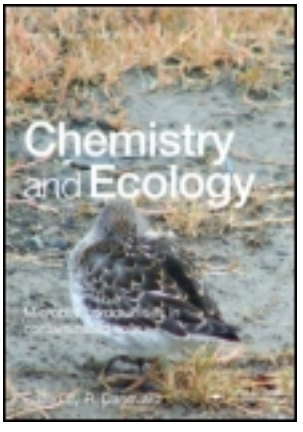

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gche20

Influence of 1,2,4,5-tetrazine derivatives on growth of bacterial consortium isolated from soil

Andrzej Borkowski [a], Mateusz Szala [b] & Dorota Wolicka [c]

[a] Department of Environmental Protection and Natural Resources, University of Warsaw, Warsaw, Poland

[b] Military University of Technology, Warsaw, Poland

[c] Department of Geochemistry, Mineralogy and Petrology, University of Warsaw, Warsaw, Poland

Available online: 16 Feb 2011

To cite this article: Andrzej Borkowski, Mateusz Szala & Dorota Wolicka (2011): Influence of 1,2,4,5-tetrazine derivatives on growth of bacterial consortium isolated from soil, Chemistry and Ecology, 27:1, 57-68

To link to this article: http://dx.doi.org/10.1080/02757540.2010.522998

Chemistry and Ecology
Vol. 27, No. 1, February 2011, 57–68

Taylor & Francis
Taylor & Francis Group

Influence of 1,2,4,5-tetrazine derivatives on growth of bacterial consortium isolated from soil

Andrzej Borkowski[a]*, Mateusz Szala[b] and Dorota Wolicka[c]

[a]*Department of Environmental Protection and Natural Resources, University of Warsaw, Warsaw, Poland;* [b]*Military University of Technology, Warsaw, Poland;* [c]*Department of Geochemistry, Mineralogy and Petrology, University of Warsaw, Warsaw, Poland*

(*Received 26 April 2010; final version accepted 13 September 2010*)

This article presents the results of laboratory studies of the influence of tetrazine derivatives on the growth kinetic parameters of soil bacteria. 3,6-Dihydrazinotetrazine (DHTz), 3,6-*bis*(3,5-dimethylpyrazol-1-yl)-dihydro-1,2,4,5-tetrazine (DMPDHT) and *N*,*N*′-*bis*(1,2,4,5-tetrazine-6-(3,5-dimethylpirazylo))hydrazine (BDMPT) were applied. 3,6-Dihydrazinetetrazine had the largest influence on the growth of bacteria, reflected in a significant lengthening of the lag-phase and a decrease in the specific growth rate. Dehydrogenase activity was also determined in bacterial cultures exposed to tetrazine derivatives. Dehydrogenases remained active even at DHTz concentrations of 80 mg $\cdot$ L^{-1}, which completely inhibited bacterial growth. The compounds studied variously influence the kinetics of growth in the bacterial consortium; at the same time, they undergo biodegradation in soil by autochthonous microflora.

Keywords: tetrazines; high-energy materials; bacterial activity

1. Introduction

Industrial development and the accompanying technical progress are linked to the appearance of various organic and inorganic compounds not present under natural conditions. Chemical compounds that are intermediate products of various technological processes accumulate after utilisation in soil or water in an unchanged state or are transformed into other compounds, thus becoming anthropogenic pollutants.

The search for new high-energy materials is one of the most important topics of fundamental investigation in military and civilian areas. The use of such materials by armies is obvious, but civil applications of explosives have increased in recent years. Explosives are the molecular containers of chemical energy, which can be used at any moment and at any time. The oilpatch (petroleum perforators) and automotive (airbags) industries are particularly interested in high-energy and high-nitrogen compounds. For the correct operation of an airbag during a traffic accident, several litres of gases must be evolved within a very short time ($\sim$10 ms). This effect can be gained only by using a molecular explosive or special pyrotechnic mixtures. High-nitrogen compounds

*Corresponding author. Email: andrzejborkowski2@gmail.com

ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757540.2010.522998
http://www.informaworld.com

combine the properties of pyrotechnic gas generators with a very high speed of decomposition of molecular explosives. For example, during a car crash, a mixture based on a high-nitrogen compound is ignited by an impulse from a gauge placed in the bumper and harmless nitrogen is transferred to a nylon bag. However, the compounds mentioned are environmentally friendly as opposed to classical gas generators that involve sodium azide which is very toxic.

We are normally unaware of the ubiquity of explosives. They are used as ingredients in special pyrotechnics (airbags and to increase the tension in pyrotechnic-powered seat belts in cars, library fire extinguishers, etc.) [1], in industry for explosion welding, cutting, reinforcing metals and in strictly military applications.

Typical military explosives are organic compounds which contain a nitro group (-NO_2) connected to carbon, nitrogen or oxygen atoms. Commonly known explosive compounds from each group are: 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) and 1,2,3-propanetriol trinitrate (NG). These compounds are built up of carbon, hydrogen, nitrogen and oxygen, and have a relatively small oxygen content which during intermolecular oxidation leads to the production of elemental carbon. When some carbon atoms in explosive molecules are replaced by nitrogen and the amount of the other elements in the compound remains constant, elemental carbon does not occur because it can be fully oxidised to carbon dioxide. Full gasification of the explosive results in better utilisation of the chemical energy stored in the compound molecule and gives higher pressures. More energetic materials can be used in lower quantities, which reduces costs and is environmentally friendly.

A high nitrogen content gives compounds with a high density, a high positive enthalpy of formation and a low hydrogen content. During intermolecular combustion, nitrogen-rich compounds react without an additional oxidant and the rates of gas evolution are very high. Combustion of the above-mentioned compounds occurs without flames and ash [2].

