactive minerals (Khatiwada *et al.* 2018), adsorption (Zhang *et al.* 2011), and biological transformation in soils (Hawari *et al.* 2015; Olivares *et al.* 2016) and sludges (Olivares *et al.* 2013). However, parameters for optimization of biological treatment have not yet been completely elucidated. DNAN is a nitroaromatic with two nitro groups (–NO₂). Due to the nitro groups' electron-withdrawing character, nitroaromatics are generally resistant to aerobic biodegradation (Amaral *et al.* 2009). Conversely, nitroaromatics can be used as electron acceptors by microorganisms under anaerobic conditions (Field *et al.* 1995). In this process, the nitro groups are reduced to amino groups (–NH₂). Such biotransformation via electron transfer processes catalyzed by microorganisms is also dependent on the presence of electron donors. Although the residual organic matter in sludge can supply a limited number of electrons for the reduction of the nitro groups, the use of external electron donors is expected to stimulate the microbes to produce more reducing equivalents for the process (Olivares *et al.* 2016; Jog *et al.* 2020), affecting the biotransformation kinetics.

Another crucial parameter influencing microbial metabolism rates is temperature (Nedwell 1999). The enzymatic mechanisms are highly dependent on temperature, interfering with the growth and substrate-utilization rates of microorganisms. Thus, there is a need to determine the efficiency of different electron donors and operational temperatures for DNAN biotransformation by anaerobic sludge.

In this study, we investigated anaerobic sludge's performance in reducing DNAN using different electron donors (ethanol, acetate, pyruvate, H₂, H₂, and pyruvate together, and sludge's residual organic matter) at two different temperatures (20 and 30 °C). Our results aimed to provide valuable information for the design of

biological treatments for DNAN-contaminated industrial wastewaters.

MATERIALS AND METHODS

Chemicals

2,4-Dinitroanisole (DNAN, $C_7H_6N_2O_5$, molecular weight: 198.13 g/mol, CAS # 119-27-7, 98% purity) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 2-Methoxy-5-nitroaniline (MENA, $C_7H_8N_2O_3$, molecular weight: 168.15 g/mol, CAS # 99-59-2, 98% purity) and 2,4-diaminoanisole (DAAN, $C_7H_{10}N_2O$, molecular weight: 138.17 g/mol, CAS # 615-05-4, analytical standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Inoculum and mineral medium

The inoculum was a mixture in a 1:2 proportion of an anaerobic–microaerophilic sludge from a laboratory-scale sequencing batch reactor treating textile effluent (Menezes *et al.* 2019) and an anaerobic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) treating petrochemical wastewater (Suape Port, Ipojuca, PE, Brazil), respectively. A sludge concentration of 1.5 g VSS L^{-1} was used in the microcosms.

The mineral medium used in this study was prepared as previously described (Menezes *et al.* 2021b) and composed of ($mg L^{-1}$): K_2HPO_4 (250), $CaCl_2 \cdot 2H_2O$ (10), $MgSO_4 \cdot 7H_2O$ (100), $MgCl_2 \cdot 6H_2O$ (100), NH_4Cl (280), $NaHCO_3$ (1100) and 1 mL L^{-1} of trace elements solution. The trace elements solution was composed of (mg/L): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (2000), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (50), $CuCl_2 \cdot 2H_2O$ (100), EDTA (1000), resazurin (2000), HCl 36% (1 mL/L). We used ultra-pure water in the preparation.

Anaerobic bioassays

The biotransformation of DNAN (150 μM) (Olivares *et al.* 2013) on anaerobic sludge microcosms was studied in the presence of five added electron donors, namely: pyruvate (1,000 $mg L^{-1}$), ethanol (500 $mg L^{-1}$), acetate (1,000 $mg L^{-1}$), hydrogen gas (1.5 atm overpressure in the headspace), and hydrogen gas (1.5 atm overpressure in the headspace) + pyruvate (200 $mg L^{-1}$). We also assayed a condition without added electron donors. All bioassays were

conducted in continuous mode in 100-mL serum flasks (microcosms) with a liquid volume of 80 mL, sealed with butyl rubber stoppers and aluminum caps. The liquid and the headspace in the bottles were flushed with a mixture of N_2/CO_2 (80:20, v/v) for 4 min to remove oxygen. The microcosms were kept in a glove box (818-GB, Plas-Labs, Lansing, MI, USA) filled with the same N_2/CO_2 mixture. The assays were performed at two different temperatures, $20 \pm 2^\circ C$ and $30 \pm 3^\circ C$. Abiotic controls with heat-killed sludge with and without added electron donors were also assayed. Heat-killed sludge was autoclaved for three consecutive days (1 h in the first and 30 min the following days). All assays were performed in triplicates.

Analytical methods

The samples were filtered in 0.22 μm membranes, diluted in acetonitrile (1:3), and immediately analyzed for DNAN, MENA, and DAAN using high-performance liquid chromatography (HPLC). A Shimadzu LC-20AT HPLC (Kyoto, Japan) coupled to a diode array detector (DAD) was equipped with a Zorbax Eclipse XDB-C18 column (5 μm , 4.6×250 nm) (Agilent, Santa Clara, CA, USA). The mobile phase consisted of acetonitrile (42% v/v) and H_2O with 5 mM phosphate buffer (58% v/v) running isocratically (0.6 mL min^{-1}) at $30^\circ C$. The injection volume was 50 μL . The method detected DNAN, MENA, and DAAN at 300, 254, and 210 nm, and 16.5, 12.0, and 5.5 min, respectively.

RESULTS AND DISCUSSION

Inoculum microbial community

The sludge used as inoculum in this work was the same one used as inoculum by Menezes *et al.* (2021b). Results of 16S rRNA sequencing of the inoculum can be found in Menezes *et al.* (2021b). Some of the most abundant genera in the inoculum are known to reduce nitroaromatic compounds. Some species of *Clostridium*, one of the most abundant genera in our inoculum, were observed to anaerobically biotransform TNT (Hughes *et al.* 1998) to 2,4-dihydroxylamino-6-nitrotoluene and the subsequent the formation of a polar product. Additionally, a consortium with *Methanobacterium* and *Clostridium* transformed several mono- and dinitroaromatic compounds into their corresponding amino derivatives (Gorontzy *et al.* 1993). Other genera found in our sludge also reported for the transformation of nitroaromatics were *Paenibacillus* (Khan *et al.* 2020),

Methanosaeta (Lin et al. 2013), *Bacteroides* (Carlier et al. 1997). Overall, the microbial community structure indicated that industrial sludge was a potential source of DNAN-reducing microorganisms. Thus, the next step was to investigate DNAN biotransformation by the sludge.

DNAN anaerobic biotransformation

The sludge was able to reduce DNAN without a lag phase. Such innate ability of the sludge to reduce the nitro group can be explained by the ubiquitous presence of nitro reductases in a wide variety of microorganisms. Nitro reductases are non-specific enzymes that carry electrons from the degradation of an electron donor to reduce nitro groups to amino groups (Spain 1995). Indeed, in all biotic conditions tested, DNAN was first reduced to 2-methoxy-5-nitroaniline (MENA), which resulted from the reduction of the nitro group in the ortho position (Figure 1). Then, the reduction of the MENA nitro group in the para position produced the aromatic amine 2,4-diaminoanisole (DAAN) as described by previous studies (Olivares et al. 2016). The minor formation of 4-methoxy-3-nitroaniline (iMENA) as a product of the reduction of DNAN's nitro group in the para position has also been reported, however, in abiotic systems using reactive minerals (Khatiwada et al. 2018). iMENA is not produced in quantifiable amounts by DNAN biological reduction (Olivares et al. 2016).