Organic compounds known commonly as ‘high-nitrogen compounds’ are represented in many chemical groups, such as tetrazines (and their N-oxides), tetrazoles, furoxanes and furazanes (Figure 1). Derivatives of symmetric tetrazine have good thermal and chemical stability, e.g. 3,3′-azobis(6-amino-1,2,4,5-tetrazine) and 2,6-dihydrazinotetrazine, and represent high-energy compounds [3–5].

Military requirements for the storage of explosives require stability without any undesirable transformations for a minimum of 25 years. The explosives in ammunition, missiles, bombs and torpedoes can be isolated from the chemical agents in many ways, but separation from bacteria is practically impossible. For this reason, bacteriological investigations of explosives and their intermediates are legitimised, because in extreme cases bacterial growth can lead to damage of a weapon or make it dangerous to use.

Nitrocompounds, e.g. nitrobenzene, nitrophenol and TNT, are produced on a massive scale [6]. They are used in the production of polyurethanes, herbicides, insecticides and explosives.

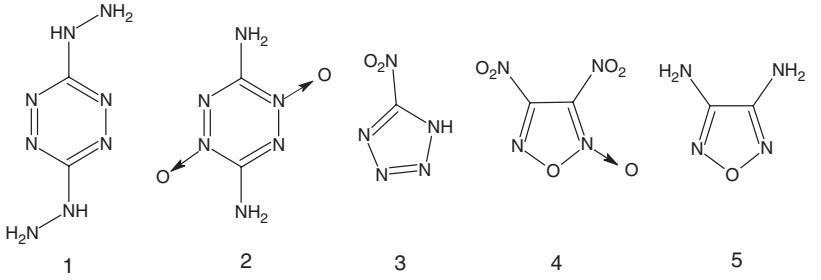

Figure 1. Examples of high-nitrogen compounds: tetrazine (3,6-dihydrazinotetrazine) (1), tetrazine N-oxide (2), tetrazole (3), furoxan (4), and furazan (5).

All of these compounds are toxic and pose a health hazard to humans; some are poisonous. The biodegradation of chemical compounds used as explosives has been studied in TNT, RDX, 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane (HMX) and 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaizowurtzitane (CL-20) [7–9]. Biodegradation of these compounds by various bacteria was noted under both aerobic and strictly anaerobic conditions, e.g. in bacteria representing *Pseudomonas*, *Clostridium* and *Desulfovibrio* [10,11].

This study focused on evaluating the influence of selected derivatives of 1,2,4,5-tetrazine on the growth of bacteria isolated from soil. The question is how these compounds affect the kinetic parameters of bacterial growth and metabolic activity? These compounds have not been studied in detail with regard to their interactions with microorganisms; only a few articles indicate possible antibacterial properties of compounds containing a tetrazine ring [12,13]. 3-Amino-6-(3,5-dimethylpyrazol-1-yl)-1,2,4,5-tetrazine (ADMPT) is useful as a precursor in the synthesis of compounds having antibiotic, herbicidal, fungicidal and insecticidal properties [14]. Such studies are particularly important in the context of utilising microorganisms in the bioremediation of environments polluted by the storage or use of explosives.

2. Material and methods

2.1. *Synthesis of investigated compounds*

The following tetrazine derivatives were used in the experiments: 3,6-dihydrazinotetrazine (DHTz), 3,6-bis(3,5-dimethylpyrazol-1-yl)-dihydro-1,2,4,5-tetrazine (DMPDHT) and N,N′-bis(1,2,4,5-tetrazine-6-(3,5-dimethylpirazylo))hydrazine (BDMPT). The tetrazine-based compounds were prepared following published methods [3–5]. An outline of the synthesis route is shown in Figure 2. All applied reagents were of high purity (minimum 98%). The structures of the products were confirmed by ^{1}H and ^{13}C NMR spectroscopy (Bruker, DRX 500). The purity was determined by HPLC (Shimadzu LC 20A, RP column, UV detector, eluent: acetonitrile/water 1:1) using the internal standard method.

The reagents differ in their water solubility; DHTz and BDMPT are relatively soluble ($\sim 150\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ for DHTz and $< 100\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ for BDMPT), resulting in coloured solutions. DMPDHT is barely soluble but is easily oxidised in water to the more soluble 3,6-bis(3,5-dimethylpirazylo)1,2,4,5-tetrazine (DMPT), resulting in a pinkish-red solution. The visible absorption spectra of DHTz, BDMPT and DMPT water solutions are presented in Figure 3.

2.2. *Isolation of soil bacteria*

A bacterial consortium was isolated from garden soil ($pH_{KCl} = 6.5$) on a medium with glucose comprising: 4.0 g Na_2HPO_4, 3.5 g KH_2PO_4, 0.1 g NH_4Cl, 0.1 g $MgSO_4$, 0.1 g $CaCl_2$, 5.0 g $C_6H_{12}O_6 \cdot H_2O$, 1000 mL distilled water and pH 6.8–7.0. Garden soil (1.0 g) was introduced into 50 mL of the medium and incubated for 48 h at 25 °C in aerobic conditions. Next, 1 mL of the culture was reintroduced to 50 mL of the medium and incubated for 24 h; after that, 1 mL of the culture was sieved on a fresh medium and incubated. This culture was subjected to genetic studies (16*S* rRNA) to identify the bacterial strains in the consortium (Table 1). DNA was isolated from the culture and fragments of the 16*S* rRNA gene were sequenced according to the procedure: the bacterial genomic DNA from culture was extracted by using a commercially available kit (Genomic Mini Prep isolation, A&A Biotechnology) following the manufacturer's instructions. The purity and concentration of the DNA preparation were determined spectrophotometrically at 260 nm. Furthermore, the DNA was used as a template for PCR. Universal