DNAN's reduction to MENA was faster than MENA's reduction to DAAN (Figure 2), probably because DNAN has two nitro groups, presenting a stronger electron-withdrawing character than MENA (with only one nitro group). The reduction of the first nitro group to an amino group in polynitrated compounds is known to imply a greater difficulty for the other nitro groups to be reduced (Van Beelen & Burris 1995). In all conditions, DAAN concentration fluctuated until it disappeared completely, impacting the mass balance. Previous works with DNAN's bioreduction also showed poor mass balances due to the formation of azo oligomers from the coupling reaction of DAAN and nitroso intermediates of DNAN's reduction. The azo oligomers can be reversibly reduced to DAAN, creating a fluctuation in DAAN's concentration (Olivares et al. 2016). DAAN can also react with quinone moieties of

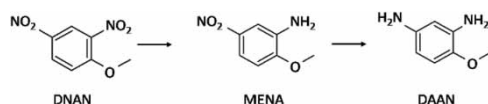


Figure 1 | Observed DNAN anaerobic biotransformation pathway in the sludge.

natural organic matter, getting irreversibly incorporated into humic-like polymers (Menezes et al. 2021a).

In our controls with heat-killed sludge, DNAN concentration was stable, not being reduced to MENA or DAAN. Olivares et al. (2016) reported that Fe(II)-containing minerals present in the sludge may still be active after biological inactivation and cause some reduction in nitroaromatic compounds. In our experiments, however, the process was utterly dependent on biological activity. The lack of any abiotic transformation in our study suggests that DNAN can be very persistent unless reducing biological activity is stimulated. In accordance with our study, Jog et al. (2020) showed that other insensitive munitions compound, 3-nitro-1,2,4-triazol-5-one (NTO), did not undergo abiotic transformation in anaerobic sludge either. Thus, since the biological activity is required, the next step was to determine which electron donor and temperature effectively stimulate the microorganisms.

Effects of different electron donors

The DNAN consumption data obtained during the experiment (Figure 2) could be adjusted to kinetic models of both zero and first order, with R^2 values higher than 0.82 and 0.86, respectively. The conditions with ethanol, acetate, pyruvate, or without added electron donors (endogenous) adjusted better to the zero-order kinetic model ($R^2 \geq 0.93$). This indicates that the production of reducing equivalents from the electron donor (residual organic matter in the sludge or pyruvate) to reduce DNAN, and not DNAN availability, was the limiting step for DNAN reduction. The conditions with H₂ and H₂ + pyruvate adjusted to a first-order kinetic model ($R^2 \geq 0.94$). A better fit in the first-order kinetic model indicates that the production of reducing equivalents from hydrogen exceeded the amount necessary for DNAN's reduction. Hence, the process became independent from the production of reducing equivalents and became dependent on the DNAN's concentration. However, since both kinetic models presented a proper fit to the data, both are presented in this study. The zero-order kinetic model results are shown in Figure 3 to illustrate DNAN specific consumption rate by the sludge, whereas Table 1 presents the first-order consumption rate and the corresponding half-life of DNAN in the sludge.

The endogenous condition (without electron donor) (Figure 2, panels A and B) reduced DNAN using the sludge's residual organic matter as the electron donor. Ethanol (Figure 2, panel C and D) and acetate (Figure 2, panel E and F) did not contribute e-donor in this sludge beyond

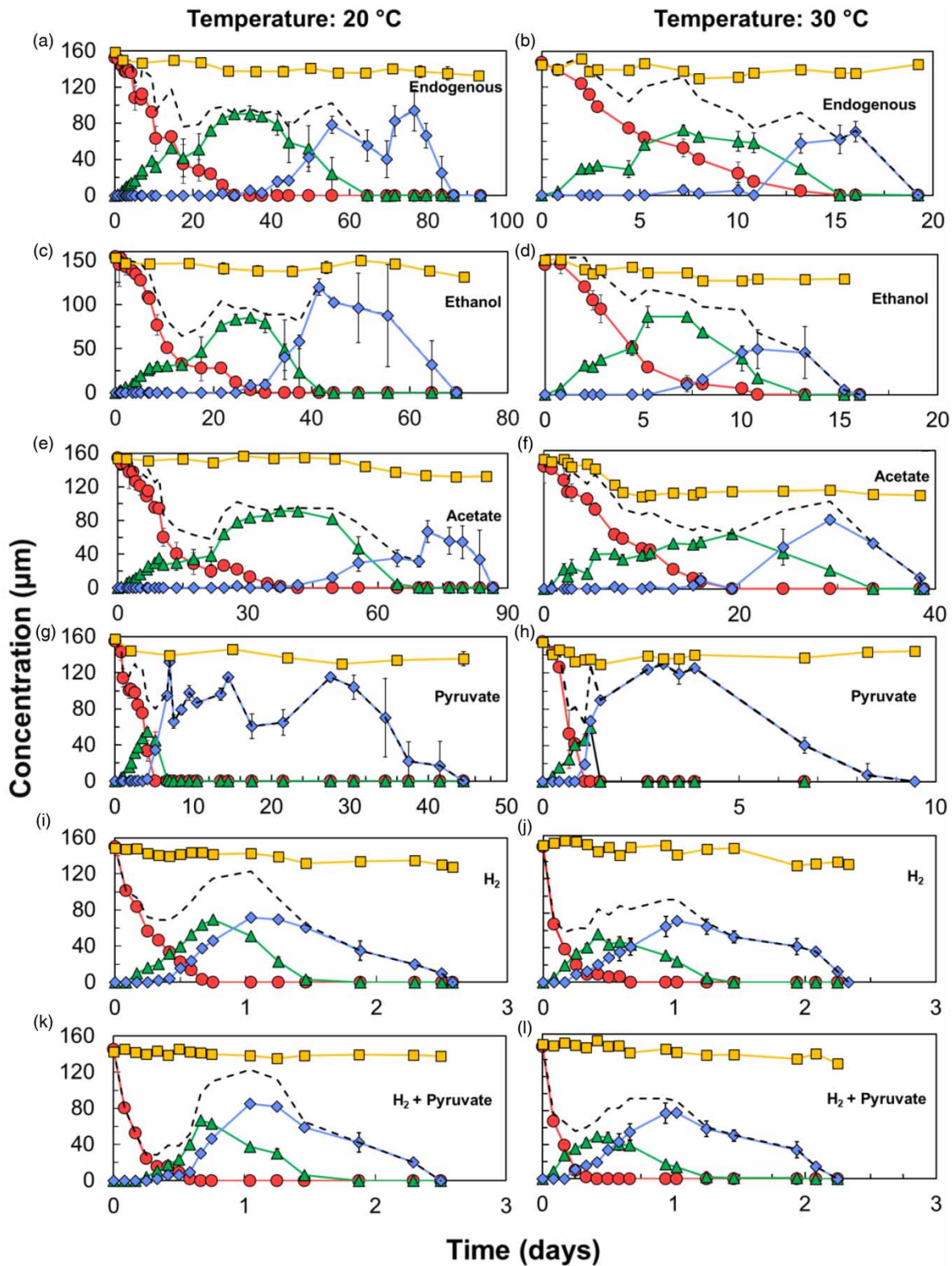


Figure 2 | DNAN reduction by sludge in different electron donor conditions. Legend: DNAN (●), MENA (▲), DAAN (◆), concentrations of DNAN, MENA, and DAAN summed (—), and DNAN concentration in the abiotic controls (■). Symbols represent the average of three replicates, and error bars represent the standard deviation.

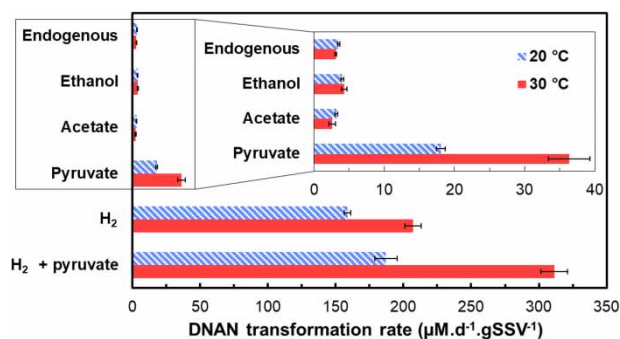


Figure 3 | Comparison of DNAN biotransformation rates in different electron donors expressed per dry mass of inoculated sludge.