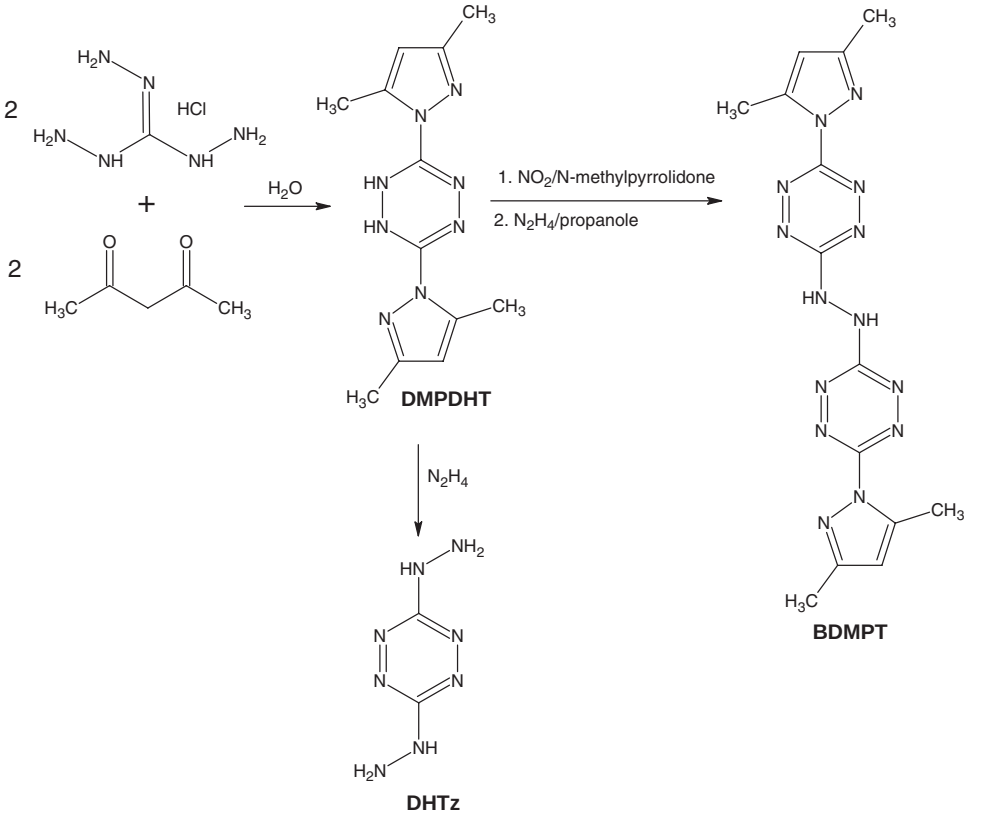

Figure 2. Synthesis route of DHTz, DMPDHT and BDMPT.

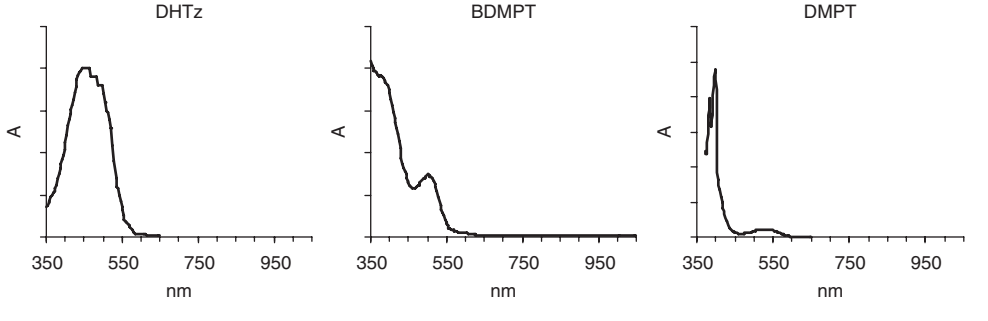

Figure 3. Visible absorption spectra of DHTz, BDMPT, DMPT.

primers for eubacterial 16*S* rRNA, 27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′, were used to amplify a 1540 bp segment from the 16*S* rRNA gene. The PCR was performed using the Gene Amp PCR reagent kit and AmpliTaq DNA polymerase (Invitrogen). A 50 mL PCR mixture contained 5 μ L of the template DNA (∼ 100 ng of DNA), 5 mL of 10 × PCR buffer, 25 mM $MgCl_2$; 200 mM mix of each dNTPs – containing all four deoxyribonucleosides (Sigma), 20 pmol each of forward (27F) and reverse (1492R) primers, and 2.0 $U \cdot \mu L^{-1}$ of *Taq* DNA polymerase. All PCRs were carried out by using a Mini Cycler (MJ Research). For 16*S* rRNA primers, the PCR conditions consisted of (i) 94 °C for 5 min,

Table 1. Bacterial consortium isolated from soil (16*S* rRNA analysis).

Strain	Similarity (%)
Bacillus halodurans C-125	100
Geobacillus thermodenitrificans NG80-2	98
Geobacillus kaustophilus	95
Pseudomonas sp. B728a	80
Pseudomonas stutzeri A1501	78

(ii) 94 °C for 1 min, (iii) 55 °C for 1 min, and (iv) 72 °C for 2 min. All PCR were performed for 35 cycles. An initial denaturation step at 94 °C for 5 min and a final extension of 72 °C for 5 min were included in all reactions. Subsequently, the amplification products were column-purified using the Wizard Purification System (Promega) and analysed by electrophoresis in 1.0% (w/v) agarose gels in 1 × TBE buffer as the running buffer containing ethidium bromide ($0.5\,\mathrm{mg}\cdot\mathrm{mL}^{-1}$) at $4.8\,\mathrm{V}\cdot\mathrm{cm}^{-1}$ for 1 h. A 100-bp DNA ladder (Invitrogen) was used as a marker to estimate the size of the DNA bands. The sequences obtained were compared with all bacterial sequences available in the GenBank database by using the Blast 2.0 program of National Center for Biotechnology Information (NCBI). The analysed sequences showed close similarities to the sequences of typical soil bacteria, e.g. *Geobacillus*, *Bacillus* and *Pseudomonas* (Table 1).