what could be supplied by the endogenous treatment. Indeed, acetate was found to be not effective as an electron donor for nitro group bioreduction by previous studies (Donlon et al. 1996; Razo-Flores et al. 1999), in which the reduction of nitrobenzene or trinitrobenzene was not observed in anaerobic sediment when acetate was provided as an electron donor. An important and surprising empirical observation from our study was the inefficacy of ethanol as an electron donor since ethanol has been previously reported to work well as an electron donor for the reduction of TNT and 1,3,5-trinitro-1,3,5-triazinane (RDX) (Adrian et al. 2003), and NTO (Jog et al. 2020). Such results demonstrate the importance of using an effective electron donor to speed up DNAN anaerobic biological treatment. Differently from what was observed for the other organic electron donors, the addition of pyruvate (Figure 2, panel G and H) significantly accelerated the reduction of the nitro group compared to the endogenous condition (Figure 3).

DNAN reduction rates immensely increased when H₂ was used as the electron donor (Figure 2, panel I and J), being 88-fold faster than the endogenous treatment. H₂ seems to act as an immediate electron donor for the

biotransformation of DNAN. Other explosive compounds, such as RDX, TNT, and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), were also more readily reduced when H₂ was employed (Beller 2002; Adrian et al. 2003). H₂ was shown to be the direct electron donor for dichlorination processes, whereas other electron donors serve as precursors for hydrogen formation by the microbial communities (Distefano et al. 1992). Our results suggest that a similar process probably occurred during the reduction of DNAN by the anaerobic sludge, explaining the high efficiency of H₂ as an electron donor. Additionally, an interesting empirical observation of this study was that, although not affecting the overall duration of DNAN conversion to DAAN, the addition of a small concentration of pyruvate (200 mg L⁻¹) to the treatment with H₂ (Figure 2, panels K, L) significantly increased the reduction rate of DNAN to MENA compared with the treatment with H₂ only.

In addition, when assessing the overall duration of treatment, conditions with H₂ and H₂ + pyruvate as an electron donor required significantly less time, just 2.5 days at 30 °C. The endogenous treatment, and the treatments with ethanol, acetate, and pyruvate lasted 19, 16, 39 and 9 days, respectively, at 30 °C.

Effects of different temperatures

First, the bioassays were conducted at 20 °C, an average operational temperature broadly applied in industrial wastewaters treatment. Slow rates of biotransformation were observed. Thus, to maximize sludge's metabolic functions, the assays were also conducted at 30 °C. The temperature increase favored the processes, significantly improving DNAN biotransformation rate in the treatments with pyruvate, H₂, and H₂ + pyruvate. For the conditions with pyruvate, DNAN's consumption rates more than doubled.

Table 1 | First-order consumption rate (k_1) and corresponding half-life in all biotic conditions tested

Electron donor	Temperature = 20 °C		Temperature = 30 °C	
	k_1 (d ⁻¹)	Half-life (d)	k_1 (d ⁻¹)	Half-life (d)
Endogenous	0.09 ± 0.01	7.70 ± 2.64	0.08 ± 0.00	8.66 ± 0.00
Ethanol	0.09 ± 0.01	7.70 ± 1.93	0.12 ± 0.01	5.78 ± 1.47
Acetate	0.10 ± 0.01	6.93 ± 0.65	0.06 ± 0.01	11.55 ± 6.16
Pyruvate	0.25 ± 0.04	2.77 ± 0.02	0.58 ± 0.13	1.20 ± 0.61
H ₂	4.23 ± 0.29	0.16 ± 0.04	7.02 ± 1.27	0.10 ± 0.04
H ₂ + pyruvate	6.42 ± 0.10	0.11 ± 0.00	11.03 ± 1.22	0.06 ± 0.01

There was no DNAN reduction in the abiotic conditions.

In treatments with H₂ and H₂ + pyruvate, rates increased by about 1.5 with increasing temperature. For the other conditions (endogenous, with ethanol, and with acetate), the temperature increase did not present a positive effect.

Implications

The same sludge used as inoculum in this study was also shown to keep a sustainable removal of DAAN (the final aromatic amine from DNAN's reduction) under micro-aerobic conditions (Menezes et al. 2021b). This indicates that a complete treatment of DNAN-containing wastewater may be archived using the anaerobic sludge under a combination of anaerobic and micro-aerobic conditions, which can be further investigated.

Furthermore, the use of H₂ as an electron donor may seem a technical challenge at first. Nevertheless, Adrian & Arnett (2007), working with other nitroaromatic compounds, showed that this issue could be worked around using organic electron donors that allow the production of high concentrations of H₂ by microorganisms, such as propylene glycol. Future studies can be developed to optimize the application of H₂ (direct or biogenic) in DNAN-contaminated soils or bioreactors treating DNAN-containing wastewater. Future work can also evaluate the efficiency of the electron donors tested in this study for the full-scale treatment of DNAN-containing wastewater.

The application of nitroaromatics is not limited to the explosive industry. These compounds are also used in the pharmaceutical, pesticide, cosmetic, and dye industries. Although focused on the biotransformation of the emerging contaminant DNAN, this work's results can potentially contribute to optimizing the engineered biotransformation of different nitroaromatics, especially polynitroaromatic compounds.

CONCLUSIONS

The use of ethanol or acetate as electron donors did not accelerated the process compared to the endogenous condition. Among the conditions tested, the use of H₂ as an electron donor showed the best performance (88-fold faster than the endogenous treatment at 30 °C). Adding pyruvate as an organic carbon source to microcosms with H₂ accelerated the first step of DNAN's biotransformation (reduction to MENA), but it did not affect the overall process duration (complete reduction to DAAN). Pyruvate used alone was the only effective organic electron donor

tested. Finally, raising the temperature to 30 °C only caused a significant improvement when pyruvate, H₂, or H₂ + pyruvate were used as electron donors. The technical challenges involved in using H₂ as an electron donor can be worked around using organic substrates that can support the formation of high concentrations of biogenic H₂. The results can be applied in the optimization of the anaerobic treatment of DNAN-containing wastewaters.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

- Adrian, N. R. & Arnett, C. M. 2007 Anaerobic biotransformation of explosives in aquifer slurries amended with ethanol and propylene glycol. *Chemosphere* **66** (10), 1849–1856.
- Adrian, N. R., Arnett, C. M. & Hickey, R. F. 2003 Stimulating the anaerobic biodegradation of explosives by the addition of hydrogen or electron donors that produce hydrogen. *Water Research* **37** (14), 3499–3507.
- Amaral, H. I., Fernandes, J., Berg, M., Schwarzenbach, R. P. & Kipfer, R. 2009 Assessing TNT and DNT groundwater contamination by compound-specific isotope analysis and ³H-³He groundwater dating: a case study in Portugal. *Chemosphere* **77** (6), 805–812.
- Beller, H. R. 2002 Anaerobic biotransformation of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) by aquifer bacteria using hydrogen as the sole electron donor. *Water Research* **36** (10), 2533–2540.
- Carlier, J.-P., Sellier, N., Rager, M.-N. & Reyssset, G. 1997 Metabolism of a 5-nitroimidazole in susceptible and resistant isogenic strains of *Bacteroides fragilis*. *Antimicrobial Agents and Chemotherapy* **41** (7), 1495–1499.