2.3. *Influence of the studied tetrazine derivatives on bacterial growth*

The influence of tetrazine derivatives on microorganism growth was tested using the isolated soil bacterial consortium. The laboratory system comprised three cultures in three repetitions; the studied tetrazine derivative was introduced to each of them to reach final solutions of 10, 20 and $40\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ in cultures with DHTz and BDMPT, and additionally $1000\,\mathrm{mg}\cdot\mathrm{L}^{-1}$ in the culture with DMPDHT. Next, 1 mL of the 12-h culture of the isolated bacterial consortium was added to 50 mL of the medium. The set was incubated at 25 °C in aerobic conditions for a maximum of 100 h, during which the optical density (OD) was tested using a spectrophotometer (Thermo Scientific) at 550 nm wavelength in relation to a culture solution after filtering through a 0.45 μm filter (Millipore). In addition, the absorption of the culture solutions was tested at 400 nm wavelength after filtering through a 0.45 μm filter (Millipore). The absorption of the filtered solution at 400 nm can be regarded as a measure of the concentration of the compounds in solution. The controlling systems were: a culture without the chemical compounds – biotic control, and a sterile (filtered) solution of the medium with the addition of the studied tertrazine derivative – abiotic control. In cultures with DMPDHT, the content of NH_4^+ ions was tested photometrically after reaction with Nessler's reagent. NH_4^+ can potentially appear in solution as an effect of decomposition of DMPDHT. In the remaining cultures with DHTz and BDMPT, the content of ammonium ions was not determined due to interaction of the studied compounds with Nessler's reagent.

2.4. *Kinetics of bacterial growth*

On the basis of measurements of culture turbidity (as an absorbance at 550 nm), growth of the bacterial consortium on a liquid medium was calculated with the following equation [15]:

$$\frac{dX}{dt} = \mu X, \tag{1}$$

where X is proportional to the density of bacterial growth in the exponential phase (culture turbidity) and μ is the specific growth rate of the studied bacterial consortium. Parameter μ was

calculated after integrating the above equation:

$$\mu = \frac{\ln X_1 - \ln X_0}{\ln t_1 - t_0}, \tag{2}$$

where t is the time in hours (h), t_0 is the beginning of the exponential growth, and t_1 is the end of the exponential growth.

Generation time (T_g, half-time) was calculated according to the following equation:

$$T_g = \frac{\ln 2}{\mu}, \tag{3}$$

as well as the length of the lag-phase (T_l), i.e. the time from inoculation to the beginning of exponential growth. This parameter was calculated from a half-logarithmic plot.

2.5. *Activity of dehydrogenases*

The activity of dehydrogenases in bacterial cultures in the presence of tetrazine derivatives was determined in triplicate using triphenyltetrazolium chloride (TTC). TTC can be used by microorganisms as an electron acceptor and the production of insoluble formazan from TTC may reflect dehydrogenase activity [16,17]. Five millilitres of the 12-h culture was inserted into a test-tube, to which one of the studied compounds was added: DHTz and BDMPT or additionally 3,5-dimethylpyrazole (DMP) for comparison (DMP can appear in solution as an effect of the decomposition of DMPDHT and BDMPT), to a final concentration of 8, 16, 33, 50 or 80 mg $\cdot$ L^{-1}. Next, 0.5 mL of 3% TTC and 50 mg of $CaCO_3$ were added to the test-tube ($CaCO_3$ in order to maintain neutral pH). The incubation was carried out for 1 h at 25 °C; next 0.5 mL of 37% formaldehyde was added, and the solution was filtered through a 0.45 μm filter (Millipore). The formazan retained on the filter was extracted in 96% ethanol and the content of the colouring was determined using spectrophotometry. The measure of dehydrogenase activity was the content of reduced TTC to formazan per millilitre of culture per hour (μg $\cdot$ mL$_{cult}^{-1}$ $\cdot$ h^{-1}). The control batch was a culture incubated without tetrazine addition.

3. Results

3.1. *Influence of DHTz and BDMPT on the growth of the microbial consortium*

The experiment showed distinct differences in the length of the lag-phase in cultures in the presence of DHTz and BDMPT, depending on the concentration of the compound being studied (Table 2). A concentration of 10 mg $\cdot$ L^{-1} DHTz caused lengthening of the lag-phase in cultures to 20 h, and a concentration of 20 mg $\cdot$ L^{-1} to 50 h. After this period, exponential growth took place, and the specific growth rate of the bacterial consortium (μ) was slightly lower than that determined in the control culture without DHTz. In turn, DHTz at 40 mg $\cdot$ L^{-1} inhibited bacterial growth and in the 80-h study period no increase in the optical density was noted (Figure 4). DHTz is a coloured substance that gives orange aqueous solutions, with an intensity depending on the concentration. In cultures where it was present at concentrations of 10 and 20 mg $\cdot$ L^{-1}, a decrease in colour intensity was observed (absorption at 400 nm), followed by its complete disappearance. A slightly smaller absorption decrease was noted also in the culture with 40 mg $\cdot$ L^{-1} DHTz although there was no increase in optical density. In the abiotic control culture, in which DHTz was present at 40 mg $\cdot$ L^{-1}, there was only a slight decrease in absorption, much smaller than in the remaining cultures (Figure 4).

Table 2. Parameters characterising the growth kinetic of bacterial consortium in cultures with DHTz and with BDMPT.