- Davies, P. J. & Provatas, A. 2006 *Characterisation of 2, 4-Dinitroanisole: An Ingredient for Use in Low Sensitivity Melt Cast Formulations*. Science Defence Technology Organization, Edinburgh, Australia.
- Distefano, T. D., Gossett, J. M. & Zinder, S. 1992 Hydrogen as an electron donor for dechlorination of tetrachloroethene by an anaerobic mixed culture. *Applied and Environmental Microbiology* **58** (11), 3622–3629.
- Dodard, S. G., Sarrazin, M., Hawari, J., Paquet, L., Ampleman, G., Thiboutot, S. & Sunahara, G. I. 2013 Ecotoxicological assessment of a high energetic and insensitive munitions compound: 2,4-dinitroanisole (DNAN). *Journal of Hazardous Materials* **262**, 143–150.
- Donlon, B. A., Razo-Flores, E., Lettinga, G. & Field, J. A. 1996 Continuous detoxification, transformation, and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering* **51** (4), 439–449.
- Field, J. A., Stams, A. J., Kato, M. & Schraa, G. 1995 Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie Van Leeuwenhoek* **67** (1), 47–77.
- Gorontzy, T., Küver, J. & Blotevogel, K.-H. 1993 Microbial transformation of nitroaromatic compounds under anaerobic conditions. *Microbiology* **139** (6), 1331–1336.
- Hawari, J., Monteil-Rivera, F., Perreault, N. N., Halasz, A., Paquet, L., Radovic-Hrapovic, Z., Deschamps, S., Thiboutot, S. & Ampleman, G. 2015 Environmental fate of 2,4-dinitroanisole (DNAN) and its reduced products. *Chemosphere* **119**, 16–23.
- Hughes, J., Wang, C., Yesland, K., Richardson, A., Bhadra, R., Bennett, G. & Rudolph, F. 1998 Bamberger rearrangement during TNT metabolism by *Clostridium acetobutylicum*. *Environmental Science & Technology* **32** (4), 494–500.
- Jog, K. V., Sierra-Alvarez, R. & Field, J. A. 2020 Rapid biotransformation of the insensitive munitions compound, 3-nitro-1,2,4-triazol-5-one (NTO), by wastewater sludge. *World Journal of Microbiology and Biotechnology* **36** (5), 67.
- Kennedy, A. J., Laird, J. G., Lounds, C., Gong, P., Barker, N. D., Brasfield, S. M., Russell, A. L. & Johnson, M. S. 2015 Inter- and intraspecies chemical sensitivity: a case study using 2,4-dinitroanisole. *Environmental Toxicology and Chemistry* **34** (2), 402–411.
- Khan, M. S., Gao, J., Chen, X., Zhang, M., Yang, F., Du, Y., Munir, I., Xue, J. & Zhang, X. 2020 Isolation and characterization of plant growth-promoting endophytic bacteria *Paenibacillus polymyxa* SK1 from *Lilium lancifolium*. *BioMed Research International* **2020**, Article ID 8650957, 17 pp.
- Khatiwada, R., Root, R. A., Abrell, L., Sierra-Alvarez, R., Field, J. A. & Chorover, J. 2018 Abiotic reduction of insensitive munition compounds by sulfate green rust. *Environmental Chemistry* **15** (5), 259–266.
- Koutsospyros, A., Pavlov, J., Fawcett, J., Strickland, D., Smolinski, B. & Braid, W. 2012 Degradation of high energetic and insensitive munitions compounds by Fe/Cu bimetal reduction. *Journal of Hazardous Materials* **219–220**, 75–81.
- Liang, J., Olivares, C., Field, J. A. & Sierra-Alvarez, R. 2013 Microbial toxicity of the insensitive munitions compound, 2,4-dinitroanisole (DNAN), and its aromatic amine metabolites. *Journal of Hazardous Materials* **262**, 281–287.
- Lin, Y., Han, X., Lu, H. & Zhou, J. 2013 Study of archaea community structure during the biodegradation process of nitrobenzene wastewater in an anaerobic baffled reactor. *International Biodeterioration & Biodegradation* **85**, 499–505.
- Menezes, O., Brito, R., Hallwass, F., Florêncio, L., Kato, M. T. & Gavazza, S. 2019 Coupling intermittent micro-aeration to anaerobic digestion improves tetra-azo dye Direct Black 22 treatment in sequencing batch reactors. *Chemical Engineering Research and Design* **146**, 369–378.
- Menezes, O., Kadoya, W. M., Gavazza, S., Sierra-Alvarez, R., Mash, E. A., Abrell, L. & Field, J. A. 2021a Covalent binding with model quinone compounds unveils the environmental fate of the insensitive munitions reduced product 2,4-diaminoanisole (DAAN) under anoxic conditions. *Journal of Hazardous Materials* **413**, 125459.
- Menezes, O., Melo, N., Paraiso, M., Freitas, D., Florencio, L., Kato, M. T. & Gavazza, S. 2021b The key role of oxygen in the bioremoval of 2,4-diaminoanisole (DAAN), the biotransformation product of the insensitive munitions compound 2,4-dinitroanisole (DNAN), over other electron acceptors. *Chemosphere* **267**, 128862.
- Nedwell, D. B. 1999 Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. *FEMS Microbiology Ecology* **30** (2), 101–111.
- Olivares, C. I., Jidong, L., Abrell, L., Sierra-Alvarez, R. & Field, J. A. 2013 Pathways of reductive 2,4-dinitroanisole (DNAN) biotransformation in sludge. *Biotechnology and Bioengineering* **110**, 1595–1604.
- Olivares, C. I., Abrell, L., Khatiwada, R., Chorover, J., Sierra-Alvarez, R. & Field, J. A. 2016 (Bio)transformation of 2,4-dinitroanisole (DNAN) in soils. *Journal of Hazardous Materials* **304**, 214–221.
- Razo-Flores, E., Lettinga, G. & Field, J. A. 1999 Biotransformation and biodegradation of selected nitroaromatics under anaerobic conditions. *Biotechnology Progress* **15** (3), 358–365.
- Schroer, H. W. 2018 *Biotransformation and Photolysis of 2,4-Dinitroanisole, 3-Nitro-1,2,4-Triazol-5-one, and Nitroguanidine*. PhD thesis, University of Iowa, Iowa. <https://doi.org/10.17077/etd.rn31166x>.
- Shen, J., Ou, C., Zhou, Z., Chen, J., Fang, K., Sun, X., Li, J., Zhou, L. & Wang, L. 2013 Pretreatment of 2,4-dinitroanisole (DNAN) producing wastewater using a combined zero-valent iron (ZVI) reduction and Fenton oxidation process. *Journal of Hazardous Materials* **260**, 993–1000.
- Spain, J. C. 1995 Biodegradation of nitroaromatic compounds. *Annual Review of Microbiology* **49** (1), 523–555.
- Sviatenko, L., Kinney, C., Gorb, L., Hill, F. C., Bednar, A. J., Okovytyy, S. & Leszczynski, J. 2014 Comprehensive investigations of kinetics of alkaline hydrolysis of TNT (2,4,6-trinitrotoluene), DNT (2,4-dinitrotoluene), and DNAN (2,4-dinitroanisole). *Environmental Science & Technology* **48** (17), 10465–10474.

- Taylor, S., Dontsova, K., Walsh, M. E. & Walsh, M. R. 2015 [Outdoor dissolution of detonation residues of three insensitive munitions \(IM\) formulations](#). *Chemosphere* **134**, 250–256.
- Van Beelen, P. & Burris, D. R. 1995 [Reduction of the explosive 2,4,6-trinitrotoluene by enzymes from aquatic sediments](#). *Environmental Toxicology and Chemistry: An International Journal* **14** (12), 2115–2123.
- Walsh, M. R., Walsh, M. E., Taylor, S., Ramsey, C. A., Ringelberg, D. B., Zufelt, J. E., Thiboutot, S., Ampleman, G. & Diaz, E. 2013 [Characterization of PAX-21 insensitive munition detonation residues](#). *Propellants, Explosives, Pyrotechnics* **38** (3), 399–409.
- Zhang, J., Lin, X., Luo, X., Zhang, C. & Zhu, H. 2011 [A modified lignin adsorbent for the removal of 2,4,6-trinitrotoluene](#). *Chemical Engineering Journal* **168**, 1055–1063.

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