DHTz	Biotic control without DHTz	Culture I $10\,mg \cdot L^{-1}$	Culture II $20\,mg \cdot L^{-1}$
μ (h^{-1})	0.36	0.29	0.31
T_g (h)	1.94	2.41	2.21
T_l (h)	0.5	20	50

BDMPT	Biotic control without BDMPT	Culture I $10\,mg \cdot L^{-1}$	Culture II $20\,mg \cdot L^{-1}$	Culture III $40\,mg \cdot L^{-1}$
μ (h^{-1})	0.40	0.28	0.23	0.31
T_g (h)	1.73	2.50	2.97	2.27
T_l (h)	0.5	6	6	14

Notes: μ, specific growth rate; T_g, generation time; T_l, length of lag-phase in culture.

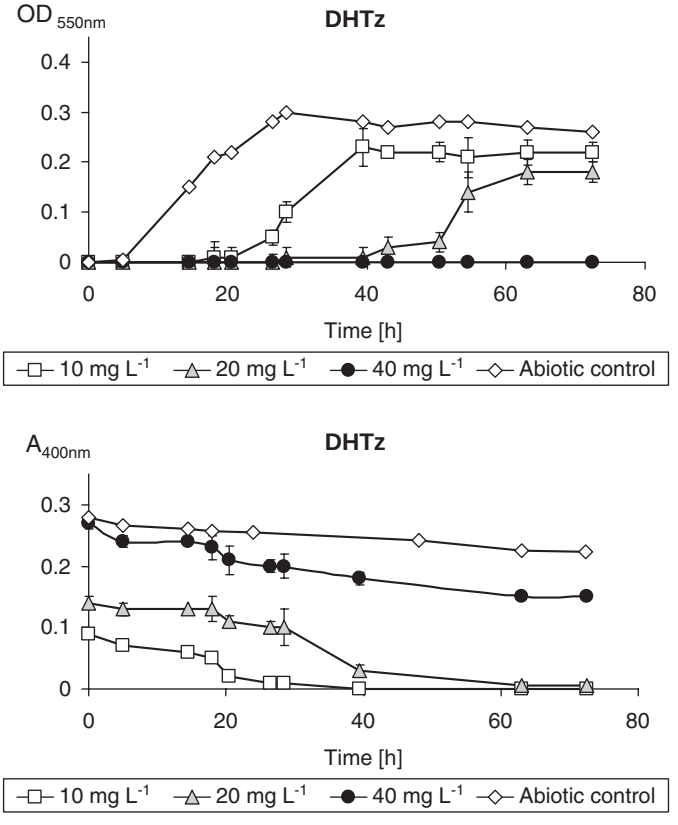

Figure 4. Changes in optical density (OD) in cultures of bacterial consortium amended with DHTz (upper), and changes in the absorption of filtrated solutions from cultures (lower). Abiotic control with $40\,mg \cdot L^{-1}$ DHTz. Error bars indicate standard deviations.

In the second system, in which BDMPT was added to the cultures, the influence of the tetrazine derivative on bacterial growth was also observed (Table 2 and Figure 5). This influence was, however, much lower and reflected in a slight lengthening of the lag-phase and a slightly longer specific growth rate in relation to the control culture (μ). Even in the culture in which the BDMPT concentration reached $40\,mg \cdot L^{-1}$, intense bacterial growth took place after a lag-phase lasting 14 h. As in the system with DHTz, absorption changes took place in the culture solutions. BDMPT

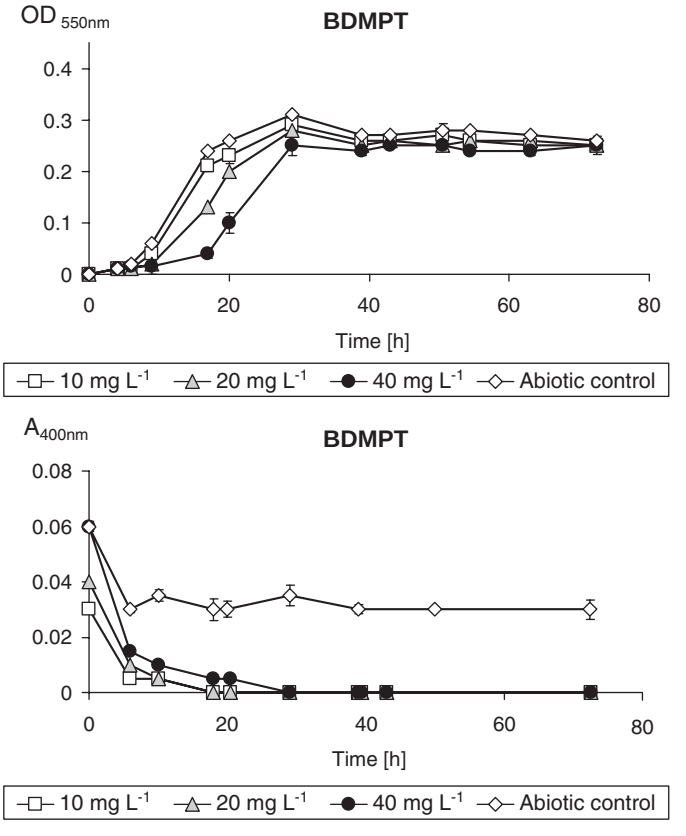

Figure 5. Changes in optical density (OD) in cultures of bacterial consortium amended with BDMPT (upper), and changes in the absorption of filtrated solutions from cultures (lower). Abiotic control with 40 mg $\cdot$ L^{-1} BDMPT. Error bars indicate standard deviations.

gives solutions with a colour similar to that of solutions with DHTz, and the colouring disappeared completely in all cultures after a maximum of 30 h. In the abiotic control with BDMPT at 40 mg $\cdot$ L^{-1}, a decrease in absorption was also noted, although only at a rate of 50% in the initial incubation, after which the absorption did not change.

3.2. *Influence of DMPDHT on the growth of the microbial consortium*

At low concentrations (10–100 mg $\cdot$ L^{-1}), DMPDHT does not completely inhibit microbial growth; a larger effect was noted at 1000 mg $\cdot$ L^{-1} (Figure 6). The inhibiting was reflected in a longer lag-phase in relation to the control culture by $\sim$12 h and a decrease in the specific growth rate to 0.20 h^{-1}. In the control culture, μ was determined at 0.35 h^{-1}. Despite a slightly longer lag-phase and a decreased specific growth rate, bacterial growth was intense and reached a rate similar to that of the control culture. A decrease in the concentration of ammonium ions was noted in the cultures; they were utilised by the developing bacteria as a nitrogen source. Ammonium ions did not appear in solution as an effect of the decomposition of DMPDHT.

DMPDHT is poorly soluble in water but quite easily oxidised to the more soluble DMPT, resulting in pinkish-red solutions. The intensity of the colour increased in the abiotic control culture to a stable level after $\sim$ 70 h. In the culture, in turn, as for the previous systems, a complete

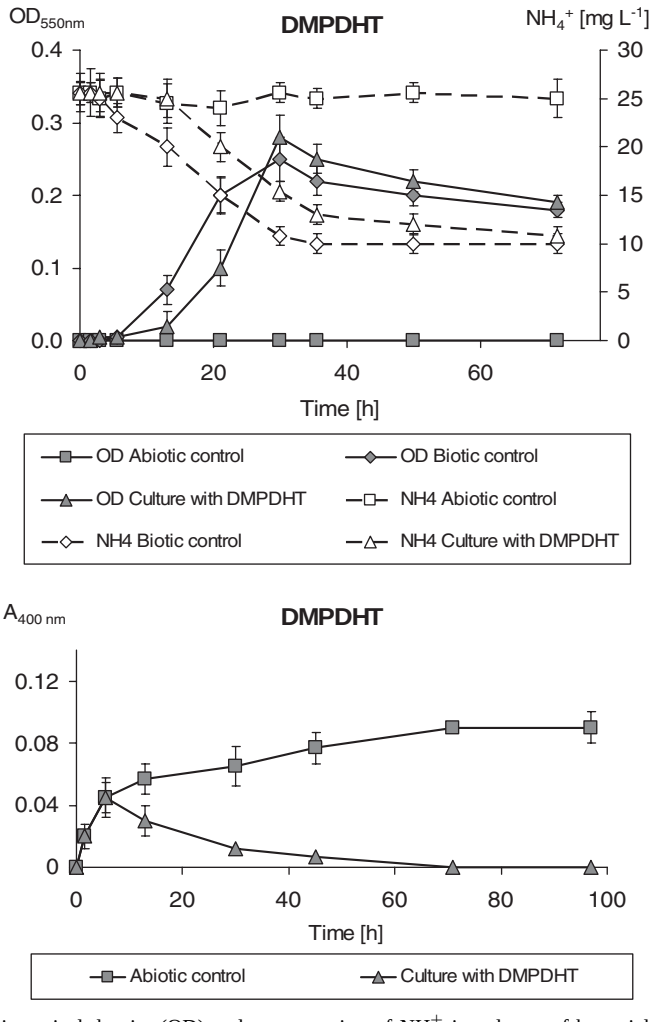

Figure 6. Changes in optical density (OD) and concentration of NH_4^+ in cultures of bacterial consortium amended with DMPDHT (upper). Changes in the absorption of filtrated solutions from cultures (lower). Abiotic control with $1000\,mg \cdot L^{-1}$ DMPDHT. Error bars indicate standard deviations.

disappearance of solution absorption was observed after an initial slight increase, as in the abiotic control (Figure 6).

3.3. *Dehydrogenase activity*

Dehydrogenase activity in the culture of soil bacteria was measured in the presence of DHTz, BDMPT and additionally DMP. The chart presented in Figure 7 shows the decrease in dehydrogenase activity with an increase in DHTz or BDMPT concentration in the culture. The most marked decrease was in the presence of DHTz; at $80\,mg \cdot L^{-1}$, the activity reached only $0.7\,\mu g \cdot mL^{-1} \cdot h^{-1}$ of formazan, whereas at the same concentration of BDMPT, the activity reached almost $2\,\mu g \cdot mL^{-1} \cdot h^{-1}$ of formazan. The control culture without any compounds showed dehydrogenase activity at a formazan level of $4.2\,\mu g \cdot mL^{-1} \cdot h^{-1}$. Moreover, in the case of BDMPT, dehydrogenase activity in the culture did not decrease at BDMPT concentrations not exceeding $30\,mg \cdot L^{-1}$.

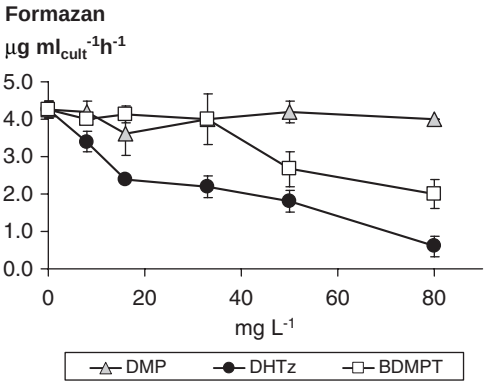

Figure 7. Activity of dehydrogenases in 12-h cultures in relation to a different concentration of DHTz, BDMPT and DMP. Error bars indicate standard deviations.

For comparison, Figure 7 also shows dehydrogenase activity in a culture with DMP, which did not significantly inhibit soil bacteria growth at concentrations of 10–100 mg · L^{-1}. No significant changes of activity were detected; a slight decrease was observed only at 16 mg · L^{-1} – probably a result of a measurement error during one of the three determinations.

4. Discussion

Heterocyclic nitrogen compounds may influence microbiological processes, as suggested in earlier articles. For example, studies of 3,5-dimethylpyrazole and its phosphate salts showed inhibition of nitrification processes in soils, a fact often utilised in agriculture [18–20]; by contrast, studies by Muller et al. [21] noted a lack of its influence on denitrification enzymes. Heterocyclic compounds utilised as explosives can be biodegraded by various bacterial strains. Studies on this topic have focused on, for example, RDX and TNT [7–9,15,22,23] and indicated the possibility of using bacteria in the bioremediation of soils polluted by the storage and use of explosives.

The results of this research show that the tetrazine derivatives differ in toxicity with regard to the applied bacterial consortium isolated from the soil environment. Of the three compounds studied, DHTz showed the strongest activity; it is interesting, however, that despite complete inhibition of growth by DHTz at 40 mg · L^{-1}, a decrease in the absorption of the coloured solution in relation to the control batch was observed. Thus, it can be assumed that DHTz at the given concentration does not completely inhibit bacterial metabolism. It may, however, weaken the ability of cell division, as in the case of β-lactamase antibiotics, although the mechanism of this process may be entirely different. The determined dehydrogenase activity which is linked solely with live cells, confirms that even at DHTz concentrations exceeding 40 mg · L^{-1} there are still live cells in the culture capable of respiration. Microbially induced decolourisation of substances in the water solution may be caused both by use of some compounds as final electron acceptors in the respiration chain (similarly to methylene blue), and by partial or complete biodegradation of a given coloured substance. Khehra et al. [24] described a series of bacteria capable of the decolouring of water solutions of some nitrogen dyes. A similar case was described by Selvam et al. [25] in the case of fungi representing *Fomes lividus* (Basidiomycetes). These authors indicated the possibility of using microorganisms in the removal of dyes in the management of industrial sewage. Partial or complete biodegradation of cyclic nitrogen compounds, as mentioned above in the case of RDX, cannot be excluded. It is interesting, however, that in the studied cultures, the decrease in absorption of the medium solution with the relevant compound took place mainly during the

lag-phase, and was later followed by an exponential growth of the culture. This is particularly notable in cultures with DHTz.

The highest toxicity of DHTz may be related to properties of its molecule. Dihydrazinotetrazine is the smallest molecule in the range studied. The transport ability of DHTz in the bacterial cell is probably much faster than for the relatively large molecules of DMPDHT and BDMPT. Of the compounds investigated, only DHTz has a two-reactive hydrazine group. A hydrazine chemical substituent can react with many critical compounds present in bacterial cell [26–28]. A split-off hydrazine group (in ionic form) from DHTz caused the appearance of free hydrazine which represents a high toxicity and mutagenity for live organisms.

When a tetrazine ring is substituted in carbon by a large substituent, strain bonds between the groups tend to dissociate, especially in a strongly basic environment (e.g. the reaction of DMPT with hydrazine). At room temperature in dilute solutions, the tetrazine derivatives investigated are relatively stable chemically. DMP can, however, appear in solution as an effect of the partial decomposition of DMPT and BDMPT. DMP is known as a nitrification inhibitor [19–21], but this compound does not have bactericidal properties.

5. Conclusions

Our experiments have shown that among the tetrazine derivatives DHTz had the largest influence on the growth of the soil bacterial consortium. Its presence in cultures caused lengthening of the lag-phase and a decrease in the specific growth rate of the bacterial consortium. BDMPT had a much smaller influence on the growth of the bacterial consortium; in this case, the main effect was also a longer lag-phase in the culture. In all cultures with tetrazine derivatives, inhibition of absorption of the initially coloured solution was observed, even in cultures in which an increase in optical density was not noted during the experiment. Based on this and on dehydrogenase activity studies, it can be assumed that the compounds in the applied concentrations did not completely inhibit respiration bacterial activity and could take part in biotic processes with microorganisms from the culture. The results indicate the potential of biodegradation of the compounds studied here by bacteria isolated from soil.

Acknowledgements

The study was supported by the Ministry of Science and Higher Education through the Institute of Chemistry, Military University of Technology, Grant No. O N204-000834. We thank for Professor Raymond Macdonald for checking the language of the manuscript.

References

[1] A.P. Hardt, *Pyrotechnics*, Pyrotechnica Publications, Austin, TX, 2001.
[2] R. Damse and A. Sikder, *Suitability of nitrogen rich compounds for gun propellant formulations*, J. Hazard. Mat. 166(2–3) (2009), pp. 967–971.
[3] H. Marcus and A. Remanick, *The reaction of hydrazine with 3,6-diamino-*S*-tetrazine*, J. Org. Chem. 28(9) (1963), pp. 2372–2375.
[4] M. Coburn, M. Hiskey, K. Lee, D. Ott, and M. Stinecipher, *Oxidation of 3,6-diamino-1,2,4,-tetrazine and 3,6-bis(*S,S*-dimethylsulfilimino)-1,2,4,5-tetrazine*, J. Heterocyc. Chem. 30 (1993), pp. 1593–1595.
[5] D. Chavez, M. Hiskey, and D. Gilardi, 3, 3′*-Azobis(6-amino-1,2,4,5-tetrazine): A novel high-nitrogen energetic material*, Ang. Chem. Int. Ed. 39 (2000), pp. 1791–1793.
[6] T. Urbanski, *Chemistry and Technology of Explosives*, Pergamon Press, Oxford, 1967.
[7] B. Bhushan, A. Halasz, J. Spain, and J. Hawari, *Diaphorase catalyzed biotransformation of RDX via N-denitration mechanism*, Biochem. Biophys. Res. Commun. 296 (2002), pp. 779–784.
[8] D. Fournier, A. Halasz, J. Spain, P. Fiurasek, and J. Hawari, *Determination of key metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine with* Rhodococcus *sp. strain DN22*, Appl. Environ. Microbiol. 68(1) (2002), pp. 166–172.

[9] F.H. Crocker, K.J. Indest, and H.L. Fredrickson, *Biodegradation of the cyclic nitramine explosives RDX, HMX, and CL-20*, Appl. Microbiol. Biotechnol. 73 (2006), pp. 274–290.
[10] B. Bhushan, L. Paquet, J.C. Spain, and J. Hawari, *Biotransformation of 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane (CL-20) by denitrifying* Pseudomonas *sp. strain FA1*, Appl. Environ. Microbiol. 69 (2003), pp. 5216–5221.
[11] J-S. Zhao, J. Spain, and J. Hawari, *Phylogenetic and metabolic diversity of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-transforming bacteria in strictly anaerobic mixed cultures enriched on RDX as nitrogen source*, FEMS Microbiol. Ecol. 46 (2003), pp. 189–196.
[12] M.W.Y. Khan, J. Mustafa, A. Rauf, M.T. Saeed, and S.M. Osman, *Long-chain derivatives with a hexahydrothioxotetrazine moiety as potential antimicrobial agents*, J. Am. Oil Chem. Soc. 69(11) (1992), pp. 1160–1161.
[13] S.K. Pandey, A. Singh, A. Singh, and Nizamuddin, *Antimicrobial studies of some novel quinazolinones fused with [1,2,4]-triazole, [1,2,4]-triazine and [1,2,4,5]-tetrazine rings*, Eur. J. Med. Chem. 44 (2009), pp. 1188–1197.
[14] G.A. Kemp, H.J. Bachmann, G. Berkelhammer, and G. Asato, United Kingdom Patent GB 1245443, 1971.
[15] P. Chulhwan, T. Kim, S. Kim, J. Lee, and S. Kim, *Biokinetic parameter estimation for degradation of 2,4,6-trinitrotoluene (TNT) with* Pseudomonas putida *KP-T201*, J. Biosci. Bioeng. 94(1) (2002), pp. 57–61.
[16] L.E. Casida, D.A. Klein, and T. Santoro, *Soil dehydrogenase activity*, Soil Sci. 98 (1964), pp. 371–376.
[17] Praveen-Kumar and J.C. Tarafdar, *2,3,5-Triphenyltetrazolium chloride (TTC) as electron acceptor of culturable soil bacteria, fungi and actinomycetes*, Biol. Fertil. Soils 38 (2003), pp. 186-189.
[18] G.W. McCarty and J.M. Bremner, *Inhibition of nitrification in soil by heterocyclic nitrogen compounds*, Biol. Fertil. Soils. 8 (1989), pp. 204-211.
[19] W. Zerulla, T. Barth, J. Dressel, K. Erhardt, K. Horchler von Locquwnghien, G. Pasda, M. Radle, and A.H. Wissemeier, *3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrificator inhibitor for agriculture and horticulture*, Biol. Fertil. Soils 34 (2001), pp. 79–84.
[20] Q. Yu, Y. Chen, X. Ye, G. Tian, and Z. Zhang, *Influence of the DMPP (3,4-dimethyl pyrazole phosphate) on nitrogen transformation and leaching in multi-layer soil columns*, Chemosphere 69 (2007), pp. 825–831.
[21] C. Muller, R.J. Stevens, R.J. Laughlin, F. Azam, and J.C.G. Ottow, *The nitrification inhibitor DMPP had no effect on denitrifying enzyme activity*, Soil Biol. Biochem. 34 (2002), pp. 1825–1827.
[22] J. Hawari, A. Halasz, T. Sheremata, S. Beaudet, C. Groom, L. Paquet, C. Rhofir, G. Ampleman, and S. Thiboutot, *Characterization of metabolites during biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) with municipal anaerobic sludge*, Appl. Environ. Microbiol. 66(6) (2000), pp. 2652–2657.
[23] A. Suszek and D. Wolicka, *Possibility of application of indigenous soil organisms in bioremediation of the nitrogen compounds* (in Polish), in *Environmental Biotechnology*, K. Miksch, ed., Department of Environmental Biotechnology, Silesian University of Technology, Gliwice, Poland, 2008, pp. 139–145.
[24] M.S. Khehra, H.S. Saini, D.K. Sharma, B.S. Chadha, and S.S. Chimni, *Decolorization of various azo dyes by bacterial consortium*, Dyes Pigments 67 (2005), pp. 55–61.
[25] K. Selvam, K. Swaminathan, and Keon-Sang Chae, *Microbial decolorization of azo dyes and dye industry effluent by* Fomes lividus, World J. Microbiol. Biotechnol. 19 (2002), pp. 591–593.
[26] S. Parodi, S.D. Flora, M. Cavanna, A. Pino, L. Robbiano, C. Bennicelli, and G. Brambilla, *DNA-damaging activity* in vivo *and bacterial mutagenicity of sixteen hydrazine derivatives as related quantitatively to their carcinogenicity*, Cancer Res. 41 (1981), pp. 1469–1482.
[27] E.G. Rogan, B.A. Walker, R. Gingell, D.L. Nagel, and B. Toth, *Microbial mutagenicity of selected hydrazines*, Mutat. Res. 102(4) (1982), pp. 413–424.
[28] A. Poso, A. von Wright, and J. Gynther, *An empirical and theoretical study on mechanisms of mutagenic activity of hydrazine compounds*, Mutat. Res. 332 (1995), pp. 63–71.